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The mechanism of glucocorticoid induced murine thymocyte and peripheral T cell apoptosis

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1.   Introduction

1.1 Glucocorticoids

The term steroid refers to a group of small lipophilic compounds derived from a common precursor, cholesterol. The four major types of steroids: progestins, androgens, estrogens, and corticoids, differ in the number of carbon atoms they contain, the receptors they bind, and the biological activities they possess. One can further divide the corticoids into two groups: mineralocorticoids, which regulate ion transport and thus fluid and electrolyte balance, and glucocorticoids, which have many activities, including mediating the stress response, regulation of intermediary metabolism, and immunosuppressive and anti-inflammatory effects.

The conversion of cholesterol to the various steroids is performed by an array of dehydrogenases and cytochrome P450 enzymes, membrane-bound and hemecontaining monooxygenases that catalyze dehydroxylation-oxidation reactions. The first and rate-limiting step in steroid biosynthesis is the cleavage of the side chain of cholesterol by P450scc to generate the first steroid, pregnenolone. P450scc expression seems to be limited to steroidogenic tissues such as the adrenals, placenta, gonads, brain, and thymus (Mintami F, 1982; Geuze HJ, 1987; Payne AH, 1990; LeGoascogne, 1987; Vacchio, 1994). Pregnenolone is hydroxylated at position 17 by P450c17, resulting in two possible parallel pathways of corticoid synthesis. Although rodents express P450c17 in the gonads, in adult animals it is not detectable in the adrenal cortex (Keeney, 1995), so the major circulating glucocorticoid in mice (corticosterone) differs slightly from the preponderant circulating glucocorticoid in most species, including human (cortisol). Progesterone (or its 17-OH form) is hydroxylated in the endoplasmic reticulum by P450c21 to yield 11-deoxycorticosterone (or 11-deoxycortisol), which has little glucocorticoid activity, and is then converted in mitochondria to the active glucocorticoid corticosterone (or cortisol) by P450c11. A small amount of corticosterone undergoes a series of intermediate steps that result in conversion to the mineralocorticoid aldosterone. Because steroids are lipophilic they are transported in the blood in a reversible complex with protein. The major high-capacity low-affinity carrier is albumin; the major low-capacity high-affinity protein is

transcortin, also known as corticosteroid-binding globulin (CBG). Only a small fraction (in the order of 1–10%) of glucocorticoids is free and available to mediate biological functions, so the effective glucocorticoid concentration in plasma is considerably lower than the total that is typically measured. The synthetic glucocorticoid dexamethasone (Dex) is particularly potent in vivo because of its high affinity for the GR and relatively low level of binding to plasma proteins (Meikle, 1977; Vinson GP, 1992).

Secretion of glucocorticoids by the adrenals is under the control of the hypothalamo-pituitary axis. Adrenocorticotrophic hormone (ACTH) produced by the anterior pituitary causes an immediate increase in the secretion of glucocorticoids as well as an increase in the production of steroid biosynthetic enzymes (Simpson, 1988). Glucocorticoid levels are maintained, in part, by a feedback loop with the hypothalamus and anterior pituitary in which low systemic glucocorticoid levels increase, and high levels suppress, ACTH secretion (Vinson GP, 1992). Under normal conditions, secretion of ACTH occurs with a circadian pattern, in humans peaking prior to waking and reaching a nadir in the evening. Perhaps of special note to immunologists, in nocturnal animals such as rodents this pattern is reversed, with the peak in ACTH secretion occurring in the late afternoon (Vinson GP, 1992). The resulting changes in glucocorticoid secretion typically yield fluctuations in plasma concentration, with a range of threefold to as much as tenfold over the course of the day (Vinson GP, 1998). The major stimulus to increased ACTH, and thus glucocorticoid, secretion is ''stress,'' a term that covers a wide range of physiologic (e.g. exercise, emotional disturbance, etc) and pathologic (e.g. trauma, hemorrhage, fever, etc) situations (Jones, 1988).

1.2 Glucocorticoid receptor

The glucocorticoid receptor (GR) is a phosphoprotein of Mr 85,000—97,000 that is present at low concentration in most mammalian cells (Yamamoto, 1985). Nonprotein-bound corticosteroids passively diffuse across the plasma membrane into the cell where they encounter the GR. The GR is a member of a large superfamily that includes receptors for other steroid hormones, thyroid hormone, vitamin D3, retinoic acid, and a number of orphan receptors, including Nur77. Receptors of this superfamily have many

conserved structural elements, including a COOH-terminal ligand-binding domain (which also contains residues required for dimerization and hormone-dependent gene transactivation), a nearby hinge region containing nuclear localization signals, a central zinc-finger-containing DNA-binding domain, and an NH_2-terminal variable region important for ligand-independent gene transactivation (Beato,1995). Although many of these receptors are nuclear-resident, the GR exists in the cytosol in a complex with heat shock proteins such as hsp90, hsp70, and the immunophilins hsp56 and CyP-40 (Owens-Grillo,1995), and it translocates to the nucleus when it is occupied by ligand (Abraham, 1991). Once in the nucleus, the GR binds as a homodimer to specific DNA sequences (glucocorticoid responsive elements, or GREs), where it acts to enhance or inhibit transcription of corresponding genes. The classic consensus GRE consists of two conserved 6-nucleotide halves separated by three no conserved bases, and it typically acts as an enhancer element (Beato, 1991). However, ''negative'' GREs that suppress gene transcription have also been described (Beato, 1991; Zhang, 1997), and some GREs contain sites for other transcription factors embedded in the GR binding region, yielding a ''composite'' response element that may have enhancing or repressing activity (Diamond, 1990). Thus, the GR is a ligand-regulated transcription factor. The members of this receptor superfamily interact with a cohort of molecules to mediate their function as transcriptional regulators, and the GR is no exception. For example, GRs are thought to stabilize the formation of a preinitiation complex that contains components of the basal transcriptional machinery (Shibata, 1997). Furthermore, the ligand-binding domain contains a region (AF-2) that binds a number of proteins important for GR (and other members of this receptor superfamily) function. One group of co-activators includes steroid receptor coactivator- 1 (SRC-1), activator of thyroid hormone and retinoid receptors (ACTR), and transcriptional intermediary factor 2 (TIF2)/glucocorticoid receptor interacting protein 1 (GRIP1) gene products. A second co-activator group includes proteins such as CREB binding protein (CBP) and its homolog p300, and p300/CBP-associated factor (P/CAF). One of the most exciting developments in this area in recent years is the observation that SRC-1, ACTR, p300/CBP, and P/CAF all have intrinsic histone acetytransferase (HAT) activity (Bannister, 1996; Chen, 1997; Ogryzko, 1996; Spencer, 1997;Yang,1996). Acetylation of core histones alters nucleosomal packing

to allow increased access of transacting factors and components of the basal transcriptional machinery to the local DNA (Wade, 1997). Thus, chromatin remodeling in response to recruitment of HATs by the liganded GR is at least one mechanism by which glucocorticoids enhance gene transcription.

Five different splice variants of the GR gene have been identified: GR-α, GR-β, GR-γ, GR-P, and GR-A. The GR-α isoform is responsible for GC-mediated transcriptional activation, whereas the other isoforms contain alterations in either the hormone binding domain or the DNA binding domain and may modulate GR-α(Hollenberg,1985; Moalli,1993; Rivers,1999; Bamberger,1995). Humans have two GR isoforms: α (the classic GR) and β (Baughman, 1991). These receptors share the first 727 amino acids (encoded by the first 8 exons of the GR gene and containing the transactivating and DNA-binding domains), but due to alternate mRNA splicing the receptors contain carboxy-terminal residues encoded by either exon 9α or 9β, respectively. Thus, in GRβ the last 50 amino acids of GRα are replaced with a unique 15 amino acid sequence that renders the molecule incapable of binding glucocorticoids and therefore transcriptionally inactive. Unlike GRα, GRβ constitutively resides in the nucleus (Chapman, 1995). Indeed, GRβ can bind a GRE consensus sequence, and overexpression of GRβ can inhibit gene transactivation mediated by GRα in a dominant negative fashion. Screens for mRNA expression found that GRα and GRβ are widely expressed in the same tissues (Baughman, 1991; Chapman, 1995), leading to the speculation that these GR isoforms might interact, perhaps via heterodimerization, to regulate the transcriptional effects of glucocorticoids. This possibility was supported by the finding that peripheral blood mononuclear cells from patients with glucocorticoid-resistant asthma had decreased binding of the GR to DNA, accompanied by elevated numbers of cells in which GRβ was detected by immunohistochemistry (Medh, 2003). Binding analyses indicated that the decrease in GR-DNA binding was due to lowered GR affinity for the GRE. Moreover, overexpression of GRβ in HepG2 cells (hepatocytes) had the same effect on binding of the endogenous GRα to DNA. GR DNA binding activity increased in peripheral blood mononuclear cells cultured in medium but not those cultured in IL-2 and IL-4, and culture of cells from normal donors with IL-2 and IL-4 resulted in increased GRβ expression. Together, these

data suggest that GRβ expression can be regulated by cytokines and that there may in fact be situations in vivo in which the interplay between GRα and GRβ has significant biological consequences. However, the finding that the GRβ is absent in mice questions the in vivo relevance of their isoform in humans (Christiane Otto, 1997).

1.3 GR regulates gene expression through different mechanism

Glucocorticoids can regulate gene expression directly or through the cross-talk with other transcription factors. The first such interaction described was between the GR and AP-1 (Diamond MI, 1990; Schüle, 1990; Yang, 1990; Jonat, 1990). Typically, these factors antagonize each other's transcription enhancing activity, although AP-1 consisting of c-Jun dimers can also enhance GRE-mediated transactivation (reviewed in Zacharchuk, 1990; Gottlicher, 1998). Most, although not all, studies that examined the issue detected a direct physical interaction between the GR and AP-1, suggesting at least one direct molecular basis for cross-talk, although simple sequestration of these factors into inactive complexes does not seem to be likely to explain the phenomenon. Other nuclear factors that have been found to bind the GR and modify its activity are NF-κB (Stein, 1993; Ray, 1994; Caldenhoven, 1995; Scheinman, 1995), the cAMP response element binding protein CREB (Imai, 1993), and the signaling and transcription factors STAT3 and STAT5 (Zhang, 1997; Stocklin, 1996). Another potential mechanism is competition for co-activators (''squelching''). Both AP-1 and the GR are co-activated by CBP/p300, and in fact overexpression of CBP or p300 reverses the antagonism between AP-1 and the GR (Zhang, 1997). Similarly, overexpression of CPB or SRC-1 reverses the transcriptional antagonism between the GR and NF-κB (Stocklin, 1996). These results support the notion that under some circumstances cross-talk between the GR and other transcription factors is due to competition for limiting co-activators of transcription. In the case of NF-κB, yet another mechanism for cross-talk with the GR has been proposed: Glucocorticoids increase the transcription and synthesis of IκBα and thus may inhibit NF-κB by promoting its retention in the cytosol (Auphan, 1995; Scheinman, 1995). Although the possibility that glucocorticoids inhibit NF-κB by upregulating IκBα is attractive, its biological relevance is uncertain at best, as there is a steadily increasing number of examples in which inhibition of NF-κB occurs in the absence of IκBα upregulation

(Brostjan, 1996; Heck, 1997; De Bosscher, 1997; Wissink, 1998). Regardless of the particular mechanism involved, the extensive degree of cross-talk between the GR and other transcription factors provides a rich framework for mutual regulation (positive or negative) between glucocorticoids and other signaling pathways.

1.4 Glucocorticoid and apoptosis

At least since the end of the nineteenth century it has been known that adrenal insufficiency in humans (Star, 1895) and adrenalectomy of animals result in thymic hypertrophy that cannot be reversed by the adrenal medullary product epinephrine (Boinet,1899; Jaffe,1924; Selye,1936), and that stress and drug-induced involution of the thymus is prevented by adrenalectomy (Selye,1936). These observations were followed by the findings that administration of ACTH to mice caused a marked reduction in thymus and lymph node mass (Dougherty, 1943), and that a purified corticosteroid caused the regression of a lymphosarcoma (Heilman, 1944). It is now appreciated that lymphoid cells, especially CD4+CD8+ (double positive, or DP) thymocytes, are among the few cell types that undergo apoptosis in response to corticosteroids (Mitchell, 1998). Although GCs have been widely used in the treatment of inflammation, hematological malignancies and autoimmune diseases for many years, the mechanism that GCs induced cell apoptosis is still not fully understood. As far as we know now, this process could be divided into three stages arbitrarily, an initiation stage that involves GR-mediated gene regulation, a decision stage that involves the counterbalancing influence of prosurvival and proapoptotic factors, and the execution stage which involves caspase and endonuclease activation.

1.4.1 Glucocorticoids induce apoptosis via gene transactivation in the initiation stage

In the initiation stage, it has been well established that gene regulation is involved in this process, but we still don't know which genes mediate cell death although many attempts have been made.   In part, the difficulty is to know what to look for: the induction of a gene or set of genes that cause cell death as a direct or indirect consequence of their

biological action, or the repression of genes necessary for cell survival, or a combination of both? More and more evidences show both are involved.

Evidence from several laboratories indicates the transactivation activity of the GR is required to mediate GC-induced apoptosis. Chapman (Chapman, 1996) found that chimeric receptors containing the potent VP16 and E1A viral transactivation domains in place of the GR amino terminus enhanced the sensitivity of lymphoid cells to GC-induced apoptosis, supporting a role for transactivation in apoptosis induction. Ramdas (Ramdas, 1998) addressed the question by comparing human leukemic T cells that express either wild type corticosteroid receptor or receptor that has a mutation that is activation deficient, but that retains the ability to repress AP-1 activity. This mutant failed to mediate corticosteroid-induced apoptosis, indicating that the transactivation function of the receptor is essential for corticosteroid-induced apoptosis. Reichardt (Reichardt, 1998) engineered a point mutation on the GR in mice that impairs GR dimerization, and hence inactivates the transactivation function of the receptor. In mice with this receptor mutation, GC-induced apoptosis of thymocytes was inhibited. Since the mutant receptor retains the transrepression activity, these studies provide strong evidence that the transactivation function of GR is necessary for GC-induced apoptosis.

The concept that GR-induced apoptosis is mediated by the transrepression activity of the GR is largely based on the work of Helmberg (Helmberg, 1995). They reported that mutant GR defective in transactivation, but fully competent at interfering with AP-1 activity, mediated apoptosis induction by GC. Since the AP-1 regulates expression of genes involved in cell growth, differentiation and transformation (Karin, 1997; Wisdom, 1999), these findings suggest that interference with transcription factors required for cell survival, may contribute to apoptosis induction. With the introduction of gene chip technology, more and more GR associated genes have been or will be found and this problem will be solved sooner or later.

1.4.2 Bcl-2 family members, proteasome, calcium and the crosstalk with other signaling pathways are the main effecting factors in the decision stage

In the decision stage, people already have known the proteasome, cross talk with other signaling pathways, calcium and Bcl-2 family members have been acknowledged as main players of this process.

1.4.2.1 The role of proteasome in glucocorticoid induced apoptosis

The proteasome is a multicatalytic protease complex located in both the cytoplasm and the nucleus that degrade proteins by polyubiquitination. Much less is known about the role of the proteasome in apoptosis, compared to the vast body of information regarding caspases, evidence in primitive organisms has suggested a fundamental involvement of the proteasome in apoptosis. For example, in the hawk moth, Manduca sexta, apoptosis of intersegmental muscles is associated with elevated ubiquitin gene expression and proteasome activity (Schwartz, 1990; Jones, 1995; Dawson, 1995). Genetic evidence for an involvement of the proteasome in apoptosis has been provided by investigation of a dominant mouse mutation `fused toes', characterized by partial syndactyly of the limbs and thymic hyperplasia(Lesche ,1997). Both morphological abnormalities are attributed to impaired regulation of programmed cell death, due to decreased expression of a novel gene, Ft1, which encodes a protein related to ubiquitin-conjugating enzymes. In mammalian cells, evidence that proteasome activity plays a role in apoptosis induction comes primarily from work on the induction of apoptosis in thymocytes by corticosteroids. Grimm et al (Grimm, 1996) demonstrated that proteasome inhibitors block corticosteroid-induced cleavage of poly-ADP ribose polymerase (PARP), a downstream caspase target, and apoptosis in thymocytes. These findings raised the possibility that the proteasome may either degrade regulatory proteins that normally inhibit the apoptotic pathway, or may proteolytically activate proteins that promote cell death. In corticosteroid-induced apoptosis of thymocytes, proteasome activity appears to be involved at a step preceding mitochondrial changes and caspase activation. Beyette (Beyette, 1998) have shown that thymocytes are rich in proteasomes, and that a chymotryptic component of proteasome activity decreases following dexamethasone

treatment. Moreover, proteasome inhibitors prevented disruption of the mitochondrial transmembrane potential and also prevented exposure of phosphatidylserine and nuclear DNA fragmentation (Hirsch, 1998). Pharmacological stabilization of the mitochondrial permeability transition pore, or inhibition of caspases, did not prevent the activation of proteasomes (Hirsch, 1998; Dallaporta, 2000). Proteasome activation in dexamethasone-treated thymocytes is inhibited by overexpression of Bcl-2, as well as by protein synthesis inhibitors and antioxidants (Dallaporta, 2000). Involvement of the proteasome in apoptosis induction is a distinguishing characteristic of the corticosteroid-induced death pathway versus the Fas-mediated cell death pathway where the proteasome appears dispensable.

Although proteasome activity appears to play a proapoptotic role in corticosteroid-treated lymphocytes, proteasome targets in this context are just now being identified. One of these targets, as mentioned in the preceding section, is the transcription factor c-Fos. In a T cell lymphoma line, degradation of the transcription factor c-Fos by the proteasome was found to be a relatively early step in corticosteroid-induced apoptosis that preceded caspase-3 activation and DNA fragmentation and was inhibited by Bcl-2 overexpression (He, 1998). A mutant form of c-Fos that evades degradation by the proteasome inhibited corticosteroid-induced apoptosis, suggesting that c-Fos degradation contributed to apoptosis induction (He, 1998). Moreover, Ivanov et al (Ivanov, 1998) found that the inhibition of corticosteroid induced apoptosis in thymocytes by the proteasomespecific inhibitor lactacystin is associated with stabilization of AP-1, NF-κB and NUR-77 against proteasome-mediated degradation. The role of the proteasome in corticosteroid-induced apoptosis is not limited to the degradation of prosurvival transcription factors. Another concept regarding the role of the proteasome in corticosteroid-induced apoptosis comes from the work of Grassilli (Grassilli, 1998). They found that polyamine levels decrease in thymocytes undergoing apoptosis in response to dexamethasone treatment due to accelerated degradation of ornithine decarboxylase by the proteasome. Inhibition of proteasome function preserves polyamine synthesis in association with inhibition of corticosteroid apoptosis (Grassilli, 1998). Another target of the proteasome in corticosteroid induced apoptosis is the cyclin dependent kinase

inhibitor, p27Kip1 (Gil-Gomez, 1998). Following treatment with dexamethasone, the level of p27Kip1 in thymocytes decreases due to proteasome-mediated degradation, a process that is inhibited by Bcl-2 overexpression and accelerated by Bax. Finally, degradation of apoptosis inhibitory proteins, c-IAP1 and XIAP, by the proteasome has been described in thymocytes undergoing apoptosis in response to dexamethasone (Yang, 2000). Moreover, mutant forms of IAP that are not degraded by the proteasome had an inhibitory effect on apoptosis induction by dexamethasone, suggesting that IAP degradation promotes dexamethasone-induced apoptosis in thymocytes. The IAPs appear to bind directly to caspases and inhibit their activation (Green, 2000). Hence, the proteasome may contribute to apoptosis induction by abrogating the apoptosis inhibitory effect of IAPs. In summary, the multicatalytic proteasome appears to play an important role in the decision of a lymphoid cell to die following corticosteroid treatment. Recognized targets of the proteasome include transcription factors that regulate genes necessary for cell proliferation (e.g., c-Fos), enzymes whose activity is essential for cell proliferation (e.g., ornithine decarboxylase), cell cycle regulatory proteins (e.g., p27Kip1) and proteins that normally repress caspases (e.g., IAPs). Bcl-2 has been reported to inhibit the increase in proteasome activity associated with corticosteroid-induced apoptosis, as well as the degradation of c-Fos and p27Kip1. Therefore, regulation of proteasome-mediated degradation of factors essential for cell survival may be a point of control for Bcl-2 in the apoptotic pathway.

1.4.2.2 The role of Bcl-2 family members in glucocorticoid induced apoptosis

GC-induced apoptosis is both positively and negatively regulated by Bcl-2 family members. The Bcl-2 protein is encoded by the B-cell lymphoma/leukemia-2 gene and is a member of a family of well conserved regulatory proteins involved in the regulation of apoptosis. The Bcl-2 family includes both proapoptosis (Bak, Bax) and prosurvival members (Bcl-2, Bcl-xl).These molecules include both multidomain proteins (Bak and Bax) and the BH3-only proteins (Bad, Noxa, and PUMA). The multidomain molecules can be activated following interaction with BH3-only proteins. The Bcl-2 proteins are involved in control of mitochondrial permeability by forming pores in the outer membrane or by regulating the opening and closing of the permeability pores. Disruption

of Bcl-2 in mice accelerates GC-induced thymocytes apoptosis, whereas lack of Bax and Bak prevents it (Veis, 1993; Rathmell, 2002). A Bcl-2 family-regulated link between cell cycle and cell death has been proposed, based on the evidence that Bax and Bcl-2 modulate Cdk2 (cyclin dependent kinase) activation during dexamethasone-induced apoptosis in thymocytes (Gil-Gomez, 1998). Following treatment with dexamethasone, the level of p27Kip1 in thymocytes decreases and the level of Cdk2 kinase activity increases. Cdk2 activity is crucial for induction of apoptosis in thymocytes by corticosteroids (Hakem, 1999).The degradation of p27Kip1 by the proteasome is regulated by the Bcl-2 family. Bcl-2 overexpression delays the degradation of p27Kip1, whereas Bax overexpression accelerates its degradation. Moreover, Cdk2 activation during apoptosis is a highly regulated process under the control of known apoptosis regulators such as Bax and Bcl-2. Bcl-2 overexpression delayed the increase in Cdk2 kinase activity associated with dexamethasone-induced apoptosis, whereas Bax overexpression had the opposite effect. Thus, induction of thymocyte apoptosis by dexamethasone activates the biochemical machinery that is shared with the normal cell cycle, i.e, Cdk2 and p27Kip1. Bax and Bcl-2 are able to modulate the levels of p27Kip1 and Cdk2 identifies both of these factors as downstream components of a common biochemical pathway leading to apoptosis. Furthermore, these findings draw a mechanistic distinction between corticosteroid-induced apoptosis and apoptosis induction in thymocytes by Fas/CD95, as the latter does not involve changes in Cdk2 and p27Kip1 and is not inhibited by Bcl-2. Bcl-2 may also function by regulating proteasome mediated degradation of prosurvival transcription factors. Evidence that Bcl-2 inhibits dexamethasone-induced degradation of c-Fos by the proteasome, published by He (He, 1998), was mentioned earlier. Feinman (Feinman, 1999) reported that dexamethasone induced apoptosis of multiple myeloma cells is accompanied by decreased NF-κB DNA binding activity which is preserved by Bcl-2 overexpression. In this situation, Bcl-2 did not prevent I-κBα induction by dexamethasone; thus, it is possible that Bcl-2 stabilizes NF-κB by inhibiting proteasome-mediated degradation.

In short, both antiapoptotic and proapoptotic members of the Bcl-2 family are involved in regulating corticosteroid-induced apoptosis. However, their site of action has yet not

been determined. Although in many forms of apoptosis we have learned that Bcl-2 family members act primarily at the level of mitochondria, in the case of corticosteroid-induced apoptosis it appears that Bcl-2 family members may rather regulate degradation of cell cycle factors and transcription factors by the proteasome.

Bcl-2 family proteins could also regulate the death signal stimulated by GC. It has been suggested that Bcl-2 regulates apoptosis via an interaction with the outer mitochondrial membrane to preserve mitochondrial function (Gross A, 1999, 2001). Several reports have documented the ability of GCs to regulate the expression of Bcl-2 proteins and modulate apoptosis. It has been shown that overexpression of Bcl-2 in human pre-B lymphocytes (Alnemri, 1992) and in myeloma cell lines (Moreaux, 2004) protects against GC-induced apoptosis. Also, Bcl-2 knockout mice display accelerated apoptosis of thymocytes in response to treatment with GCs (Veis, 1993). The antagonism of apoptosis afforded by prosurvival Bcl-2 proteins appears to be specific for the GCs, because Bcl-2 (and Bcl-xl) block only GC-induced apoptosis and not death receptor-induced apoptosis (Memon,1995) and only partially block calcium ionophore or cycloheximide mediated apoptosis in a mouse lymphoma cell line (Caron,1994). GCs promote apoptosis through induction of expression of proapoptotic Bcl-2 proteins such as Bad in thymocytes (Mok,1999) or through a decrease in the expression of prosurvival proteins like Bcl-2 and Bcl-xL in leukemic cells (Broome,2002) and other cell types (Rogatsky,1999). The suppression of prosurvival Bcl-2 members by GCs and abrogation of GC induced apoptosis by overexpression of Bcl-2 suggest a causal relation between GC-induced apoptosis and Bcl-2 proteins via a mechanism that may require generation of reactive oxygen species or altered mitochondrial transmembrane transport (Tonomura, 2003). Hartmann et al. (Hartmann, 1999) reported that Bcl-2 protects cells against GC-induced apoptosis in the human T-ALL line (CCRF-CEM) for as long as 48 hours. However, when cultured for another 24 hours, these cells undergo massive apoptosis. Bcl-2 did not affect GC-mediated growth arrest, hence separating the antiproliferative and apoptosis-inducing effects of GCs (Hartmann, 1999). This finding was confirmed in other studies that showed that GCs in the presence of high levels of Bcl-2 have only an antiproliferative effect, but not an apoptotic effect; whereas in the presence of low Bcl-2 expression, GCs

induce inhibition of cellular proliferation and apoptosis (Caron, 1994; Smets LA, 1994,1996). It has been shown that GCs enhance the expression of the BH3-only protein Bim, which in turn facilitates the mitochondrial translocation of Bax, complexing of Bax with Bak, and activation of caspase-9, leading to apoptosis (Wang Z, 2003). It has been proposed that GCs interact with BH3- only proteins via 14-3-3 proteins. In normal cells, Bad exists in the inactive phosphorylated form in association with cytoplasmic 14-3-3 protein (Wang HG, 1999). Wang showed that 14-3-3 proteins are capable of binding to the C-terminus of the GR, forming a complex (Kino T, 2003). It is feasible that sequestration of 14-3-3 by GCs releases Bad, allowing for its downstream effect on the Bax-Bak complex, resulting in apoptosis (Zha J, 1996). Brunet(Brunet CL,1998) have shown that the loss of clonogenicity in CEM C7A human leukemia cell line, which represents commitment to cell death, is a separate event from caspase activation and subsequent apoptotic markers. Moreover, caspase inhibition did not prevent commitment to cell death, but overexpression of Bcl-2 did, suggesting that Bcl-2 acts upstream of caspase activation to inhibit commitment to cell death, and that the survival functions of Bcl-2 are not at the level of caspase inhibition. The involvement of the Bcl-2 family in GC-induced apoptosis at the level of cell cycle regulation has also been proposed. It has been established that the activity of cyclin dependent kinase 2 (or Cdk2) is crucial for GC induced apoptosis. In GC-treated thymocytes, Bcl-2 overexpression was associated with a delay in increase of Cdk2 activity, whereas overexpression of Bax enhanced Cdk2 activity (Gil-Gomez, 1998). In the WEHI7.2 mouse lymphoma cell line, Bcl-2 stabilized c-Fos, a transcription factor that promotes cell growth, differentiation, and transformation, against GCs induced proteasome-mediated degradation (He H, 1998). In addition, overexpression of Bcl-2 in the ARP-1 myeloma cell line prevents GC-mediated repression of NF-κB activity and subsequent apoptosis. However, Bcl-2 did not prevent I-κBα induction by GCs, suggesting that Bcl-2 stabilizes NF-κB by acting upstream of I-κBα in the NF- κB signaling pathway, possibly by stabilizing NF-κB DNA binding through inhibition of its degradation by the proteasome (Feinman R, 1999).

In summary, both antiapoptotic and proapoptotic members of the Bcl-2 family are involved in regulating corticosteroid-induced apoptosis. However, their site of action is

yet to be determined. Although in many forms of apoptosis we have learned that Bcl-2 family members act primarily at the level of mitochondria, in the case of corticosteroid-induced apoptosis it appears that Bcl-2 family members may also regulate degradation of cell cycle factors and transcription factors by the proteasome.

1.4.2.3 The role of calcium and potassium in glucocorticoid induced apoptosis

Next, changes in calcium and potassium homeostasis are also involved in the decision stage. Calcium has been implicated as a mediator of corticosteroid induced thymocyte apoptosis for a number of years. Basically, two types of evidence support a role of calcium in GC induced apoptosis. First, inhibitors of calcium activated proteases, or calpain, could inhibit Dex induced thymocytes apoptosis (Squier, 1997). Second, alterations of intracellular calcium homeostasis have been detected in lymphoid cells undergoing GC-induced apoptosis (Bian, 1997; Orrenius, 1991; Distelhorst, 1998; Lam, 1993; Hughes, 1997). The major intracellular reservoir of calcium in nonmuscle cells is the endoplasmic reticulum (ER). Corticosteroid treatment is associated with a decline in the calcium concentration within the ER lumen, which contributes to a decrease in total cellular calcium (Bian, 1997; Lam, 1993; Hughes, 1997). Moreover, recent findings have implicated the inositol trisphosphate receptor (IP3R) in corticosteroid-induced apoptosis. The IP3R is an IP3-gated calcium release channel in the ER membrane. Lymphocytes undergoing apoptosis in response to dexamethasone were found to have increased levels of IP3R expression, and antisense-mediated repression of IP3R expression was reported to inhibit dexamethasone induced apoptosis (Khan, 1996). Also, IP3R-deficient T cells are resistant to apoptosis induction by dexamethasone (Jayaraman, 1997). Therefore, these findings suggest that calcium release from the ER, via the IP3R, produces cytoplasmic calcium elevation and ER calcium pool depletion that triggers downstream effector pathways of apoptosis. An alternative theory is that dexamethasone treatment induces expression of a plasma membrane calcium channel, the P2X receptor, resulting in an elevation of cytosolic calcium (Chvatchko Y, 1996). However, recent findings suggest that P2X receptor expression is not altered by dexamethasone treatment, that the P2X

receptor agonist, ATP, does not induce apoptosis in thymocytes, and that the P2X receptor agonist do not block corticosteroid-induced apoptosis (Jiang S, 1996).

Bcl-2 family proteins emerge as major regulators of cellular calcium handling. Recent evidence suggests that overexpression of Bcl-2 results in a reduced ER Ca2+ load and, whereas down-regulation of Bcl-2 yields an increase in [Ca2+] ER (P. Pinton, 2000; R. Foyouzi, 2000; F. Vanden, 2002). To promote a decrease in ER calcium, Bcl-2 seems to increase the passive Ca^{2+} leak through the ER membrane without changing the activity of the SERCA pumps (P. Pinton, 2000; R. Foyouzi, 2000). However, it is not clear whether Bcl-2 conducts Ca^{2+} per se or activates an endogenous leak. Furthermore, Bcl-2 expression has also been reported to induce a decreased expression of the lumenal Ca^{2+} binding chaperone, calreticulin, and SERCA2b pumps, providing additional mechanisms that may be important to lower $[Ca^{2+}]ER$ (F. Vanden,2002). In Bcl-2 overexpression cells, mobilization of ER Ca^{2+} evoked by IP3-linked agonists or thapsigargin, an inhibitor of the SERCA pumps, results in a relatively small Ca^{2+} signal that may be a consequence of a reduction in ER Ca^{2+} loading. Furthermore, overexpression of Bcl-xl has been reported to evoke a decrease in IP3R protein (types 1 and 3) and this mechanism may also account for a decrease in the IP3-dependent $[Ca^{2+}]c$ signal (C. Li,2002).

Similar to overexpression of Bcl-2, enforced expression of Bax or Bak has been reported to induce depletion of the ER $[Ca^{2+}]$ store (Z. Pan, 2001; L.K, 2002), but has no effect in apoptosis (S. Aiba-Masago, 2002). In Bax or Bak overexpression cells, an early increase in mitochondrial calcium has also been observed and under conditions of inhibited mitochondrial Ca^{2+} uptake the Bax/Bak-induced apoptosis was also inhibited (L.K, 2002). These results indicate a Bax/Bak-induced early redistribution of Ca^{2+} from ER to the mitochondria.

In addition, apoptotic factors released from the mitochondria and activated caspase enzymes may also control Ca^{2+} transport proteins (e.g., control of IP3 receptors and SERCA by ROS or caspase-3). These effects on calcium handling often provide an

amplification loop for the apoptotic cascade or enhance the security of antiapoptotic mechanisms.

The potassium ion is also implicated in corticosteroid induced apoptosis. The potassium ion contributes to maintenance of cell volume, and volume loss is a characteristic feature of apoptosis in thymocytes (Bortner, 2000).Two phases of volume loss have been identified in CEM human T cell leukemia cells treated with dexamethasone: the first is a reversible phase, associated with net loss of potassium ions, while the second phase coincides with chromatin condensation (Benson, 2000). Potassium efflux enhances apoptosis in thymocytes (Bortner, 1997). Potassium at normal intracellular levels inhibits both apoptotic DNA fragmentation and caspase-3 activation (Hughes, 1997). Recent findings indicate that thymocyte apoptosis is accompanied by gross perturbations of plasma membrane potential related to loss of cytosolic potassium (Dallaporta, 1999). Furthermore, an inhibitor of plasma membrane potassium channels (tetrapentylammonium) was found to be an effective inhibitor of dexamethasone induced apoptosis. This inhibitor prevented dissipation of mitochondrial membrane potential, loss of cytosolic potassium, phosphatidylserine exposure on the cell surface, and chromatin condensation, as well as caspase and endonuclease activation (Dallaporta, 1999). These findings suggest that potassium channels contribute significantly to the regulation of some but not all pathways leading to thymocyte apoptosis.

1.4.2.4 Other signaling pathways which could crosstalk with glucocorticoid induced apoptosis signal

At last, the cross talk between the GR signal pathway and other signal transduction pathways could also determine the fate of a cell. There are two ways by which signaling crosstalk might regulate apoptosis. Activation of prosurvival signaling pathways may repress corticosteroid induced apoptosis. Alternatively, GC may repress prosurvival signaling pathways. Hence, the balance between opposing prosurvival and prodeath signaling pathways may determine the ultimate fate of cells.

A number of different cytokines could counteract the induction of apoptosis by GC. IFN-α and interleukin-6 inhibit dexamethasone-induced apoptosis in plasma cells and myeloma cells (Ferlin, 1998; Chauhan, 1997; Xu, 1998). Interleukins-9, -4, and -6 inhibit apoptosis in dexamethasone-treated thymocytes and thymoma cells (Van, 1997; Bauer, 1998). In addition, interleukin-15 inhibits dexamethasone induced apoptosis in activated T and B cells (Bulfone-Paus, 1997) and insulin-like growth factors protect myeloma cells from dexamethasone-induced apoptosis (Xu, 1997). Interleukins may counteract corticosteroid-induced death signals by increasing expression of transcription factors that mediate expression of survival genes. Interleukin-2-mediated protection of T cell leukemia cells from dexamethasone-induced apoptosis correlates with induction of the DNA binding and transactivation functions of AP-1(Guizani, 1996). Also, interleukin-6 and interferon alpha may repress corticosteroid-induced apoptosis by activating mitogenactivated protein kinase and phosphatidylinositol 3-kinase pathways.

An example of how the balance between prosurvival signals and prodeath signal works to regulate death decisions revolved NF-κB. NF-κB is a heterodimeric transcription factor that activates more then 100 genes coding for cytokines, cytokine receptors, chemotactic proteins, and adhesion molecules (Pahl, 1999; Li Q, 2002), and is involved in the regulation of apoptosis (Rayet B, 1999). In its resting state, NF-κB is sequestered in the cytoplasm by its inhibitor IκBα. When IκBα is marked by phosphorylation and ubiquitination for proteasome mediated degradation, it undergoes a conformational change, releasing NF-κB. This allows NF-κB to translocate into the nucleus, where it exerts its influence on gene transcription (Ben,2002; Ghosh,2002).GCs promote cell death by inducing IκBα thereby inhibiting NF-κB activity or repressing NF-κB directly(Auphan,1995; Brostjan,1996; De Bosscher,1999; Heck,1997; Adcock,1999); cytokines inhibit cell death by inhibiting the induction of IκBα by GC(Xie,1997).

Another example of how GC-induced apoptosis is regulated by cross talk with other signaling pathways involves protein kinase C-PKC (Asada, 1998). PKC includes several subfamilies of enzymes including calcium dependent PKC (cPKC) and calcium independent PKC (nPKC). GC induced apoptosis was inhibited by non-isoform-selective

PKC inhibitors but not by cPKC-specific inhibitors. Thus, nPKC isoforms appear to be involved in apoptosis induction, while activation of calcineurin and cPKC are capable of inhibiting GC-induced apoptosis. Moreover, an activator of cAMP-activated protein kinase has been reported to block dexamethasone induced apoptosis and caspase-3 activation (Stefanelli, 1998).

T cell receptor signaling can also regulate GC induced apoptosis. TCR signaling and GR signaling pathways are mutually antagonistic. TCR activation of the mitogen-activated protein kinase/extracellular signal regulated kinase (mek/erk) cascade via Ras is necessary and sufficient to inhibit GC mediated cell death in thymocytes, primary T cell and immortalized T cells. Another potential explanation of how Ras represses GC-induced apoptosis may involve Ras-mediated stabilization of c-Myc protein stability (Sears, 1999). Recent work by Refojo (Refojo, 2003) has implicated cyclic AMP as a modulator of the antagonism between GR- and T-cell receptor-induced apoptosis. Cyclic AMP inhibits the T-cell receptor-mediated apoptosis via a PKA-CREB-dependent mechanism, and potentiates GR-induced apoptosis by means of a CREB-independent mechanism (Refojo, 2003).

A novel member of the gp130 family, neurotropin-1/Bcell stimulating factor-3, has recently been shown to have growth-promoting and antiapoptotic activity in human plasmacytoma cells, mediated by the gp130 receptor signaling chain and, at least in part, by the JAK/STAT3 pathway. Recombinant human B-cell stimulating factor-3 was found to have growth-stimulating activity on plasmacytoma cell lines and was able to protect cells from GC-induced apoptosis. Cell growth stimulated by B-cell stimulating factor-3 was not inhibited by neutralizing anti-IL-6 or anti-IL-6 antibodies, but was abrogated by anti-gp130 antibodies (Burger, 2003).

1.5 The execution stage of glucocorticoid induced apoptosis

1.5.1 The mitochondria is the central cell organelle of GC induced apoptosis.

If the balance between cell survival signal and cell death signal is disrupted, cells will undergo apoptosis as the result of a series of protease activities. Extensive evidence indicates that many of the apoptotic pathways converge on the mitochondria, where a series of cell death-related events take place. These cell death events are the permeability transition of the inner mitochondrial membrane, the loss of the inner mitochondrial membrane potential, and the release of apoptogenic proteins such as cytochrome c. Different models have been proposed to explain how mitochondria release apoptogenic factors. One model argues that existing pores, viz. the permeability transition pore (PTP), functioning within the context of the energy metabolism are actively opened. In favor of this model, propagating a role of PTP in apoptogenic factor release, is that pro-and anti-apoptotic Bcl-2 proteins were shown to interact with the PTP complex proteins (J.C. Martinou, 2001; N. Zamzami, 2001). A direct consequence of the loss of integrity of the inner mitochondrial membrane by PTP formation is the dissipation of the transmembrane potential. Many reports have indeed reported the loss of the transmembrane potential as a primary event in the apoptotic process (N. Zamzami, 2001). However, there are also numerous reports that have described the release of intermembrane space proteins such as cyt c without a detectable decrease in the transmembrane potential (S.L. Schendel, 1999; S.W. Fesik 2000). A second model by which mitochondria release apoptogenic factors is the direct pore forming properties of some pro-apoptotic Bcl-2 proteins in the outer mitochondrial membrane. Bax, a monomeric soluble cytosolic factor, oligomerizes, translocates, and inserts in the mitochondrial outer membrane upon induction of apoptosis (R. Eskes, 2000; M. Suzuki). Together with Bak, present in the outer mitochondrial membrane, Bax is thought to form tetrameric channels through which cyt c may escape (M.C. Wei, 2001). BH3-only proteins such as truncated Bid induce the conformational change of Bax, needed to form pores in the mitochondrial membrane. BH3-only proteins function as sensors for cellular integrity and functionality, e.g. Bim as sensor for cytoskeleton integrity (H. Puthalakath,1999),

Bad for growth factor withdrawal (L. Soane,2001), and Bid as a sensor for death domain receptor signaling (M.D. Esposti,2002). Following their activation, BH3-only proteins relocate to the mitochondria. Bid, originally discovered as a binding partner of Bax and Bcl-2(M.D. Esposti,2002, K. Wang,1996), can be cleaved by caspase-8 (M.D. Esposti,2002, M. Grinberg,2002), granzyme B (M.D. Esposti,2002, M. Barry,2000), cathepsin (M.D. Esposti,2002, V. Stoka, B, 2001)and calpain (M. Chen,2001), generating proteolytically activated Bid (truncated Bid or tBid). Although tBid has been reported to be involved in channel formation (M. Grinberg, 2002, T. Kuwana,2002), genetic studies place Bax and Bak downstream of tBid as Bax and Bak double knockout cells are completely resistant to mitochondrial cyt c release during apoptosis (M.C. Wei,2001). The conformational change and/or oligomerization of Bax and tBid can be inhibited by Bcl-2 and Bcl-xl (T. Kuwana, 2002; A. Gross, 1999)

1.5.2 The role of cytochrome c in glucocorticoid induced apoptosis

Cytochrome c, first described in 1930 by Keilin (D. Keilin, 1930) is synthesized in the cytoplasm as apocytochrome c and translocated through the OMM. During or after import in the IMS, heme is covalently attached to apocytochrome c via stereospecific thioether linkages to two cysteine residues in the protein. This complex is refolded to a more compact structure called holocytochrome c (referred to as cytochrome c or cyt c), which has an important role in the oxidative phosphorylation as electron shuttle between Complex III (cytochrome c reductase) and IV (cytochrome c oxidase). For more than 60 years, this was the only known function of cyt c, although the "cytochrome c effect," i.e., translocation of cytochrome c to the cytosol and reduced oxidative phosphorylation after γ-irradiation of cancer cells, has already been reported in the 1950s and 1960s (B. Zhivotovsky,1998). In the 1990s, it became clear that cyt c is involved in the execution of programmed cell death in more complex organisms as Xenopus laevis, mice, and humans (J.C. Ameisen, 2002; Li.P, 1997). Cellular stress can induce the release of cyt c from the inter membrane space, which will eventually lead to caspase activation (Li.P, 1997). In mammals, cyt c triggers the assembly of the apoptosome. The apoptosome is a complex composed of cyt c, Apaf-1, and dATP. Successive binding of cyt c and dATP converts Apaf-1 from a closed monomeric configuration to an open heptameric platform for

procaspase-9 assembly (D. Acehan, 2002.) After binding with Apaf-1, procaspase-9 is activated and promotes the caspase cascade, thus, leading to apoptosis.

1.5.3 Caspases are the main executioners in GC induced apoptosis

The execution phase of GC-induced apoptosis may require activation of caspases, a family of proteases that cleave substrates at aspartate residues, resulting in a conserved series of biomedical and morphologic changes (Thornberry NA, 1998). The caspase cascade can be activated via two major pathways: the death receptor mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway (Figure1. Stephanie, 2002). Most probably, GC induced caspase activation proceeds along the intrinsic pathway. The less well understood intrinsic pathway involves mitochondrial disruption by proapoptotic Bcl-2 family members and the consequent release of numbers of factors, including cytochrome c that promotes caspase-9 activation (Saleh A, 1999). Caspase-8 and caspase-10 are the initiators of the extrinsic pathway and are activated in response to death receptor engagement by ligands belonging to the tumor necrosis family (Ashkenazi A, 1998). Both pathways converge on the activation of effector caspase-3, caspase-6 and caspases-7, and cooperate to enhance apoptosis through caspase-8 mediated cleavage of Bid, a proapoptotic member of the Bcl-2 family (Li H, 1998). It has been suggested that caspase-9 and Apaf-1 are essential for GC-induced apoptosis, because thymocytes from knockout mice deficient in either one fail to undergo apoptosis (Hakem, 1998; Yoshida, 1998; Kuida, 1998). But thymocytes and peripheral T cells from Apaf-1^{-/-} or caspase-9^{-/-} reconstituted mice undergo apoptosis normally under the stress of Dex and cytokine withdrawal (Vanseea S, 2002). So, the role of Apaf-1 and caspase-9 in GC induced apoptosis is still controversial.
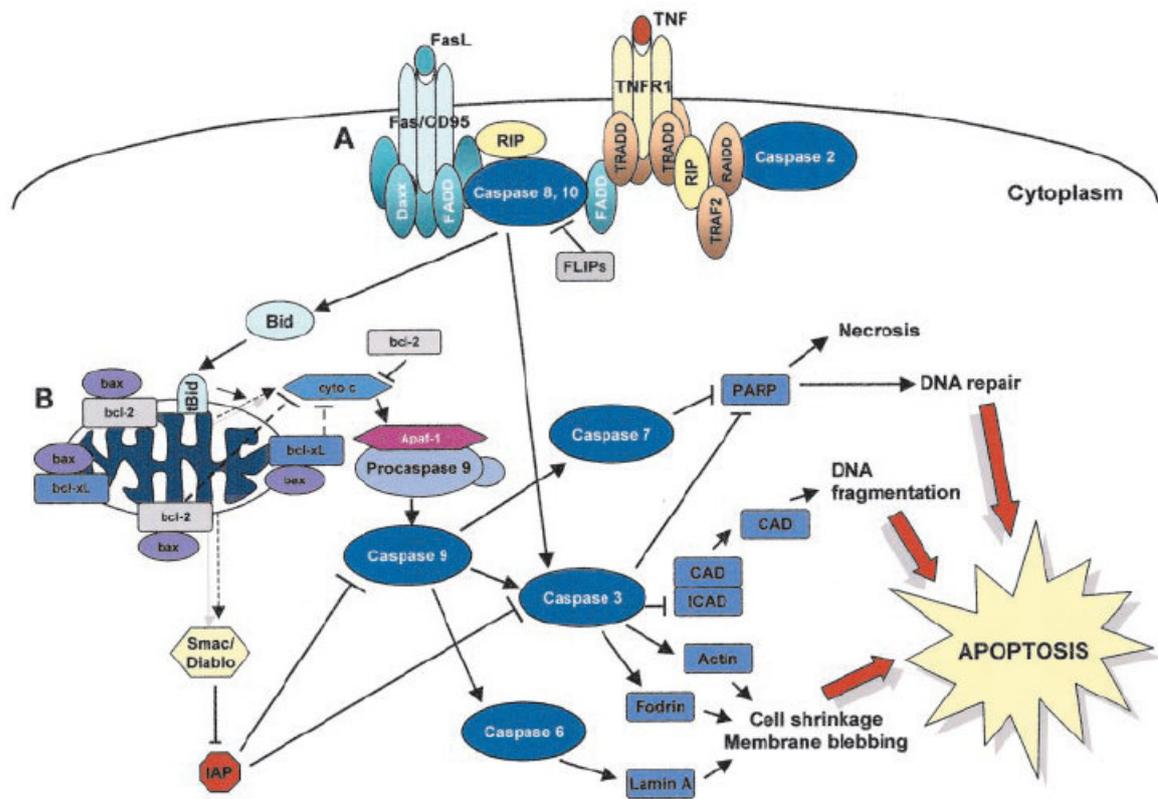
Figure1. Apoptotic signaling.

A, death receptor pathway. Apoptosis is induced by the activation of death receptors, such as Fas and TNF receptor. Ligand binding promotes death receptor oligomerization and initiates the caspase cascade via specialized adaptor proteins. Fas ligand (FasL) binds Fas and induces receptor trimerization and the recruitment of procaspase-8 via the adaptor protein FADD. Upon autocatalysis and activation, caspase-8 stimulates apoptosis through two parallel cascades; it directly cleaves and activates caspase-3, and it cleaves the proapoptotic Bcl-2 family member, Bid.   B, mitochondrial pathway. Members of the Bcl-2 family of proteins regulate apoptosis by altering mitochondrial membrane permeability and cytochrome c release. Activation of the apoptotic cascade is inhibited by the action of Bcl-2 and Bcl-xl, which inhibit the release of cytochrome c. The proapoptotic Bcl-2 protein Bax translocates to the mitochondria in response to death stimuli, including growth factor withdrawal, and promotes the release of cytochrome c. Truncated Bid (tBid) also translocates to the mitochondria upon activation by caspase-8, where it stimulates cytochrome c release and the activation of caspase-9. The subsequent activation of the effector caspases, caspase-3, caspase-6, and caspase-7, leads to the cleavage of cytoplasmic targets, causing cell shrinkage, membrane blebbing, DNA fragmentation, and eventually, apoptosis. The proapoptotic protein SMAC/DIABLO is also released from the mitochondria into the cytoplasm, where it binds IAPs and prevents the inhibitory action of this family of proteins on caspase-9 and caspase-3. Copied from Stephanie, 2002.

Caspase-3 deficiency, on the other hand, does not prevent GC-induced apoptosis, suggesting that caspase-3 may not be essential for this process (Kuida K, 1996). Studies using a specific caspase-3 inhibitor, Z-DEVD, highlightened the uncertainty regarding

the role of caspase-3 in GC-mediated apoptosis (McColl KS, 1998; Weimann E, 1999; Miyashita, 1998). Miyashita (1998) has suggested that caspase-6, rather than caspase-3, is cleaved and functionally active during GC-mediated apoptosis, and that caspase-3 like proteases are involved in DNA fragmentation, but not GC-induced death. Such a discrepancy could be due to differences in the experimental methodology. It is plausible that a caspase-3 dependent pathway could predominate in certain cell types, whereas a caspase-3 independent pathway may predominate in others.

In recent experiments, using a combination of specific caspase inhibitors and stably transfected dominant negative genes, Planey (Planey, 2003) have demonstrated that GC induced pre-B cells apoptosis is caspase-9 and caspase-10(but not caspase-8) dependent and partially caspase-3 dependent.

Based on the number of findings from knockout mice, inhibitors studies, and caspase assays, the prevailing pathways of GC-induced apoptosis appear to be caspase-9 dependent and caspase-3 independent. This pathway involves Apaf-1 mediated activation of caspase-9 followed by activation of caspase-1 and 6. An alternative pathway that is caspase-9 independent and caspase-3 dependent is less well defined. Further investigation are required to establish the precise mechanism leading to caspase activation and to determine which proapoptotic Bcl-2 family members are involved in this process.

The mitochondrial cell death pathway appears to be engaged in GCs-induced thymocyte apoptosis; however, the exact sequence of the events and other essence in GCs-mediated apoptosis remains to be clarified.

1.5.4 Other proteins released from mitochondria in glucocorticoid induced apoptosis.

Besides cytochrome c, other proteins including AIF, EndoG and Omi/HtrA2, which are released from mitochondria, may also participate in GC induced apoptosis.

AIF

The mammalian mitochondrial protein AIF (C. Cande, 2002) has been identified as a flavoprotein, which shares similarity with bacterial, plant, and fungal oxidoreductases. AIF has an N-terminal mitochondrial localization signal and is confined to the mitochondria under normal conditions. After import into the mitochondrial IMS, the mitochondrial localization signal is cleaved off to generate mature 57 kDa AIF. Mature AIF translocates to the nucleus in response to apoptogenic stimuli, poly(ADP－ribose) polymerase-1 (PARP) activation in response to DNA damage (S.W. Yu,2002). Overexpression of AIF induces peripheral chromatin condensation, dissipation of the mitochondrial transmembrane potential, exposure of phosphatidylserine on the plasma membrane, and high molecular weight (50 kbp) DNA fragmentation (S.A. Susin, 2000). The molecular mechanism as to how AIF exerts its cytotoxic activity is unknown. It has no intrinsic nuclease activity and its oxidoreductase activity is not required for its apoptogenic function (M.D. Miramar, 2001), which is in accordance with the findings in C.elegan s where WAH-1, the AIF ortholog, lacks oxidoreductase activity (Xiaochen Wang, 2002). Moreover, EGL-1, the BH3-only domain protein in C.elegans, induces the release of WAH-1 from mitochondria (Xiaochen Wang, 2002) resembling the tBid-dependent release of AIF in isolated mitochondria (G.van Loo, 2002). Heat-shock protein 70 (Hsp70), a cytoprotective factor, protects cells against the apoptogenic effects of AIF by binding and neutralizing AIF (L. Ravagnan, 2001). It is believed AIF mediated apoptosis is caspase independent (S.A. Susin, 2000). AIF appears to be involved in PCD very early in mammalian development. AIF deficient mice die at a very early stage during embryogenesis, since they lack the formation of the proamniotic cavity normally induced by death of the central core of ectodermal cells (N.Joza, 2001). This cavitation-associated cell death is a caspase-independent process, since it occurs in the presence of the pancaspase inhibitor zVAD.fmk(N. Joza, 2001).Embryonic stem (ES) cells from AIF deficient mice remain sensitive to most conventional apoptotic stimuli, including staurosporin, etoposide, and UV irradiation. While ES cells are less sensitive to cell death after growth factor deprivation (N. Joza, 2001), the role of AIF in GC induced thymocyte and peripheral T cell apoptosis has not been reported by now.

Endonuclease G

Endonuclease G is a non-specific mitochondrial nuclease that is highly conserved in the eukaryotic kingdom. Endonuclease G is encoded by a nuclear gene and was originally thought to be involved in the replication of the mitochondrial genome. Later it was shown that endonuclease G resides in the mitochondrial IMS and therefore it is less likely to participate in mitochondrial DNA replication (T.Ohsato, 2002). Its precise role in mitochondrional nucleic acid metabolism remains unclear. During apoptosis in mammalian cells, endonuclease G is released from the mitochondria and translocates to the nucleus. Endonuclease G digests nuclear DNA in the absence of caspase activity or the caspase-activated deoxyribonuclease CAD/DFF (L.Y.Li, 2001.G.van.Loo, 2002). Wang (P. Widlak, 2001) and co-workers showed endonuclease G-dependent internucleosomal DNA-laddering of isolated nuclei. Probably endonuclease G acts in concert with both exonucleases and DNase I in the nucleus to generate DNA cleavage products (P. Widlak, 2001). The finding that endonuclease G is released from mitochondria upon an apoptotic assault and translocates to the nucleus sheds some light on previous findings from genetic knockout studies. DFF40/CAD is sequestered in the cytosol by its chaperone and inhibitor DFF45/ICAD (H. Sakahira, 1998). During apoptosis, DFF45/ICAD is cleaved by caspase-3 allowing delocalization of DFF40/CAD to the nucleus (H. Sakahira, 1998). DFF45/ICAD deficient mice or transgenic mice lacking the ICAD – caspase-3 cleavage site are phenotypically normal but display impaired DNA fragmentation in in vitro cultured thymocytes in response to DNA damaging agents. Surprisingly, in vivo these mutant thymocytes underwent chromosomal DNA degradation when phagocytosed by macrophages, most likely by the lysosomal DNase II from the phagocyte (D. McIlroy, 2000). By generating a CAD deficient cell line it was shown that CAD is dispensable for the early stage chromatin condensation or high molecular weight DNA fragmentation (K. Samejima, 2001). These results indicate that CAD/ICAD is not required during murine development and that internucleosomal DNA cleavage may constitute a terminal stage of nuclear dismantling during apoptosis in the absence of phagocytes.

IAPs

Formation of the apoptosome upon release of cyt c into the cytoplasm does not always suffice to spark the caspase cascade. In insects and vertebrates endogenous inhibitor of apoptosis proteins (IAPs) are present that prevent the activation of procaspases and inhibit the activity of mature caspases (Q.L. Deveraux, 1998). The family of IAP proteins is characterized by the presence of one or more baculovirus IAP repeat (BIR) domains, an approximately 70 amino acid motif conserved from yeast to human. Murine Smac and its human ortholog, DIABLO, are 29 kDa mitochondrial precursor proteins, proteolytically processed in mitochondria to a 23 kDa mature form, and released from the IMS after an apoptotic trigger (G. Van Loo,2002; A.M. Verhagen,2000; C. Du, M,2000). Smac/DIABLO acts as a dimer and contributes to caspase activation by sequestering IAPs. Smac/DIABLO binds to several IAP proteins including XIAP, c-IAP1 and c-IAP2, baculoviral Op-IAP, and survivin, thwarting their caspase-inhibitory functions (A.M. Verhagen, 2000; C. Du, 2000). IAP binding requires the amino terminal four residues of mature Smac/DIABLO (Ala－Val－Pro－Ile) that recognize a hydrophobic groove in the BIR3 domain of XIAP (G.Wu, 2000). A similar recognition motif is present in the linker sequence of caspase-9 small subunit (Ala－Thr－Pro－Phe). Smac/DIABLO is able to compete with caspase-9 for binding to the BIR3 domain of IAPs (G. Wu, 2000, S.M. Srinivasula, 2001). The same amino terminal sequence of Smac/DIABLO binds to the BIR2 motif of XIAP, allowing competition with the XIAP-dependent inhibition of caspase-3 and-7 (J. Chai, 2001). The physiological mitochondrial function of Smac/ DIABLO is unknown and Smac/DIABLO knockout mice are apparently normal. In addition, a splice variant of Smac/DIABLO, Smac-b, which lacks the mitochondrial targeting sequence and the IAP-binding domain, is proapoptotic (D.L.Roberts, 2001). Furthermore, overexpression of a truncated Smac/DIABLO mutant lacking the IAP binding motif (IBM) induces cell death to the same extent as full-length Smac/DIABLO and Smac-b, suggesting an alternative, unknown cytotoxic mechanism of Smac/DIABLO not depending on its IBM (D.L. Roberts, 2001). In Drosophila DIAP-1 and -2 bind the proform of DRONC to block its activation and therefore play a more profound role in controlling cell death (P. Meier, 2000). The proapoptotic proteins Hid,

Grim, Reaper, and Sickle bind to and antagonize DIAP-1 and -2, thereby promoting apoptosis (L. Goyal, 2000; J.P. Wing, 2002; A. Christich, 2002). Although Smac/DIABLO does not resemble Hid, Grim or Reaper, the four amino terminal residues of Smac/DIABLO and caspase-9 that interact with the BIR3 domain of XIAP share significant homology (S.M. Srinivasula, 2001; J.W. Wu, 2001). The resemblance of the IAP interacting motif in a different protein context is an example of convergent evolution in the regulation of caspase inhibitors.

Omi/HtrA2

The mammalian serine protease Omi, also known as HtrA2 (C.W. Gray, 2000; L. Faccio, 2000), was identified as a 49 kDa serine protease, homologous to the bacterial endoprotease HtrA (high-temperature requirement). Phylogenetic analysis of the HtrA2 family supports the mitochondrial endosymbiosis theory and a mitochondrial origin of the eukaryotic HtrA-like proteases (J.C. Ameisen, 2002). The Escherichia coli DegP/HtrA endoprotease is necessary for bacterial oxidative, thermo-, and osmotic tolerance. Bacterial HtrA functions as a protease at high temperature and as a chaperone at normal temperature (C. Spiess, 1999). Whether Omi/HtrA2 exerts a similar function in mammals is unclear although it does seem to be upregulated in conditions of cellular stress such as ischemia-reperfusion, heat shock, and endoplasmic reticulum stress (C.W. Gray, 2000; L. Faccio, 2000). The proapoptotic feature of Omi/HtrA2 was first identified through its ability to bind and antagonize IAPs (Y. Suzuki, 2001; A.M, 2001; Verhagen, 2002). Unlike Smac/DIABLO however, Omi/HtrA2 does not interact with survivin. Although reported as being associated with the endoplasmic reticulum (L. Faccio, 2000) or the nucleus (P. Costantini, 2000) Omi/HtrA2is localized in the mitochondria (Y. Suzuki, 2001). Omi/Htra2 is synthesized as a 49 kDa precursor carrying an amino terminal mitochondrial localization signal (MLS). Upon mitochondrial transport an amino terminal presequence is cleaved off generating the mature 37 kDa protein. Overexpression of Omi in E.coli indicated that this maturation is dependent on its catalytic activity but whether processing during mitochondrial translocation is also due to autocatalysis is not clear (J.W. Savopoulos, 2000). Omi/HtrA2 is released from mitochondria to the cytoplasm during apoptosis where it contributes both to caspase-

dependent and caspase independent PCD (Y. Suzuki, 2001; A.M. Verhagen, 2002, G. van Loo, 2002). In the cytoplasm, Omi/ HtrA2 inter acts with cytosolic IAP proteins via its IBM similar to Smac/DIABLO (Y. Suzuki, A.M.2001; Verhagen, 2002). This feature of OMI/ Htra2 explains its caspase-dependent proapoptotic mode of action. Analogous to the N-terminus of the small subunit of mature caspase-9, mature Smac/DIABLO, and the Drosophila proteins Hid, Grim, Reaper, and Sickle, the free amino terminus of mature Omi/ HtrA2ex poses the conserved IBM AVPA and AVPS, respectively, in mouse and human (R. Hegde,2002). Using the mature Omi/HtrA2 amino acid sequence to BLAST public genomic databases, the following organisms were found to contain putative orthologs of Omi/HtrA2: the bacteria E.coli (Htra, 34% identity), the plant Arabidopsis thaliana (43% identity), the fruit fly D.mel anogaster (51% identity), the malaria mosquito Anopheles gambiae (55% identity), and the pufferfish Fugu ru-Bripes (70% identity). Surprisingly, this approach did not reveal clear Omi/HtrA2 orthologs in C.elegans or Saccharomyces cerevisiae. Nevertheless, the presence of Omi/HtrA2 likes in the organisms mentioned above contrasts sharply with Smac/DIABLO, which apparently is confined to vertebrates (J.C. Ameisen, 2002). Omi/HtrA2 and Smac/DIABLO are redundant in many features: both are mitochondrial proteins, both are released from the IMS of mitochondria, and both interact with cytosolic IAPs via a similar IBM. What could be the functional difference between these two proteins? First, Smac/DIABLO and Omi/HtrA2 display a different tissue distribution pattern. Northern blot analysis showed that Smac/DIABLO is most abundant in heart, liver, kidney, and testis with little or no expression detected in skeletal muscle, lung, thymus, and the brain (A.M. Verhagen,2000; C. Du,2000). Omi/HtrA2, however, is expressed ubiquitously (C.W. Gray, 2000; L. Faccio, 2000). Furthermore, Omi/HtrA2 has a carboxyterminal PDZ domain (L. Faccio, 2000), a motif generally involved in protein‐protein interactions, and an amino terminal serine protease catalytic domain (Y. Suzuki, A.M.2001). Cytoplasmic overexpression of mature Omi/HtrA2 induces cell death independent of caspase activation or IAP interaction but solely relying on the catalytic activity of Omi/HtrA2 (Y. Suzuki, A.M.2001; Verhagen, 2002). The crystal structure of Omi/HtrA2 revealed a trimeric structure with the PDZ domains oriented inwards and covering the catalytic center of the protease (W. Li, 2002). Mutational studies demonstrated that the PDZ domain tempers the proteolytic and

cytotoxic potential of Omi/HtrA2 (W. Li, 2002). Thus, unlike Smac/DIABLO, we exclude for a while the Smac-b splice form discussed above (D.L. Roberts, 2001), Omi/HtrA2 seems to have a dual function: one as an inhibitor of XIAP, propagating caspase cascades, and one as a serine protease, propagating atypical caspase-independent cell death. In this respect the PDZ domain may provide a targeting signal for Omi/HtrA2 once released from the mitochondria.

All in all, several proteins are released from mitochondria after apoptotic stimuli besides cytochrome c. Whether these factors are involved in GC induced thymocyte and peripheral T cell apoptosis awaits to be discovered.

1.5.5 Cathepsins may also participate in glucocorticoid induced apoptosis

Participation of lysosome in apoptosis is now well established. Formerly, cathepsins were thought to lead to cellular autolysis and damage of neighboring cells during necrosis. Accordingly, lysosomes were regarded as 'suicide bags' that would release unspecific digestive enzymes after rupturing during uncontrolled cellular stress. Recent in vitro (Deiss LP, 1996; Roberts LR, 1999; Vancompernolle K, 1998) as well as in vivo (Tetsumori, 1998) data suggest, however, that cathepsins may also act as mediators of programmed cell death. It was shown that cytosolic leakage of lysosomal enzymes following various apoptotic stimuli can initiate cell death through caspase activation (Cuervo, 2004). Moreover, activation of cathepsin B in lysosomes occasionally precedes apoptosis (Michallet, 2003). Once released into cytosol, it could activate caspase and it could even digest nuclear substrates directly (Vancompernolle, 1998; Foghsgaard, 2001; Schotte P, 1999). Although the relevance of lysosomal processes for programmed cell death in general is undoubted, it has not been studied whether it is also involved in GCs-induced cell death.

1.6 The aim of this study

A major problem of the current discussion arises from the fact that different cell types, such as thymocytes, peripheral T cells and lymphoma cells are compared without acknowledging their different characteristics and gene expression profiles. Although it is generally assumed that GCs induce apoptosis via a conserved mechanism, this is not supported by any data. In other words, it is possible that thymocytes, peripheral T cells and lymphoma cells undergo GC-induced apoptosis along different pathways. If this is true, then it will be very helpful for the clinical treatment of hematological malignancies. We therefore wondered whether a unique signal transduction pathway is engaged by GC to initiate and execute cell death in all types of T lymphocytes or whether distinct pathways exist. So, we compared the role of the proteasome, caspase, lysosome, Ca^{2+} and other factors in GC induced mouse thymocytes and peripheral T cell apoptosis.

Our data show that the initiation phase of GC-induced apoptosis is similar irrespective of the differentiation state of the cell. Apoptosis in both cell types is mediated by GR and is gene transcription dependent. In contrast, the execution of cell death in thymocyte and peripheral T cells differs significantly in the requirement for signal transduction components such as the proteasome, caspases and cathepsins. While in thymocytes, the proteasome, caspase and cathepsin B play an important role in apoptosis, these factors are dispensable for apoptosis of peripheral T cells. It is noteworthy that cathepsin B participates in thymocytes apoptosis after GCs treatment and this is the first investigation of the role that cathepsins play in GC induced apoptosis. Glucocorticoids could influence the homeostasis of the thymus and the outcome of thymocyte selection. TALL cell line share a similar apoptosis signaling pathway as thymocytes. In the future this could potentially form a basis for new anti-cancer strategies which specifically target tumor cells whereas leaving peripheral T cells of patients untouched.

2.   Materials

2.1 Animals

2.1.1 Mice

All the mice used in our experiments were C57BL/6 mice (Charles River, Germany). They were bred in our own animal facility and used for the experiments at 6-12 weeks of age.

2.1.2 Rat

Lewis rats were purchased from Charles River and used for experiments at 10-12 weeks of age. For the dexamethasone induced rat thymus atrophy and recovery assay, 4 weeks old rats were used.

All mice and rats were kept in individually ventilated cages under specific pathogen free conditions. All animal experiments were conducted in accordance with accepted standards of animal care and approved by Bavarian state authorities.

2.2 Cell line

Jurkat GR, a human acute T cell leukemia cell line, is a derivative of E6.1 (ATCC, USA). It is transduced with the mouse glucocorticoid receptor linked to eGFP via an IRES element by lentivirus. Jurkat GR was cultured in RPMI medium containing 10% FCS and 5%SSC in 50ml cell culture flask, 37°C, $5\%\,CO_2$. Cells were split every 2-3 days. The splitting ratio is 1:10.

Wehi7.1, a mouse thymoma cell line, was purchased from ATCC and cultured in RPMI1640 with 10% FCS, 5%SSC, in 50ml cell culture flask, 37°C, $5\%\,CO_2$. Cells were split every 2-3 days and the splitting ratio is 1:10.

TALL-1 is a human acute T cell leukemia cell line. It was purchased from DSMZ (Braunschweig, Germany) and cultured in RPMI1640 with 10% FCS, 5%SSC in 50ml cell culture flask, 37 ^{o}C, 5%CO$_2$. Cells were split every 3 days and the splitting ratio is 1:10.

2.3 Antibodies

Antibody for cell stimulation:

JJ316 (rCD28 Ab)   Becton Dickenson, Germany

Antibodies for FACS staining:

AnnexinV-FITC   Becton Dickenson, Germany

AnnexinV-Cy5   Becton Dickenson, Germany

Anti FITC or PE magnetic beads.   Miltenyi Biotech   Germany

Fluorescein active caspase-8 staining Kit   Biovision, USA

OX8-FITC (anti-rat CD8)   Becton Dickenson, Germany

OX38-PE (anti-rat CD4)   Becton Dickenson, Germany

OX35-Cy (anti-rat CD4)   Becton Dickenson, Germany

PE-conjugated active caspase-3 antibody apoptosis Kit   Becton Dickenson, Germany

R73-PE (anti-rat αβTCR)   Becton Dickenson, Germany

TCRβ chain antibody (H57-597)-FITC conjugated   Becton Dickenson, Germany

TCRβ chain antibody (H57-597)-PE conjugated   Becton Dickenson, Germany

Antibodies for Western-Blot:

Rabbit anti mouse GR IgG(1:1000)   Santa Cruz Biotech, Germany

Rabbit anti caspase-3 IgG(1:1000)   Santa Cruz Biotech, Germany

Rabbit anti-Bcl-xl IgG(1:500)   Santa Cruz Biotech, Germany

Rabbit anti-Bcl-2 IgG(1:200)   Santa Cruz Biotech, Germany

Rabbit anti-Bak IgG(1:1000)   Santa Cruz Biotech, Germany

Rabbit anti-Bax IgG(1:1000)   Santa Cruz Biotech, Germany

Mouse anti-β tublin polyclonal IgG (1:2000)   Sigma Aldrich GmbH, Germany

Mouse anti- β actin polyclonal IgG (1:5000)	**Becton Dickenson, Germany**
Rabbit anti mouse p56-Lck IgG(1:10000)	**Sigma Aldrich GmbH, Germany**
Goat anti rabbit IgG-HRP conjugated(1:5000)	**Santa Cruz Biotech, Germany**
Goat anti mouse IgG-HRP-conjugated (1:5000)	**Sigma Aldrich GmbH, Germany**

2.4 Chemicals

APS	**AppliChem GmbH, Germany**
BSA	**Carl Roth chemical, Germany**
CaCl$_2$	**AppliChem GmbH, Germany**
C$_2$H$_3$NaO$_2$	**MERCK, Germany**
C$_6$H$_5$Na$_3$O$_7$	**MERCK, Germany**
CaCl$_2$	**AppliChem GmbH, Germany**
Caspase inhibitors	**RnD Systems,Germany**
Cathepsin B inhibitor	**Metachem, UK**
Cycloheximide	**Sigma Aldrich GmbH, Germany**
Cyclosporine A	**Calbiochem, Germany**
Dexamethasone	**Sigma Aldrich GmbH, Germany**
Digitonin	**Sigma Aldrich GmbH, Germany**
DMSO	**Carl Roth chemical, Germany**
DTT	**Carl Roth chemical, Germany**
EDTA	**AppliChem GmbH, Germany**
Ethnoal	**AppliChem GmbH, Germany**
Glycerol	**Carl Roth chemical, Germany**
Glycin	**AppliChem GmbH, Germany**
KCl	**MERCK, Germany**
KH$_2$PO$_4$	**MERCK, Germany**
HCL	**AppliChem GmbH, Germany**
Hepes	**AppliChem GmbH, Germany**
Lactacystin	**Calbiochem, Germany**
LY294002	**Calbiochem, Germany**
Methnoal	**AppliChem GmbH, Germany**

MgCl$_2$	AppliChem GmbH, Germany
NaCl	AppliChem GmbH, Germany
Na$_2$CO$_3$	AppliChem GmbH, Germany
NaHCO$_3$	AppliChem GmbH, Germany
Na$_2$HPO$_4$	AppliChem GmbH, Germany
NaN$_3$	AppliChem GmbH, Germany
NH$_4$CL	AppliChem GmbH, Germany
NP-40	AppliChem GmbH,Germany
Pan-cathepsin inhibitor	Calbiochem, Germany
PD150606	Calbiochem, Germany
Poly-L-Lysin	Sigma Aldrich GmbH, Germany
Propidium Iodide	Sigma Aldrich GmbH, Germany
RU486	Sigma Aldrich GmbH, Germany
SDS	AppliChem GmbH, Germany
Sucrose	AppliChem GmbH, Germany
TEMED	Carl Roth chemical, Germany
Tris	AppliChem GmbH, Germany
Tween-20	AppliChem GmbH, Germany
Typan blue	Sigma Aldrich GmbH, Germany

2.5 Reagents

30% Acrylamide stock   Carl Roth chemical, Germany

Carbonate Buffer (pH9.6):

1.59 g Na$_2$CO$_3$

2.93 g NaHCO$_3$

Dissolve in 900 ml distilled H$_2$O.

Check pH and adjust to 9.6

Add water to 1 liter

FACS buffer:

1% BSA

0.05% NaN$_3$.

Solved in PBS

10 X Annexin V binding buffer:

0.1 M HEPES

1.4 M NaCl

25 mM CaCl$_2$

Adjust to pH 7.4

Dilute to 1X prior to use

Cell lysis and mitochondria intact (CLAMI) buffer:

250 mM sucrose

70 mM KCl

50 µg/ml digitonin

Solved in PBS

Universal lysis buffer:

140µM NaCl

20µM Tris

2µM EDTA

1% NP-40

pH7.5

Add protease inhibitor cocktail before use.

Protease inhibitor cocktail:

Pefabloc (1µg/ml),   Roche, Germany.

Leupeptin (10µg/ml), Roche, Germany.

Jodoacetamid (1:100), Roche, Germany.

Protein quantification reagent:

Bradford Protein Assay Reagents (BIO-RAD, USA)

Tris-glycin-SDS protein electrophoresis buffer:

25mmol/L Tris,

250mmol/L Glycin,

0.1%SDS,

pH8.3

6×SDS Protein electrophoresis loading buffer:

300mmol/L Tris.CL(pH 6.8)

600mmol/L DTT

12% SDS

0.6% Trypanblau

60% glycerol

Protein transfer buffer:

39mmol/L Glycin

48mmol/L Tris

0.037% SDS

20% methnoal

0.1% Phosphate Buffered Saline Tween-20:

Dissolve the following in 800 ml of distilled H$_2$O:

8g of NaCl

0.2g of KCl

1.44g of Na$_2$HPO$_4$

0.24g of KH$_2$PO$_4$

2ml of tween-20

Adjust pH to 7.2 with HCl.

Adjust volume to 1L with additional distilled H$_2$O.

Sterilize by autoclaving.

Add Tween-20 to 0.1％(V/V).

Balanced salt solution:

NaCl   0.64％,

KCl   0.075％,

CaCl$_2$ 0.048％,

MgCl$_2$ 0.03％,

C$_2$H$_3$NaO$_2$ 0.39％,

C$_6$H$_5$Na$_3$O$_7$ 0.17％,

Adjust pH to 7.2 with 1M HCL

HRP substrate for western -blot:	**ECL (Amersham, Freiberg, Germany)**

Cell culture medium:

RPMI1640 plus Glutamine	**Gibco, USA**
Fetal calf serum (FCS)	**Invitrogen, Karlsruhe, Germany**
Standard serum complement (SSC)	**Invitrogen, Karlsruhe, Germany**

2.6 Consumables:

12 well flat bottom culture plate for suspension cells	**Greiner-bio-one,Germany**
24 well flat bottom culture plate for suspension cells	**Greiner-bio-one,Germany**
48 well flat bottom culture plate for suspension cells	**Greiner-bio-one,Germany**
96 well flat bottom culture plate for suspension cells	**Greiner-bio-one,Germany**
3cm tissue culture dish	**Greiner-bio-one,Germany**
50ml cell culture flask	**Greiner-bio-one,Germany**
1.5ml Eppendorf centrifuge tube	**Eppendorf GMBH**
15ml centrifuge tube	**Greiner-bio-one,Germany**
50ml centrifuge tube	**Greiner-bio-one,Germany**
MACS separation LS column	**Miltenyi, Germany**
Cuvette	**Bio-rad USA**
10µl tips	**Molecular Bioproducts,USA**
200µl yellow tips	**Roth,Germany**

1000μl blue tips	**Roth,Germany**
40μM nylon cell strainer	**BD Biosciences, USA**
Syringe: 2ml and 5ml single-use syringe	**B/BRAUN Melsungen AG, Germany**
96 well flat bottom microplate	**Nunc, Denmark**
Absorbant paper for Western-Blot	**Whatman, USA**
PVDF membrane for Western-Blot	**Bio-rad USA**
Type 200L scrubbed nylon wool	**PerkinElmer Life science USA**

2.7 Apparatus

Magnetic station	**Miltenyi, Germany**
Facs calibur	**Becton Dickenson, USA**
Power supply (for protein electrophoresis and transmembrane)	**Consort E865**
UV/Visible Spectrophotometer: Ultrospe 2000	**Pharmacia Biotech,USA**
Transmembrane apparatus	**Hofer Scientific Instrument,USA**
Eppendorf centrifuge 5417R and 5804R	**Eppendorf AG,Hanmburg**
Microcentrifuge	**Roth, Germany**
Cell incubator	**Heraeus, Germany**
Heating block	**VWR, Germany**
Water bath	**Lauda, Germany**

3. Methods

3.1 Cell isolation and culture

3.1.1 Mouse thymocyte isolation

Mouse was killed by neck dislocation. Sterilize the skin of mouse chest and abdomen with 70% ethanol. Open the chest and abdomen with scissor and forceps very carefully to ensure not to damage the main blood vessel. Isolate thymus and put it in PBS in a 3cm culture dish. Keep the dish on ice before use. Put the thymus in a 40μM nylon cell strainer and press it with a 5ml syringe. Wash the cell strainer with cold PBS until all the cells have been washed out. Collect the effluent. Centrifuge at $460 \times g$, 5 minutes, pour out the supernatant and resuspend thymocytes in cold PBS.

3.1.2 Mouse spleen T cell isolation

Mouse was killed by neck dislocation. Sterilize the skin of mouse chest and abdomen with 70% ethanol. Open the abdomen with scissor and forceps very carefully to ensure not to damage the main blood vessel. Isolate spleen and put it in PBS in a 3cm culture dish. Keep the dish on ice before use. Put the spleen in a 40μM nylon cell strainer and press it with a 5ml syringe. Wash the cell strainer with cold PBS until all the cells have been washed out. Collect the effluent. Resuspend the cell pellet with 5ml Tris-NH_4Cl in a 50ml Falcon tube for 3 minutes at RT in order to delete the erythrocytes. After the treatment of Tris-NH_4Cl, most of the erythrocytes will be lysed while the spleen T cells are still intact. Add cold PBS up to 50ml and centrifuge, $460 \times g$, 5 minutes. Pour out the supernatant and repeat washing with 50ml cold PBS 2 times. Next, resuspend the cell pellet in 1ml BSS-BSA and stain the cell with FITC-conjugated H57-597 antibody (1:500) for 20 minutes at 4 $^{\circ}$C in a 5ml FACS tube. Wash the cell 3 times with BSS-BSA, 5 minutes. Resuspend the cell pellet in 0.45ml BSS-BSA and stain the cell with 50μl anti-FITC magnetic beads, vortex gently and keep it at 4 $^{\circ}$C for 20 minutes. Rinse the cell 3 times with BSS-BSA, $460 \times g$, 5 minutes. Resuspend the cell pellet in 1ml BSS-BSA and keep it on ice. Position a MACS LS column in a magnetic field and wash the column with

5ml BSS-BSA. Load the cell suspension on the MACS LS column drop by drop. Care should be taken to avoid the air bubble form. Rinse the LS column with 15ml BSS-BSA in the magnetic field. Remove the LS column from the magnetic field and wash it with 10ml BSS-BSA, and collect the elusion in a 15ml Falcon tube.

After counting, thymocytes and peripheral T cells were resuspended in RPMI medium containing 10% FCS and 5% SSC at a concentration of 1×10^6 cells/ml and cultured in 96 or 48 well plates at 37°C in an incubator with 5%CO_2.

3.1.3 The isolation of rat lymph node T cell with nylon wool column

The preparation of the nylon wool column

Weigh 1.2 gram nylon wool. Separate the nylon wool clusters into small segments and put them into a 20ml syringe. Press the nylon wool tightly to form a column. A rubber tube with an adjustable clamp was connected to the end of the syringe. After sterilization, wash the nylon wool column with 2.5 ml 37°C BSS one time and repeat washing with 37°C BSS-FCS 5% one time. Turn off the valve below the nylon wool column and add another 2.5ml 37°C BSS-FCS 5%. Keep it at 37°C and wash it with 2.5 ml 37°C BSS-FCS 5% before loading the lymph node cell suspension.

Rat lymph node T cell isolation

10-12weeks old Lewis rat was killed in an airproof box by dry ice. Sterilize the rat skin with 70% ethanol. Open the abdomen and axilla with scissor and forceps. Isolate the axillary and mesengial lymph nodes, and keep them in cold PBS before use. Rat lymph nodes were rubbed in a 40μM nylon cell strainer. Wash the strainer with cold PBS and collect the effluent in a 50ml Falcon tube. Centrifuge at 460 ×g for 5 minutes and pour out the supernatant. Resuspend the cells in 10ml TAC to lyse the erythrocytes. After 5 minutes, add PBS to the Falcon tube and wash the cell. Repeat rising two times. Resuspend cell in 1.5 ml 37°C BSS-FCS 5% and load it on the nylon wool column drop by drop. Add another 2.5 ml BSS-FCS 5% on the nylon wool column and keep it at 37°C for 40 minutes. Then, wash the nylon wool column with 10 ml 37°C BSS-FCS 5% and collect the effluent, the flowing rate should be controlled at 1 drop per 3 seconds.

3.2 Cell culture plate coating

The first step is to prepare the coating solution. Sheep anti mouse IgG was diluted with carbonate buffer (1:387.5). Then, add 500μl each well coating solution into each well and keep the plate in 37 $^{\circ}$C room for 1 hour. Next, remove the coating solution with 1ml pipette and wash each well with BSS 3times. After washing, add 1ml BSS in each well and keep the culture plate at 4 $^{\circ}$C before use.

3.3 FACS analysis

3.3.1 Apoptosis assay

Thymocyte or peripheral T cells were induced apoptosis by dexamethasone under different conditions. Cell survival after Dex treatment was determined by Annexin V-PI staining. In short, cells were collected and washed with FACS buffer one time, 460 × g, 4 $^{\circ}$C, 5 minutes. 1-5×10^5 cells were resuspended in 100μl Annexin V binding buffer with Annexin V antibody (1:100) and PI (5μg/ml) for 15 minutes in darkness at RT. Then, add another 100μl Annexin V binding buffer into each FACS tube and measure it with FACS Calibur. Annexin V-FITC fluorescence was detected in FL-1, and propidium iodide was detected in FL-2. Annexin V-Cy5 fluorescence was detected in FL-7. Usually 10^4 cells are counted. Data were analyzed by *cellquest.* Annexin V positive cells indicate dead cells.

3.3.2 The purity analysis of isolated peripheral T cells

Check the purity of isolated splentic T cell by flow cytometry (staining). Transfer 1×10^5 cell into a 5 ml FACS tube, and wash the cells with FACS buffer 1 time, 460 × g, 5 minutes. Discard the supernatant and resuspend the cell in 200μl FACS buffer. Stain the cells with anti-mTCR β chain-PE H57-597 antibody (1:1000) for 20 minutes, 4 $^{\circ}$C, in darkness. Wash the cell 1 time, 460 × g, 5 minutes. Resuspend the cells in 300μl FACS buffer and do FACS analysis with FACS Calibur. βTCR-PE fluorescence was detected at FL-2. More than 1×10^4 cells were acquired. Data were analyzed by *cellquest*. Usually the spleen T cell purity is between 80％---90％.

The purity of isolated lymph node T cell was determined by flowcytometry. 1×10^5 cells were transfered to a 5ml FACS tube and washed with FACS buffer 1 time, $460 \times g$, 5 minutes. Discard the supernatant and resuspend the cell in 200μl FACS buffer. Stain the cell with OX8-FITC and OX38-PE antibody for 20 minute in darkness, 4 ^{o}C. Wash the cell with FACS buffer 1 time, $460 \times g$, 5 minutes. Measure the purity with FACS calibur. OX8-FITC fluorescence was detected in FL-1 and OX38-PE fluorescence was detected in FL-2. At least 1×10^4 cells were acquired. Data were analyzed by *cellquest.* Usually, the purity is more than 95%.

3.4 Staining for cleaved caspase-3

Wash cells twice with cold PBS, $460 \times g$, 5 minutes, 4 ^{o}C. Then count and resuspend cells in Cytofix/Cytoperm™ solution (included in kit) at a concentration of 1×10^6 cells/0.5 ml in a 5ml FACS tube. Incubate cells for 20 minutes on ice. Pellet cells, aspirate, and discard Cytofix/Cytoperm™ solution; Wash twice with Perm/Wash™ Buffer (included in kit) at a volume of 0.5 ml buffer/1×10^6 cells at room temperature. Pour out the supernatant and add 100 μl Perm/Wash™ Buffer and 20 μl PE-conjugated monoclonal rabbit anti- active caspase-3 antibody (included in kit) to each sample. Vortex gently and incubate for 30 minutes at room temperature. Wash each test in 1.0 ml Perm/Wash™ Buffer, then resuspend the test in 0.5 ml Perm/Wash™ Buffer and analyze by flow cytometry at FL-2. At least 10^4 cells are counted. Data were analyzed by *cellquest.*

3.5 Staining for cleaved caspase-8

Aliquot 300μl each of the cultures into a 5ml FACS tube. Then, add 1μl of FITC-IETD-FMK (included in the kit) into each tube. Vortex gently and incubate for 30 minutes at 37 ^{o}C with 5% CO_2. Centrifuge cells for 5 minutes and remove supernatant. Resuspend cells in 0.5 ml wash buffer (included in the kit) and centrifuge again. Repeat washing step. Resuspend cells in 300μl wash buffer and analyze by flow cytometry at FL-1. At least 10^4 cells are counted and data were analyzed by *cellquest.*

3.6 Protein extraction

For whole cell protein extraction, cells were collected and washed with cold PBS 2 times by centrifugation, 460 × g, 5 minutes. Next, cell pellets were resuspended in ice cold universal lysis buffer and kept on ice for 1 h. After centrifugation at 10000 × g for 10 minutes at 4°C, the supernatant were collected and stored at -20 ^{o}C.

For subcellular protein extraction, 1×10^7 thymocytes or peripheral T cells were centrifuged at 1000 × g for 5 minutes at 4 ^{o}C. Then, cells were resuspended in cell lysis and mitochondria intact (CLAMI) buffer and kept on ice for 5 minutes. Aliquots from the suspensions were stained with 0.1% Trypan blue in PBS to ensure that >95% of the cells were lysed. The cells were pelleted at 1000 × g for 5 minutes at 4 ^{o}C and supernatant containing cytosolic proteins was stored at -20 ^{o}C. The pellets were incubated at 4°C for 60 minutes in Universal lysis buffer and centrifuged at 10000 × g for 10 minutes at 4°C, the supernatant containing mitochondrial protein was stored at -20 ^{o}C.

3.7 Protein quantification assay

This assay is used to quantify the protein concentration. Add 800μl distilled water into 1.5ml eppendorf centrifuge tube. Add 1-2 μl protein sample and 200μl Bradford reagents into each EP tube and vortex.   Make the protein standard curve at the same time according to Table 1. Keep them in darkness at room temperature for 20 minutes. Transfer the mixture to disposable cuvettes and measure the optical density at 595nm. Make optical density and protein concentration standard curve and calculate the protein concentration of the samples according to the equation.

Table 1

BSA(μl) 1mg/ml	1	2	5	10	20
Water(μl)	799	798	795	790	780
Bradford Reagent (μl)	200	200	200	200	200

3.8 Western blot

SDS-PAGE gel preparation

Assembling gel apparatus: Assemble two glass plates (one notched) with two side spacers, clamps. Stand assembly upright using clamps as supports. Pour some water onto glass plate to check whether the gel apparatus will leak or not. If leaked, assemble the gel apparatus again.

To make resolving Gels:

Gel concentration of 7.5% or 15% in 0.25 M Tris-HCl pH 8.8.

Reagents:	Volume (ml) to make 10ml 7.5% gel	Volume (ml) to make 10ml 15% gel
Water (distilled)	4.8	2.3
30% Acrylamide stock	2.5	5.0
1.5 M Tris-HCl pH 8.8	2.5	2.5
10% SDS	0.1	0.1
10% APS	0.1	0.1
TEMED (added last)	0.01	0.004

Gel was prepared in a 15ml Falcon tube. After adding TEMED, vortex the mixture and add it onto the cassette very carefully. Next, add 2 ml distilled water onto the gel to avoid oxidization. Keep it at RT 30 minutes.

To make stacking gel:

Gel concentration of 4.5% in 0.125 M Tris-HCl pH 6.8

Reagents:	Volume to make 5ml gel(ml)
Water (distilled)	3.4
30% Acrylamide stock	0.83
1 M Tris-HCl pH 6.8	0.63

10% SDS	0.05
10% APS	0.05
TEMED (added last)	0.005

Before adding TEMED to the stacking gel, wash the resolving gel with 5ml distilled water to delete the unpolymerized acrylamide. Then, add TEMED to the stacking gel, vortex and load it onto the resolving gel. Insert comb and mark the loading wells. Keep it at RT for 30 minutes.

Protein sample preparation

Take 50μg protein sample and mixed with 6× protein electrophoresis loading buffer in a 1.5 ml EP tube. The final concentration of the loading buffer was 1×. Adjust the volume of different group by adding distilled water in order to get the same final volume. Heat the protein sample to 100 ^{o}C for 3 minutes with a heating block. Centrifuge the protein samples with a microcentrifuge 3 seconds and wait to be loaded.

Protein electrophoresis

After the stacking gel has polymerized completely, take off the comb and fix the gel cassette in a protein electrophoresis chamber. Fill TGS in the chamber until both the two ends of the gel are covered by TGS. Next, wash each loading well with TGS to delete the unpolymerized acrylamide. Load the protein maker (10μl) and all the protein samples very carefully to ensure no mutual contamination. Connect to the power supply and start electrophoresis. The starting electronic field is 80V and 25 mA. When the protein samples move to the boundary between the resolving gel and the stacking gel, increase the current to 35mA. Stop electrophoresis until the proteins sample move to the end of the resolving gel.

Protein transmembrane

Remove stacking gel and sides of running gel beyond sample wells with a razor blade. After measuring the size of the gel, put the gel into protein transfer buffer. Next, prepare PVDF membrane and Whatman filter paper. They should have the same size as the gel. Put the PVDF membrane in methanol for 3 seconds and equilibrate membrane in protein transfer buffer for 5 minutes. Wet 3 pieces of filter paper in protein transfer buffer and place them on anode plate of blotter. Avoid trapping air between electrode and filter paper by laying filter paper on electrodes at an oblique angle. Place the PVDF membrane on top of filter papers previously placed on electrode. Make sure that the PVDF membrane and the filter paper are aligned on each side. Next place gel on top of PVDF membrane taking care not to trap air bubbles between gel and membrane. Wet 2 pieces of filter paper in protein transfer buffer and place them on top of gel. Use a clean plastic test tube to roll out air bubbles. Place cathode plate of blotter on top of transfer stack. Connect high voltage cords to power supply. Apply a constant current of 2.5 mA per cm^2 of gel area for 60 minutes.

After transfer is complete, turn off power supply and remove cathode plate of blotter. Remove transfer membrane and cut lower right corner of membrane to mark orientation of gel. Stain the PVDF membrane with porceau S for 3 minutes and wash it with distilled water. Observe the protein bands on the membrane and position the protein marker.

Blocking the PVDF membrane

Block the PVDF membrane with 0.1%PBS-T including 5% non-fat milk powder at RT for 2 hours. Wash the membrane with 0.1%PBS-T for 20 minutes and change the 0.1%PBS-T 2 times.

Staining the PVDF membrane with primary antibody

Prepare the first antibody. The primary antibody was diluted with 0.1%PBS-T including 5% non-fat milk powder. Incubate the PVDF membrane with primary antibody overnight at 4 ^{o}C.

Staining the PVDF membrane with HRP-conjugated second antibody

Prepare the HRP-conjugated second antibody. The second antibody was diluted with 0.1%PBS-T including 5% non-fat milk powder. Washing the membrane with 0.1%PBS-T for 30 minutes and change the 0.1%PBS-T 2 times during the courser of washing. Incubate the membrane with the second antibody for 1-2 hours at room temperature. Then, wash the membrane with 0.1%PBS-T for 30 minutes and change the 0.1%PBS-T 2 times when washing.

Detection the target protein with ECL

Prepare ECL solution by mixing equal parts of Detection Reagents 1 and 2. Add the mixed substrate solution on the membrane. Use 0.125 ml Working Solution per cm2 of membrane. Cover the membrane with a piece of cling film and wipe out the redundant substrate solution with absorbent paper. Next, put the membrane in the X-ray film cassette and cover it with a piece X-ray film.   Wait, from 30 seconds to 30 minutes. Take out the X-ray film and put it into developer solution for 20 seconds. Wash it with fresh water 10 seconds and put it into fixative for 20 seconds. Wash it with fresh water for 10 seconds.   All the steps mentioned above were done in dark room.

3.9 The experimental design of Dex induced rat thymus atrophy and recovery

4weeks old Lewis rats were used in this experiment. Rats were injected Dex introperitoneally 500μg/100g body weight. After injection, rats were sacrificed in a sealed plastic box filled with CO_2 at different time points. Thymus was isolated and total number of thymocytes was counted. The percentage of different thymocyte population

was determined by flowcytometry. In short, thymocytes were stained with the following antibodies:

OX8-FITC, to stain CD8

OX35-Cy,   to stain CD4

R73PE,   to stain αβTCR

Annexin V-Cy5   to stain the exposed phosphatidylserine (indicating dead cells).

Experiment group	Control	24 hours	3 days	1 week	2 weeks	3 weeks
Rat number	1	1	2	3	1	2

3.10 Statistical Analysis

All data were analyzed using the program "Statistica". Student's T test was used when two experimental groups were compared. For comparison of several groups a one-way ANOVA followed by a post-hoc Tukey HSD test was performed. The statistical significance of selected comparisons is depicted in the figures. * equals $p<0.05$, ** equals $p<0.01$, *** equals $p<0.001$. 'n.s' means there is no statistical significance.

4.   Results

4.1 GR is indispensable for GC-induced mouse thymocytes and peripheral T cells apoptosis

First, we want to prove that both GC-induced thymocyte and peripheral T cell apoptosis were mediated by GR. In the past, our group compared GC-induced apoptosis of thymocytes and peripheral T cells from wild type C57/BL6 mice and heterozygous GRN$^{+/-}$ mice whose GR expression level is significantly reduced. Thymocytes and peripheral T cells were treated with a series concentration of dexamethasone and survival rate was monitored after 20hours' culture by flow cytometry. The dose–response curve of Dex (Dexamethasone) induced apoptosis in GRN$^{+/-}$ mice was shifted to higher Dex concentration obviously in both cell types indicating that GR mediated GC-induced mouse thymocyte and peripheral T cell apoptosis. To further confirm these results, we used the pharmacological inhibitor RU486 which blocks GR by competitive binding to the ligand-binding domain (Jung-Testas, 1983) to repeat the apoptosis induction assay. As expected, RU486 prevented Dex-induced apoptosis in both thymocytes and peripheral T cells (Figure 2) which was consistent with the previous experiments. From these two experiments, we conclude that GCs induce thymocytes and peripheral T cells apoptosis through GR.
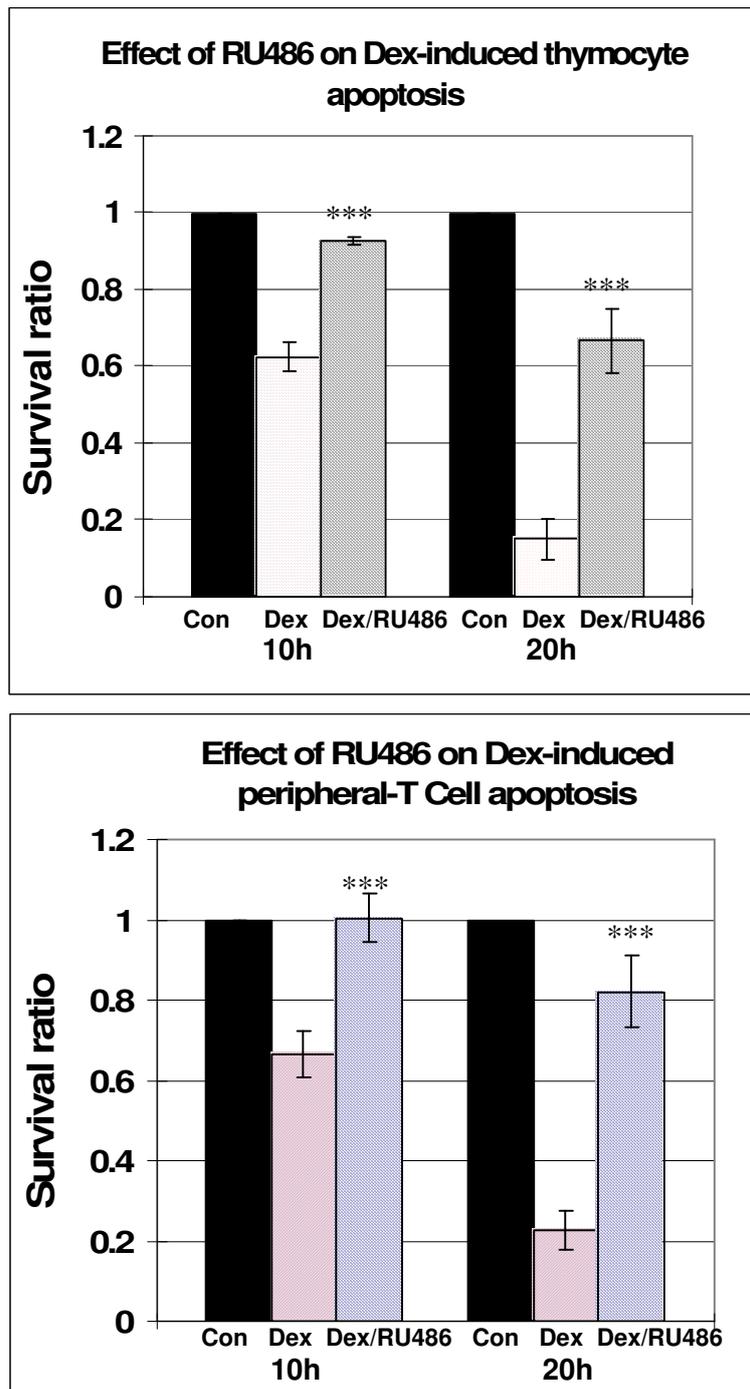
Figure2. RU486 blocks GC-induced thymocyte and peripheral T cell apoptosis

Thymocyte and peripheral T cells from C57/BL6 wild type mice were treated with Dex (1×10^{-7}M) in the presence or absence of theGR antagonist RU486(1×10^{-6}M) for 20hours. The survival rate was determined by Annexin V-PI staining at the 10 and 20 hours time points. *Student's T* test was used for statistical analysis. The error bars represent the SEM for three independent experiments with three replicates each.

GR regulates gene expression and it was reported both transactivation and transrepression were involved in GC-induced cell apoptosis. To test for the requirement of transactivation, we tested the effect of the protein synthesis inhibitor cycloheximide on Dex-induced thymocyte and peripheral T cells apoptosis. In the presence of cycloheximide, Dex-induced apoptosis in thymocytes and peripheral T cells were completely prevented within the first 10 hours after Dex treatment (Figure 3). So, gene expression was absolutely necessary for GC-induced thymocytes and peripheral T cells apoptosis although we can't exclude the possibility that transrepression regulated by GR was also necessary. Taken together, these results suggest that initiation of GC-induced apoptosis depends on GR-induced gene expression irrespective of the differentiation stage of the T cell.
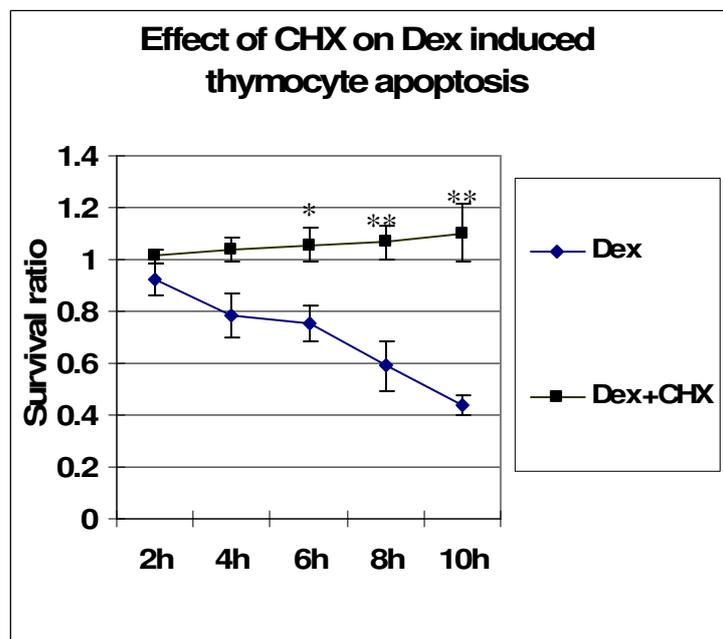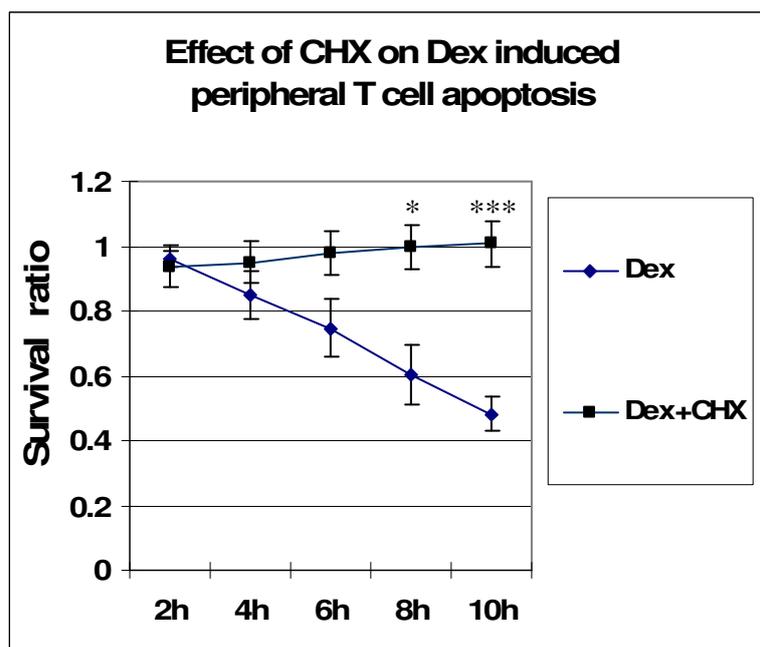
Figure3. GC induced mouse thymocyte and spleen T cell apoptosis require gene transcription.

Thymocytes and peripheral T cells from C57/BL6 wild type mice were treated with Dex(1×10^{-7}M) in the presence or absence of the protein synthesis inhibitor cycloheximide(10μM) for 10hours. Survival rate was determined by Annexin V-PI staining every 2 hours. *Student's T* test was used for statistical analysis. The error bar is the SEM for three independent experiments with three replicates each.

4.2 Caspase activity is involved in GC-induced thymocyte but not peripheral T cell apoptosis

As mentioned above caspase activity plays an important role in the execution phase of GC-induced apoptosis. More and more evidence showed that caspase-9 was indispensable for GCs induced thymocytes apoptosis, whereas the role of caspase-3 is still controversial. In addition, the role of caspases in GCs induced apoptosis of peripheral T cells has not beet investigated by now. Therefore, we treated thymocytes and peripheral T cells with 1×10^{-7}M Dex in the presence or absence of all kinds of caspase inhibitors. Cell viability was checked by flow cytometry using AnnexinV-FITC and propidium iodide at the 20h time point. In thymocytes, pancaspase inhibitor could completely rescue thymocytes from apoptosis. Caspase-3 inhibitor, caspase-9 inhibitor and caspase-8 inhibitor could also rescue thymocytes to some extent although they were not as efficient as pancaspase

inhibitor (Figure 4). In contrast to thymocytes, none of the inhibitors could prevent Dex induced peripheral T cells apoptosis. In order to exclude the possibility that the caspase inhibitors alone are toxic to peripheral T cell, we cultured peripheral T cell in the presence of three different caspase inhibitors for 20 hours and checked the viability. Our data showed that they were not toxic to peripheral T cell (data not shown).Taken together, thymocytes and peripheral T cells differ in their requirements for caspase activity to undergo apoptosis in response to GCs treatment.
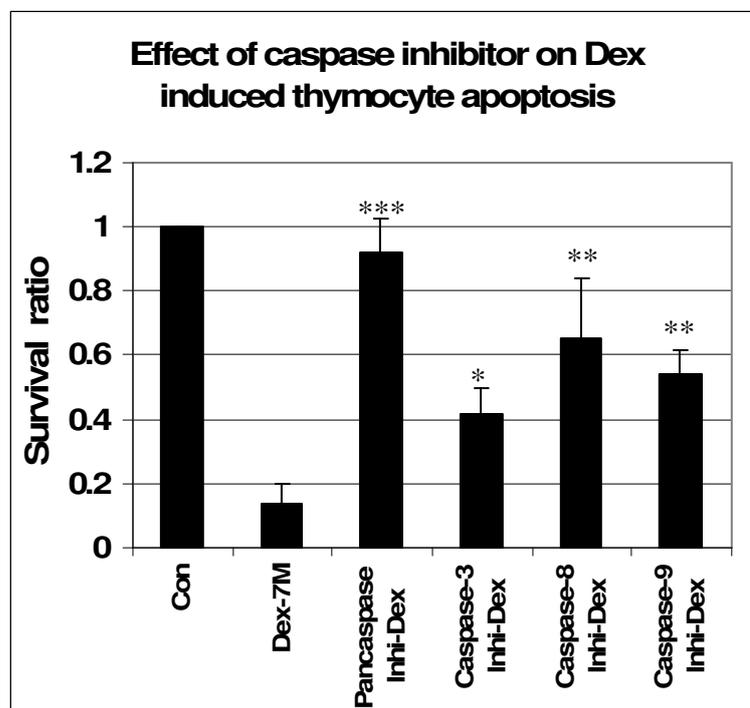
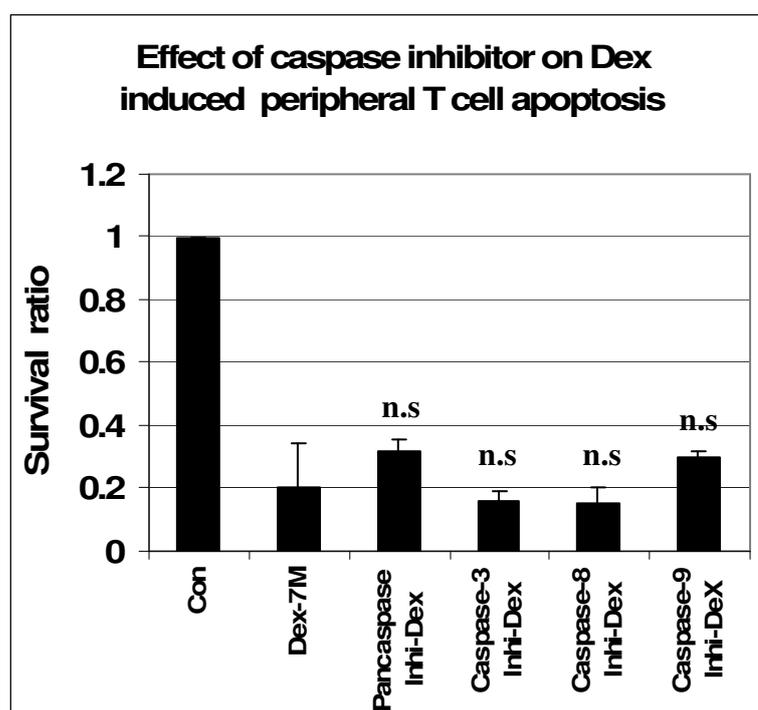**Figure4. Caspase activity is involved in GC-induced thymocyte but not peripheral T cell apoptosis .** Thymocytes and peripheral T cells from C57/BL6 wild type mice were treated with dexamethasone (1×10^{-7}M) in the presence or absence of pancaspase (100μM), caspase-3(100μM), caspase-8 (100μM) and caspase-9 inhibitor (100μM) for 20hours. Culture in the presence of vehicle alone (0.5% DMSO) served as control. Survival ratio was determined by Annexin V-PI staining. The error bars represent the SEM for three independent experiments with three replicates each. Statistical significance was determined by ANOVA as described in Methods and is only depicted for the comparison of individual inhibitor treatments with the Dex-treated cells.

Caspase-3 activation is an essential step for most forms of apoptosis (Wang, X. 2001). To obtain independent evidence for the observed-cell type specificity of caspase-3 activation, we investigated caspase-3 activity after GC-treatment by flow cytometry and Western-blot. Mouse thymocytes and peripheral T cells were cultured 8 hours in the presence or absence of 1×10^{-7}M Dex. The time-course of the cleavage of caspase-3 was studied by Western-blot and flow cytometry. A cleaved caspase-3 band (P20) was observed only in Dex treated thymocytes whereas the caspase-3 of Dex treated peripheral T cells was still intact (Figure 5A and Figure 5B). This result is consistent with the caspase inhibitor assay in which caspase-3 inhibitor could only rescue thymocytes from apoptosis. To

further confirm our conclusion, we analyzed caspase-3 cleavage by flowcytometry. A PE-conjugated caspase-3 cleaved band specific antibody was used in this assay. At the 10 hours time point, about 80% of caspase-3 was activated in Dex-treated thymocytes. In contrast, no cleaved caspase-3 was detected in Dex-treated peripheral T cells (Figure 5B), even after prolonged incubation for 24 hours (data not shown).

A

Splentic T cell   Thymocyte

Lck

Caspase-3

Lck

Caspase-3

Cleaved caspase-3

0h 2h 4h 6h 8h 2h 4h 6h 8h   0h 2h 4h 6h 8h 2h 4h 6h 8h
      Con   Dex   Con   Dex

B

Dex induced caspase-3 cleavage in mouse thymocyte and splentic T cells

C

Thymocyte Con

14.46%

Thymocyte Dex

86.48%

Cleaved caspase-3

Peripheral T cell Con

5.42%

Peripheral T cell Dex

9.23%

Cleaved caspase-3
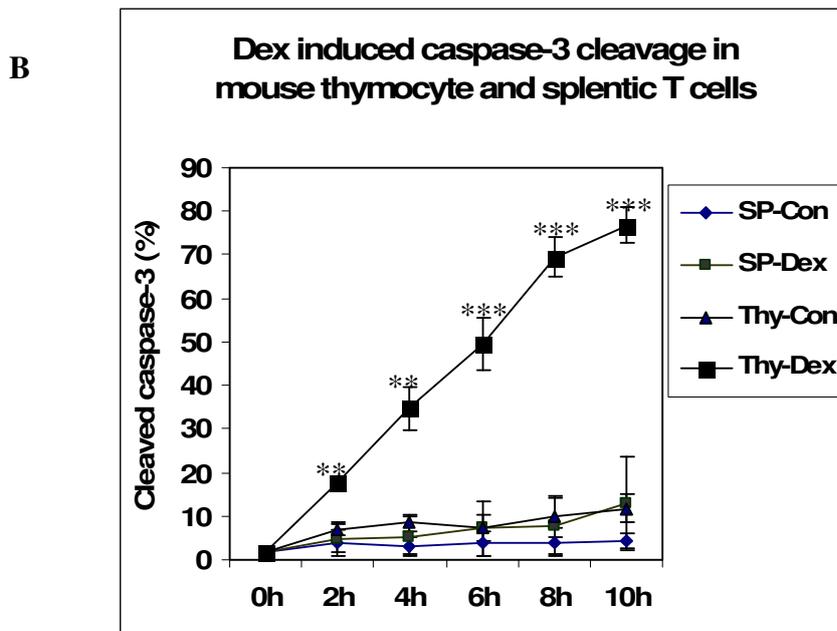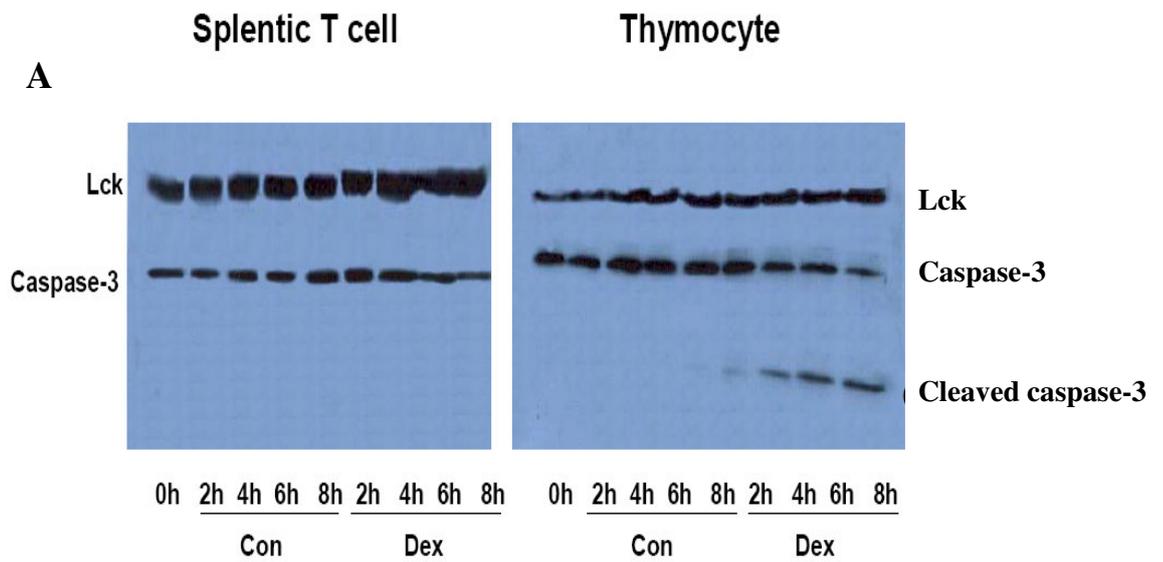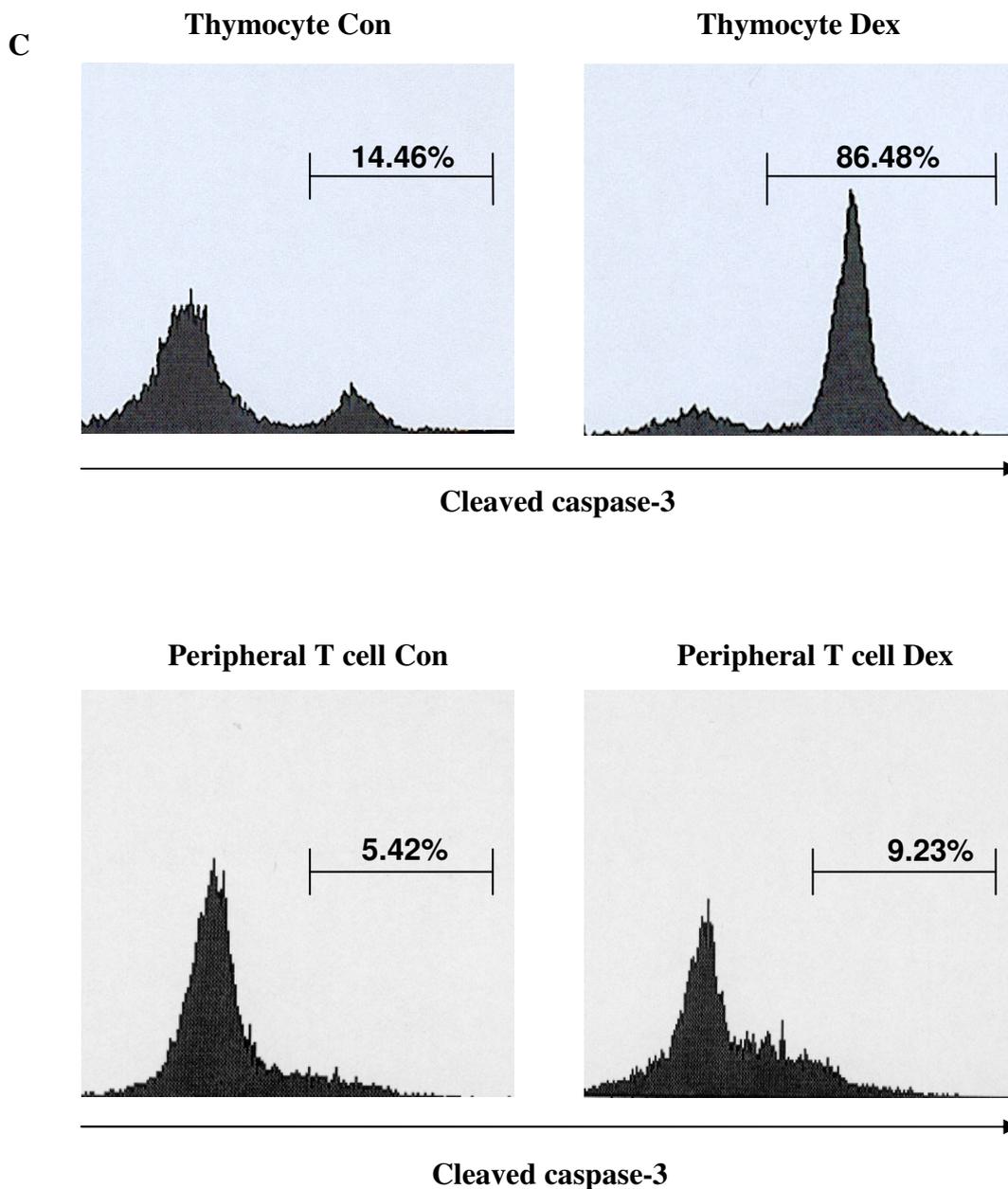
Figure5. Caspase-3 is activated in GC-treated thymocytes but not peripheral T cells.

A. Thymocyte and peripheral T cell from C57/BL6 mice were treated with Dex (1×10^{-7}M) for 8hours. Cells were collected and proteins were extracted every 2hours. Caspase-3 activation was compared by western-blot. Staining of Lck was used as loading control. One representative result out of three is shown. B. Thymocytes and peripheral T cells from C57/BL6 wild type mice were treated with dexamethasone (1×10^{-7}M) for 10hours.Cleaved caspase-3 in the live gate was measured by flow cytometry every 2 hours.

Student's T test was used for statistical analysis. The error bar is the SEM for three independent experiments with three replicates each.   C. Thymocyte and peripheral T cell from C57/BL6 wild type mice were treated with dexamethasone (1×10^{-7}M) for 10hours.Cleaved caspase-3 was measured by flow cytometry. One representative FACS result is shown. The percentage shown stands for the caspase-3 activated cells.

We found the same phenomena when we compared the caspase-8 activation of thymocytes and peripheral T cells by flow cytometry. Caspase-8 was only activated in Dex treated thymocytes but not in peripheral T cells (Figure 6). A cell permeable, non-toxic caspase-8 substrate, FITC-IETD-FMK, which could bind to the activated enzyme, was used in this assay.

A

Caspase-8 activation in Dex induced splentic T cell apoptosis

Petcentage of cleaved caspase-8 (%)

n.s

90
80
70
60
50
40
30
20
10
0

SP-T-Con   SP-T-Dex

B

Thymocyte Con   **Thymocyte Dex**

8.71%   78.27%

Cleaved   caspase-8

Peripheral T cell Con   **Peripheral T cell Dex**

0.6%   6.1%

Cleaved   caspase-8

C

Effect of caspase inhibitor on Dex induced thymocyte caspase-3 activation(24h)

Caspase-3 cleaved ratio

2

1.5

1

0.5

0

Thy-Con   Thy-Dex-7M   Thy-Dex-caspase-3 Inhi   Thy-Dex-caspase-8 Inhi

***   ***

Figure6. Caspase-8 is only activated in Dex treated thymocytes but not peripheral T cells and works upstream of caspase-3.

A Thymocytes and peripheral T cells from C57/BL6 mice were treated with Dex (1×10^{-7}M) for ten hours and the percentage of caspase-8 activated cells in the live gate was determined by flow cytometry. *Student's T* test was used for statistical analysis. The error bar is the SEM for three independent experiments with

three replicates each.　　B. Thymocytes and peripheral T cells from C57/BL6 mice were treated with dexamethasone (1×10^{-7}M) for 10hours. Cleaved caspase-8 was measured by flow cytometry. One representative FACS result is shown. The percentage shown stan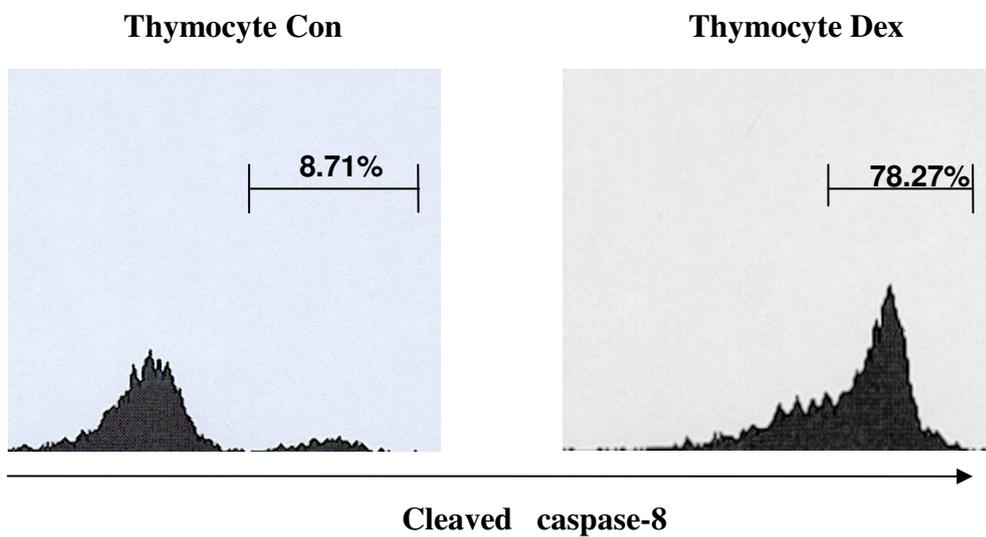ds for the caspase-8 activated cells.　 C. Thymocytes from C57/BL6 mice were treated with Dex (1×10^{-7}M) in the presence or absence of 100μM caspase-8 or caspase-3 inhibitor for 24 hours. The percentage of caspase-3 activated cells in the live gate was determined by flow cytometry. The error bars represent the SEM for three independent experiments with three replicates each. ANOVA was used for statistic analysis in this assay. Statistical significance is only depicted for the comparison of individual inhibitor treatments with the Dex treated cells.

Since both caspase-3 and caspase-8 were activated during Dex induced thymocytes apoptosis, we tried to order the activation sequence. We found that cleavage of caspase-3 in Dex treated thymocytes could be prevented by both caspase-3 and caspase-8 inhibitor (Figure 6C). Considering the specificity of caspas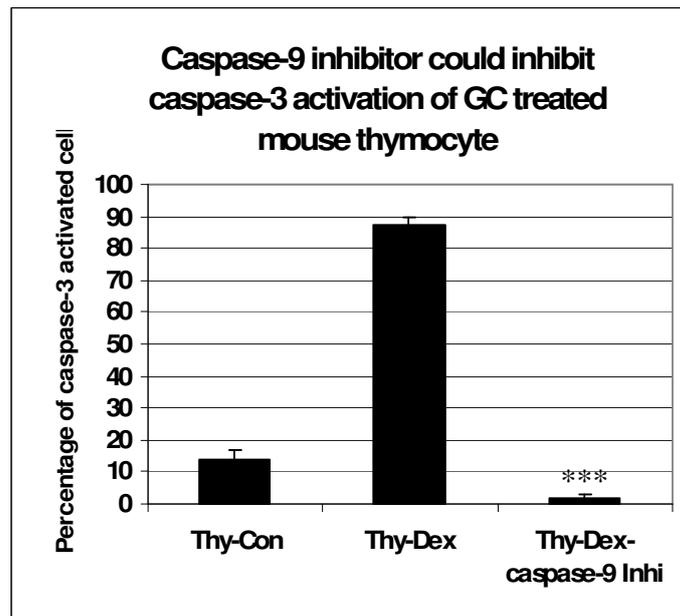e-8 inhibitor, we could draw the conclusion that caspase-8 acts upstream of caspase-3 in Dex induced thymocyte apoptosis which is consistent with the previous reports.

Previous experiments showed that caspase-9 was involved in Dex induced thymocytes apoptosis. Usually, caspase-9 is regarded as an initiator caspase and acts upstream of caspase-8 and caspase-3. So, we wondered whether it was also true in our apoptosis model. Thymocyte caspase-3 and caspase-8 activation assay was repeated in the presence of caspase-9 inhibitor. As we have expected, caspase-9 inhibitor completely inhibited the activation of caspase-8 and caspase-3(Figure 7), which indicated that caspase-9 really worked upstream of caspase-8 and caspase-3. So, from these data, we could delineate the sequence of caspase activation. Caspase-9 was the initiator caspase and activated caspase-8 and caspase-3. Caspase-8 acted upstream of caspase-3 and maybe it could activate caspase-3 directly although there was no experimental evidence.

A

Caspase-9 inhibitor could inhibit
caspase-3 activation of GC treated
mouse thymocyte

B

Caspase-9 inhibitor could inhibit
caspase-8 activation of GC treated
mouse thymocyte

Figure7. Caspase-9 works upstream of caspase-8 and caspase-3.

A. Thymocytes from C57/BL6 mice were treated with Dex (1×10^{-7}M) for ten hours and the percentage of caspase-3 activated cells in the live gate were determined by flow cytometry. The error bars represent the SEM for three independent experiments with three replicates each. ANOVA was used for statistic analysis in this assay. Statistical significance is only depicted for the comparison of caspase-9 inhibitor treatments with the Dex treated cells.   B. Thymocytes from C57/BL6 were treated with Dex (1×10^{-7}M) for ten hours and the percentage of caspase-8 activated cells in the live gate was determined by flow cytometry. The error bars represent the SEM for three independent experiments with three replicates each. ANOVA was used

for statistic analysis in this assay. Statistical significance is only depicted for the comparison of caspase-9 inhibitor treatments with the Dex treated cells.
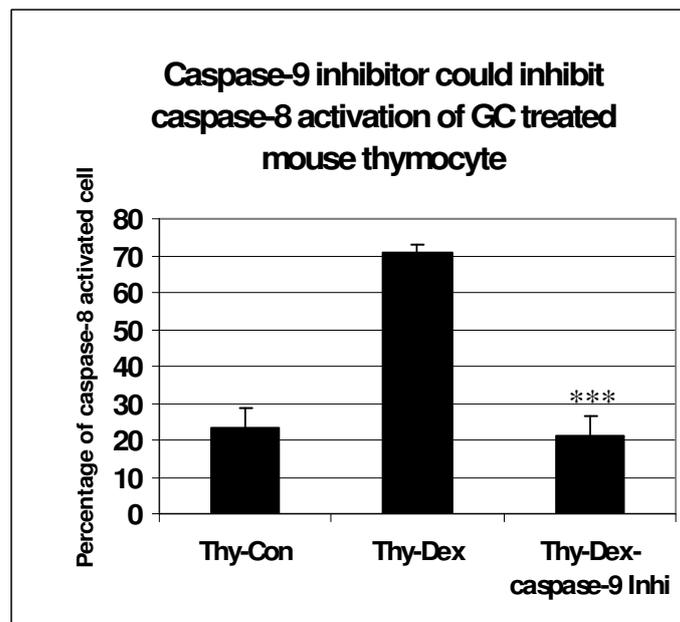
4.3 Proteasomal degradation is required for GC-induced apoptosis of thymocytes but not peripheral T cells and it acts upstream of caspase activity

It was reported that the proteasome activity was involved in GC induced thymocyte apoptosis (Tamara, 1998). Given the fact that caspase activity was only involved in GCs induced thymocytes but not peripheral T cell apoptosis, we wondered whether it was same for the proteasome activity. So, we induced mouse thymocytes and peripheral T cells apoptosis by Dex in the presence or absence of the proteasome inhibitor, lactacystin. As we have expected, lactacystin efficiently rescued Dex induced thymocytes but not peripheral T cell apoptosis (Figure 8). This experiment confirmed that the proteasome activity was only indispensable for Dex induced thymocytes apoptosis.
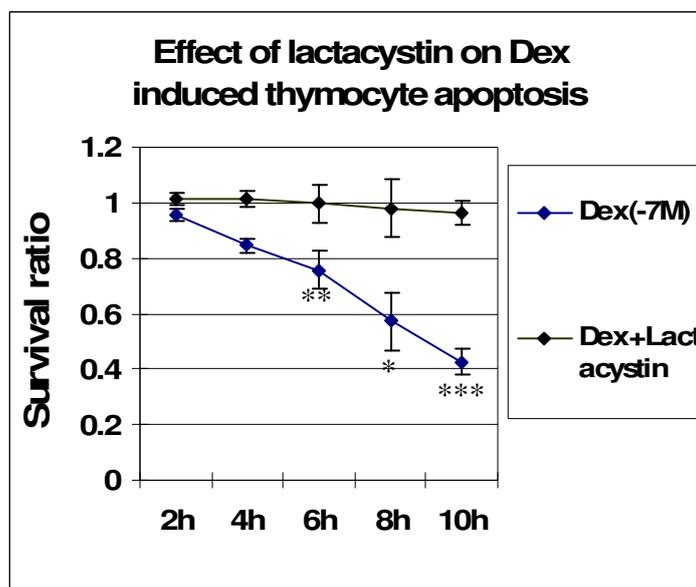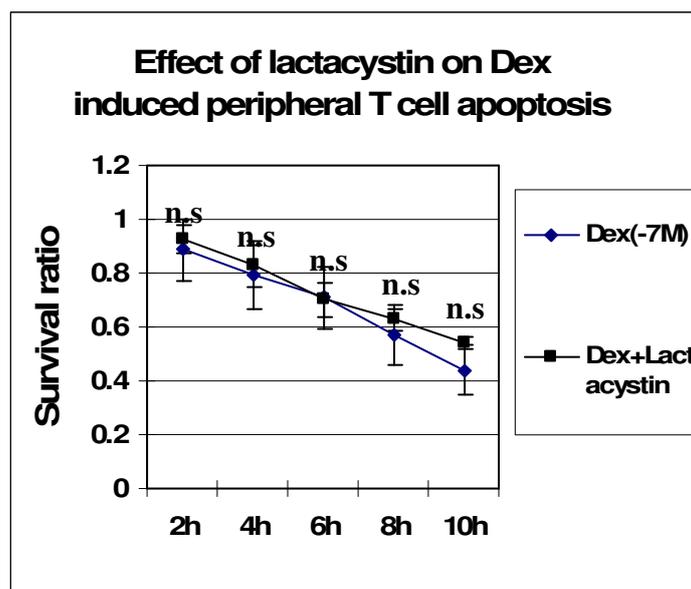
Figure 8. Proteasomal degradation is required for GC-induced apoptosis of thymocyte but not of peripheral T cell.

Thymocytes and peripheral T cells from C57/BL6 mice were treated with dexamethasone (1×10^{-7}M) in the presence or absence of proteasome inhibitor, lactacystin(8μM), for 10 hours. The cell survival ratio was determined by Annexin V-PI staining every 2 hours. *Student's T* test was used for statistical analysis. The error bar is the SEM for three independent experiments with three replicates each.

Since caspases and the proteasome are both involved in Dex induced thymocyte apoptosis, we try to delineate the connection between proteasome activity and caspase activation. We observed the caspase-8 and caspase-3 activation in the presence of lactacystin in Dex induced thymocyte apoptosis by flow cytometry. Thymocytes were treated with Dex for 10 hours in the presence or absence of lactacystin. Caspase-8 and caspase-3 activation was inhibited by lactacystin efficiently (Figure 9). To sum up, proteasomal degradation acts upstream of caspase activity.
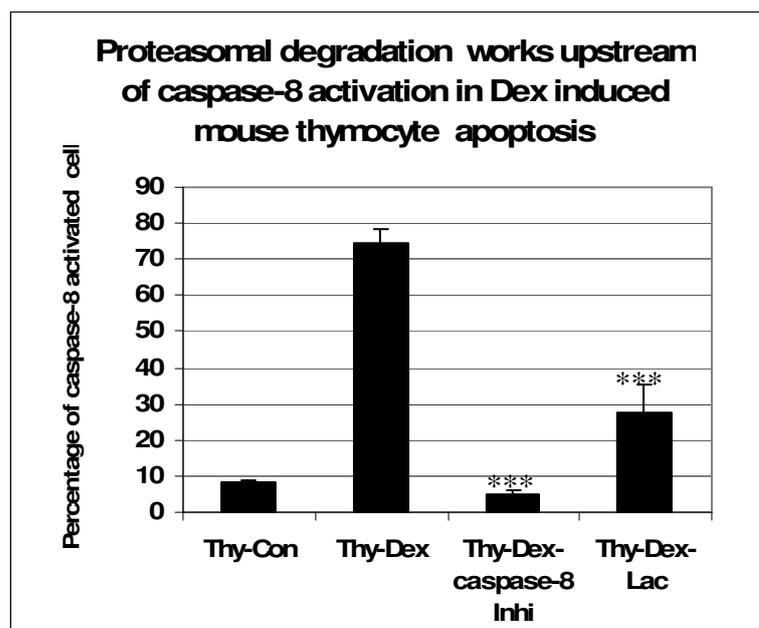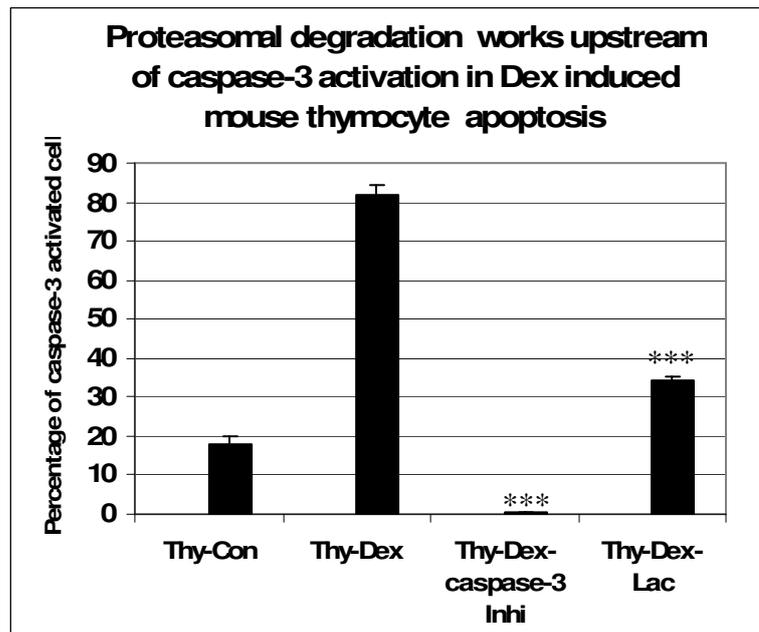
Figure9. Proteasomal degradation works upstream of caspase-8 and caspase-3.

Thymocytes from C57/BL6 mice were treated with Dex $(1\times10^{-7}\text{M})$ for ten hours in the presence or absence of 8μM lactacystin. Caspase-3 and caspase-8 inhibitor was used as negative control.The percentage of caspase-8 and caspase-3 activated cells in the live gate were determined by flow cytometry. The error bars represent the SEM for three independent experiments with three replicates each. ANOVA was used for statistic analysis as depicted in Methods in this assay. Statistical significance is only depicted for the comparison of Lactacystin treatments with the Dex treated cells

4.4 GC induced thymocyte and peripheral T cell apoptosis doesn't depend on calpain

Calpain is reported to be involved in thymocyte apoptosis induced by several stimuli (Margaret K, 1997). So, we tested the effect of calpain inhibitor, PD150606, on GC induced thymocyte and peripheral T cell apoptosis. Contrary to our expectation, PD150606 can't rescue both thymocyte and peripheral T cell apoptosis induced by Dex (Figure 10).
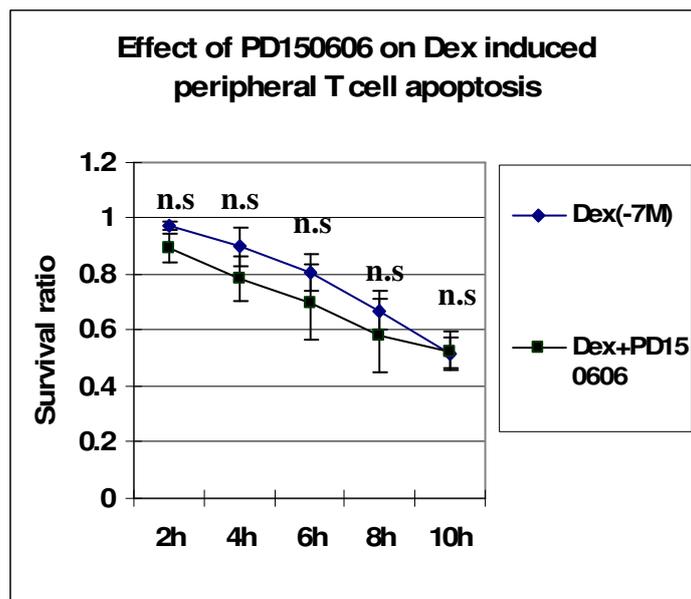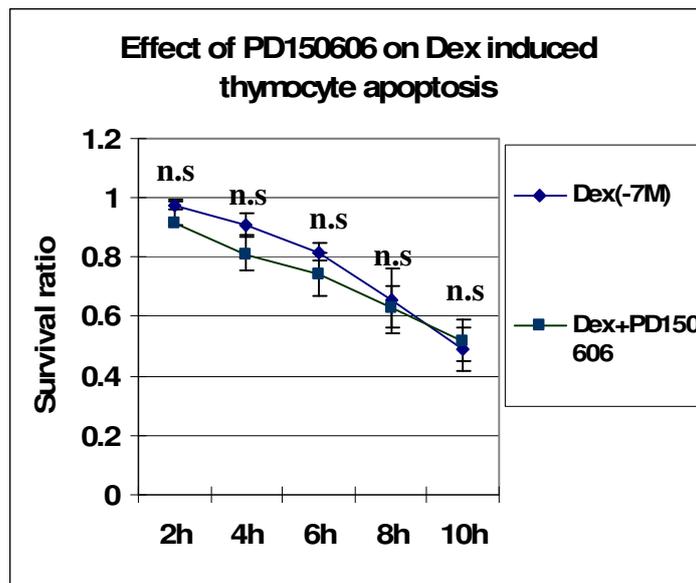
Figure10. The calcium inhibitor, PD150606, doesn't participate in GC induced thymocyte and peripheral T cell apoptosis.

Thymocytes and peripheral T cells from C57/BL6 mice were treated with dexamethasone (1×10^{-7}M) in the presence or absence of the calcium inhibitor, PD150606 (100μM) for 10 hours. The cell survival ratio was determined by Annexin V-PI staining every 2 hours. *Student's T* test was used for statistical analysis. The error bar is the SEM for three independent experiments with three replications each.

4.5 Cathepsin B activity is required for GC-induced thymocytes but not peripheral T cells apoptosis

It was reported that cathepsin activity was implicated in many types of apoptosis. This prompted us to investigate the role of cathepsin in GC-induced thymocyte and peripheral T cell apoptosis. We used the Pancathepsin inhibitor Z-FG-NHO-Bz and the cathepsin B inhibitor Z-FA-CH$_2$F in the same apoptosis model like before. Both inhibitors could prevent Dex induced thymocytes apoptosis efficiently whereas neither of them could rescue Dex induced peripheral T cells apoptosis (Figure 11). It is noteworthy that they were known for their specificities, and that the cathepsin B inhibitor was also called caspase inhibitor negative control. So they should not cross react with caspases and the rescue was the result of inhibiting cathepsin B. In addition, using confocal microscopy, our group found that cathepsin B was activated very quickly in thymocytes after being treated with GC(Wang.D, 2006) .Whereas in peripheral T cells, cathepsin B activity didn't change after being treated with GC. This is consistent with our cathepsin B inhibitor assay.

Taken together, these data reveal for the first time a role of cathepsin activity in GC-induced thymocyte apoptosis.

Effect of Pan cathepsin inhibitor on Dex induced thymocyte apoptosis

Effect of Pan cathepsin inhibitor on Dex induced peripheral T Cell apoptosis

Effect of cathepsin B inhibitor on Dex induced thymocyte apoptosis

Effect of cathepsin B inhibitor on Dex induced peripheral T cell apoptosis
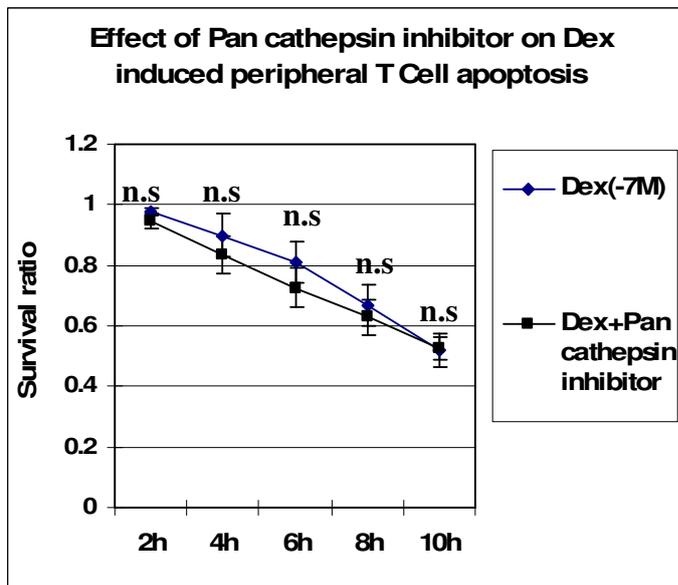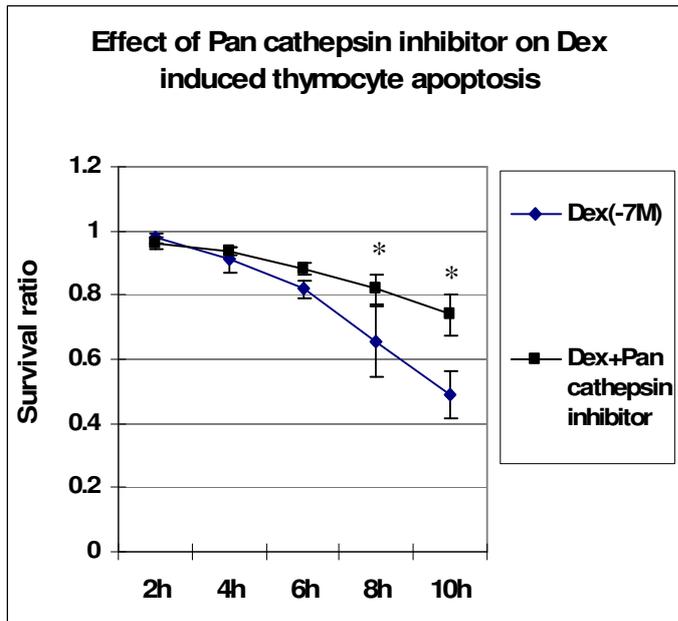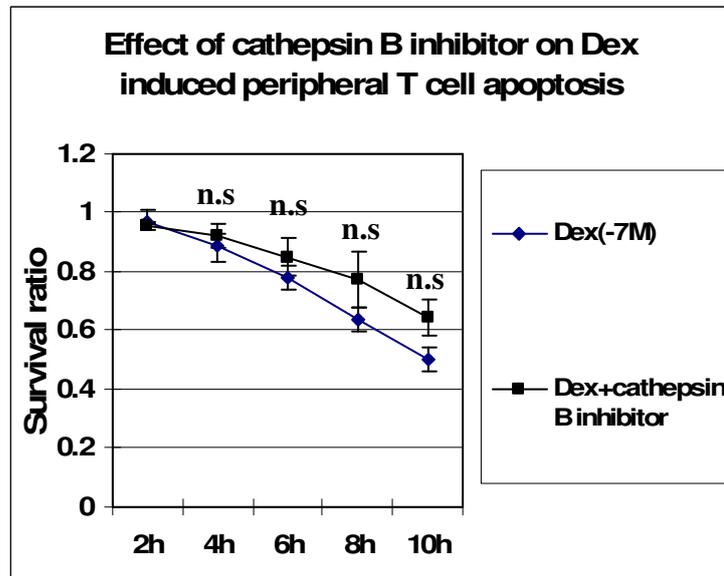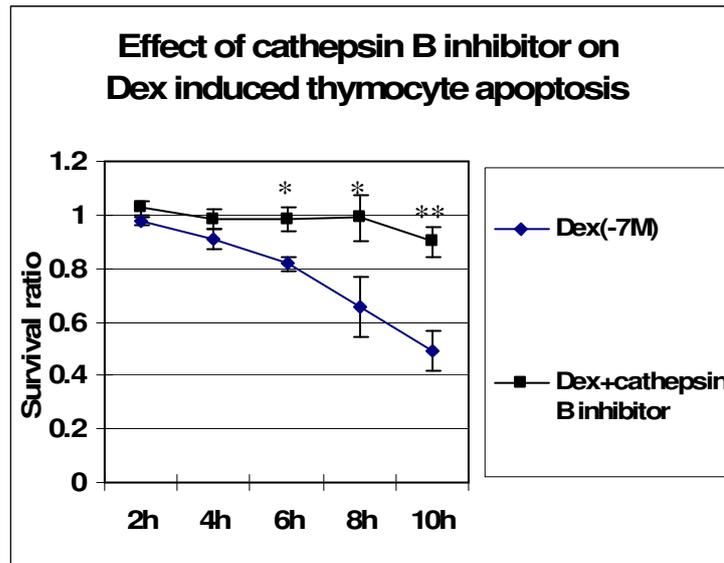
Figure11. Cathepsins play an important role in GC induced thymocyte but not peripheral T cell apoptosis.

Thymocytes and peripheral T cells from C57/BL6 wild type mice were treated with Dex $(1 \times 10^{-7} M)$ in the presence or absence of Pan cathepsin inhibitor(10μM) or cathepsin B inhibitor (100μM) for 10 hours. Cell survival ratio was determined by Annexin V-PI staining every 2 hours. *Student's T* test was used for statistical analysis. Error bar is the SEM for three independent experiments with three replications each.

Next, we tried to delineate the activation sequence of cathepsin B and caspases in Dex treated thymocytes. We tested the effect of cathepsin B inhibitor on caspase-8 and caspase-3 activation in Dex treated thymocytes. If cathepsin B acts upstream of caspase,

then the activation of caspase-8 and caspase-3 should be inhibited. As we have expected, the activation of caspases was inhibited significantly when thymocytes were cotreated with Dex and cathepsin B inhibitor (Figure 12). Our group also proved this hypothesis by another assay. Using confocal confocal microscope, we found that cathepsin B activity was not altered in Dex treated thymocytes in the presence of caspase-8 or caspase-3 inhibitor. But, caspase-9 inhibitor could inhibit cathepsin B activity completely which indicated caspase-9 works upstream of cathepsin B.
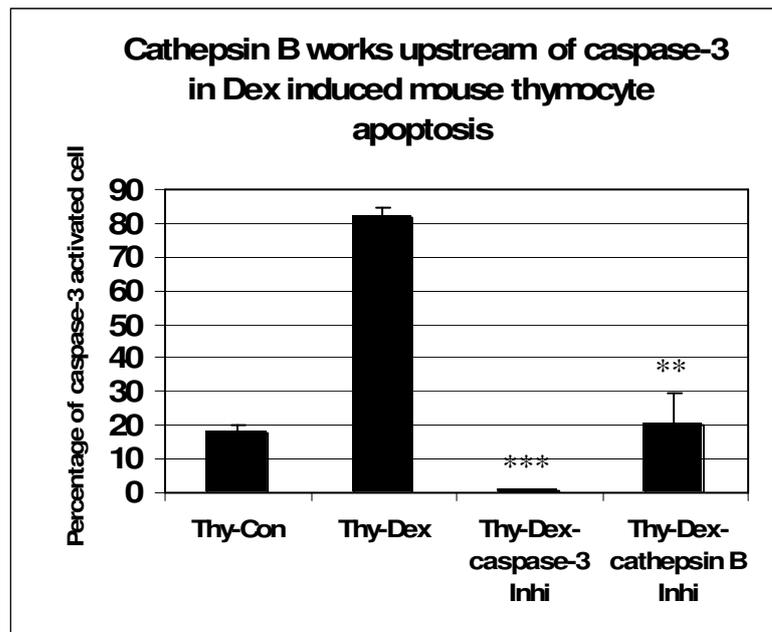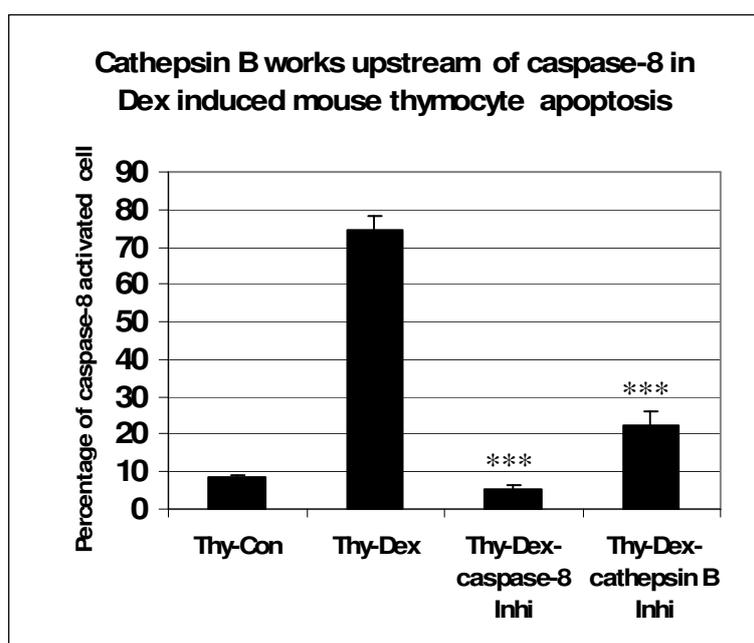
Figure12. Cathepsin B activity locates upstream of caspase-8 and caspase-3

Thymocytes from C57/BL6 mice were induced apoptosis with Dex (1×10^{-7}M) for ten hours in the presence or absence of cathepsin B inhibitor(100μM)and the percentage of caspase-8 or caspase-3 activated cells in the live gate were determined by flow cytometry. The error bars represent the SEM for three independent experiments with three replicates each. ANOVA was used for statistic analysis as depicted in the Methods in this assay. Statistical significance is only depicted for the comparison of cathepsin B inhibitor treatments with the Dex treated cells.

4.6 Expression and redistribution of Bcl-2 family members in GC induced murine thymocyte and peripheral T cell apoptosis.

4.6.1 The expression of Bak and Bax is unaltered in GC treated rat thymocytes and peripheral T cells

Bcl-2 family members play an important role in GC induced apoptosis. Bcl-2 and Bcl-xl are able to prevent apoptosis whereas Bak and Bax promote it. Therefore, we checked the expression of Bak and Bax in rat thymocytes and peripheral T cells by Western-blot.

The results are shown in Figure 13. Dex treatment didn't change the expression of Bak and Bax.
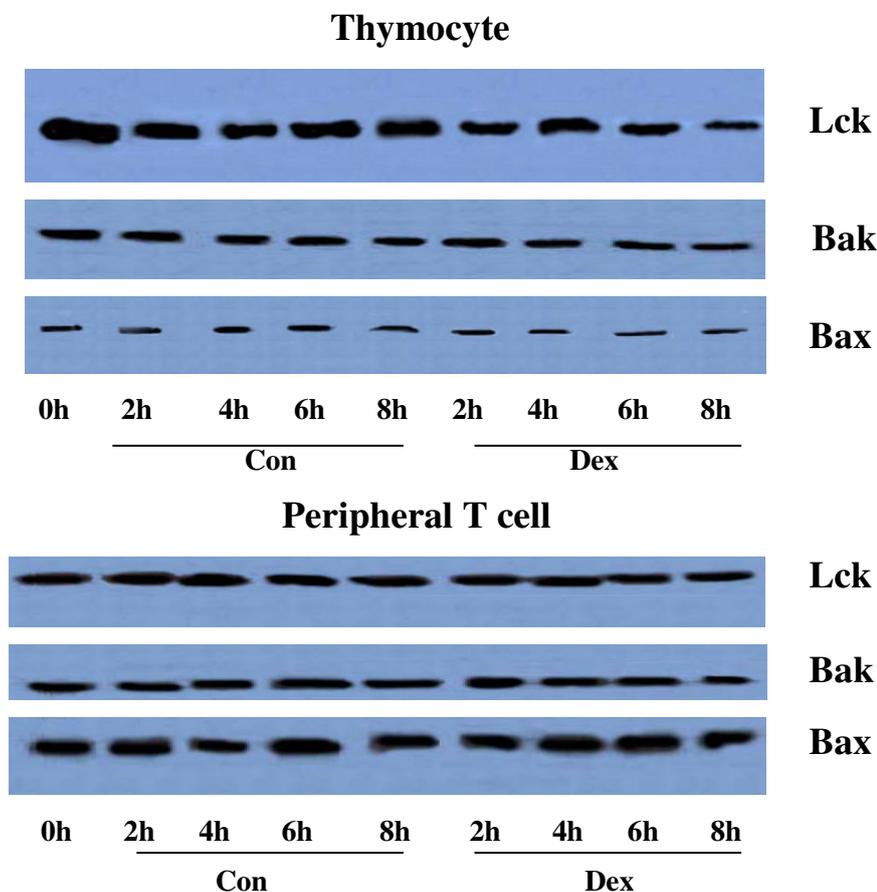
Thymocyte

Figure13. Bak and Bax expression were unchanged after Dex treatment.

Rat thymocytes and peripheral T cells were cultured in the presence or absence of Dex (1×10^{-8}M) for 20 hours. Cells were collected and total proteins were extracted by universal lysis buffer. After measuring the protein concentration, 50μg protein were separated on 15% SDS-PAGE. Bak and Bax expression were analyzed by Western-Bolt. P56-lck was used as a loading control. One representative result is shown.

4.6.2 Bax partially translocated into mitochondria upon GC-treatment in thymocytes

Usually Bax mainly resides in cytosol. Upon apoptosis induction, Bax translocates from cytosol to mitochondria. To check whether this was also true for Dex induced murine thymocyte and peripheral T cell apoptosis, we investigated the subcellular distribution of Bax in Dex treated thymocytes and peripheral T cells. In contrast to our expectation, we

could only find partial Bax translocation in Dex treated thymocytes. In peripheral T cells, there was no Bax translocation at all (Figure 14).

Thymocyte control

Bak

Bax

Cyto	Mito	Cyto	Mito	Cyto	Mito	Cyto	Mito
2h		4h		6h		8h	

Thymocyte Dex

Bak

Bax

Cyto	Mito	Cyto	Mito	Cyto	Mito	Cyto	Mito
2h		4h		6h		8h	

Peripheral T cells control

Bak

Bax

Cyto	Mito	Cyto	Mito	Cyto	Mito	Cyto	Mito
2h		4h		6h		8h	

Peripheral T cells Dex

Bak

Bax

Cyto	Mito	Cyto	Mito	Cyto	Mito	Cyto	Mito
2h		4h		6h		8h	
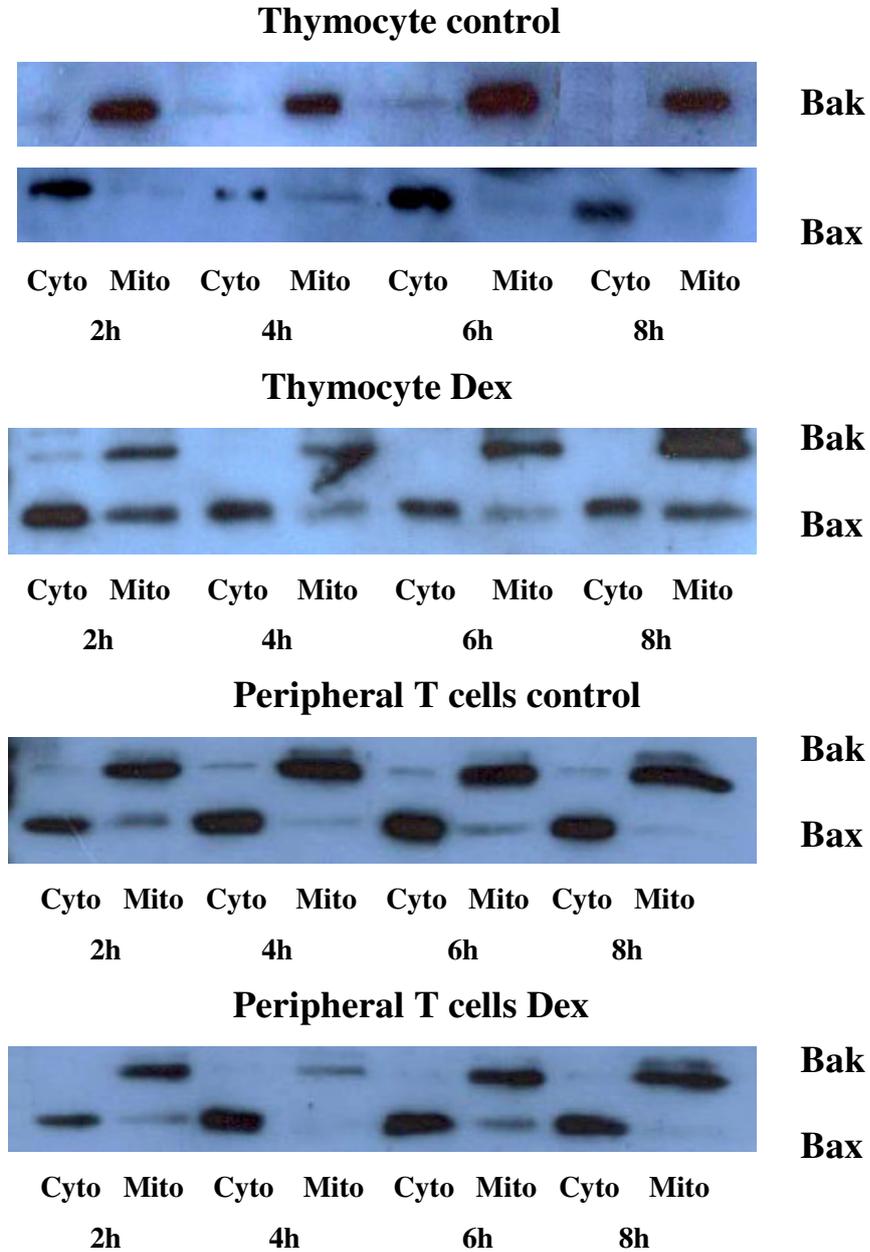
Figure14. Bax partially translocates to mitochondria upon GC treatment in thymocytes but not peripheral T cell.

Rat thymocytes and peripheral T cells were treated with 10^{-8}M Dex for 8 hours. Cells were collected and subcellular proteins were extracted every 2 hours. Protein samples were separated on a 15% SDS-PAGE gel. The distribution of Bak and Bax was checked by Western-blot. One representive result is shown.

4.6.3 Bcl-xl translocates from cytosol to mitochondrial in mouse thymocytes after GC treatment

As an antiapoptotic protein, Bcl-xl was reported to prevent the oligomerization of Bak and Bax. Therefore, we checked its distribution in murine thymocytes treated with Dex. We found that Bcl-xl translocated from the cytosol to the mitochondria upon Dex treatment (Figure 15). Before Dex treatment, we could detect some Bcl-xl in the cytosol although most of it resides in the mitochondria. After Dex treatment, Bcl-xl completely translocates to the mitochondria.

| | Bcl-xl |
| | Bak |

Cyto   Mito   Cyto   Mito

Con   Dex
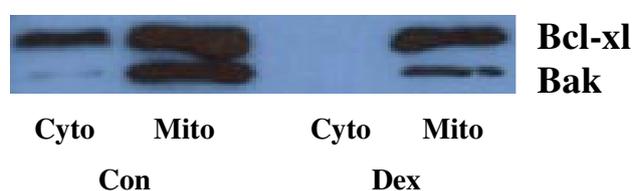
Figure15   Bcl-xl translocates from cytosol to mitochondria in thymocytes upon GC treatment.

Rat thymocytes were treated with Dex (10^{-8}M) for 8hours. Cells were collected and subcellular proteins were extracted. Protein samples were separated on a 15% SDS-PAGE gel. The distribution of Bcl-xl was checked by Western-blot. Bak was used to control the purity of the cytosol fraction since Bak is a membrane bound protein. One represerive result is shown.

4.7 Introperitoneal injection of dexamethasone induces rat thymus atrophy whilst the thymus recovers in 2 weeks

Glucocorticoids have important immunoregulatory effects on thymocytes and T cells. Ectopic production of GCs has been demonstrated in thymic epithelial cells (TECs) but the role of GCs in thymocyte homeostasis is controversial. Genetically modified mice, including those with increased or decreased GC sensitivity, due to altered GR expression , a point mutation in the GR gene or a partial or complete loss of GR protein, have generated conflicting results showing negative, positive or no effects of GCs on the number of thymocytes present in the thymus. To solve this problem, we treated young rats (4 weeks old) with dexamethasone by IP injection and monitored the total thymus cell number at different time points after injection. Also, we compared the sensitivity of different thymus cell population to dexamethasone.   The result is shown in Figure 16. The total thymocytes number decreased sharply within the first 3 days after injection and most of the thymocyte had undergone apoptosis during this time. Then, with time passing by, thymus recovered gradually. At day 7 after Dex treatment, the cell number has reached 70% of the control thymus. In 2 weeks, the total thymus cell number and the ratio among the different cell population had recovered completely. It's noteworthy that the cell number exceeded the cell number of 4 weeks old control rat. Since the rats we used are young and their thymuses are still developing, it's conceivable that after recovery the thymus cell number is higher than the cell number of the 4 weeks old control rat. Concerning the change of different thymocyte subpopulations, we found that the DP thymocytes were more sensitive to dexamethasone than CD4 or CD8 single positive thymocyte. The number of single positive thymocytes didn't decrease too much (80%) in the first day after Dex injection whereas the number of DP thymocytes decreased to 2.8% of the control thymus.

So, obviously, GC influences the homeostasis of the thymus.

Rat thymus undergoes atrophy after in vivo GC treatment and recovers in 2 weeks.

The change of different thymocyte subpopulation after in vivo GC treatment
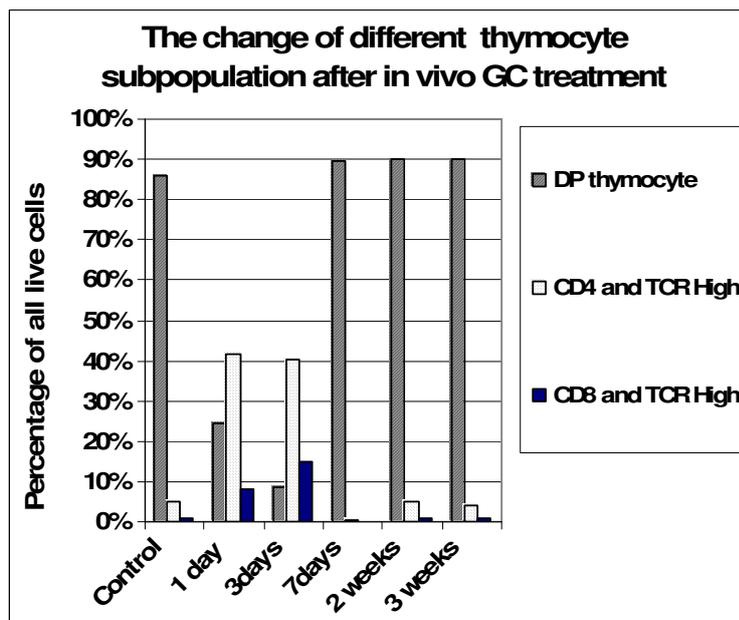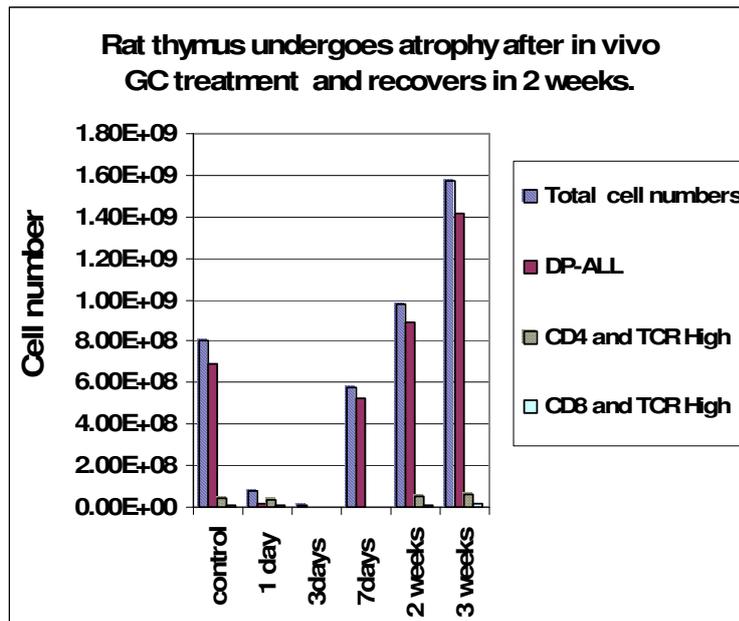
Figure16. GC treatment induces rat thymus atrophy followed by recovery.

4 weeks old inbred Lewis rats were treated with dexamethasone by IP injection (500μg/100g body weight). Then, rats were sacrificed at different time points after injection. The total cell number of thymus and the percentage of different thymocyte subpopulation were monitored. 1 rat was sacrificed and analyzed on the day of injection as control.   A. The cell number of total thymocytes and different thymocyte subpopulation at different time points after GC treatment.   B. The percentage of different thymocyte population at different time points after GC treatment.

4.8 CD28 superagonist could rescue GC induced rat peripheral T cell apoptosis

In 1996, Wagner reported that GC induced apoptosis of thymocytes could be prevented by the interaction between CD28/CTLA-4 and B7.1 /B7.2. We want to know whether this is also true in peripheral T cells. So, we induced rat peripheral T cell apoptosis in the presence or absence CD28 superagonist, JJ316. GC induced peripheral T cell apoptosis was completely prevented (Figure 17). This is consistent with our previous report that resistance of single positive thymocytes to GC induced apoptosis is mediated by CD28 signaling (Van den Brandt J, 2004). Our previous research showed that the rescue occurred by transcriptional regulation of apoptosis associated genes such as Bcl-xl via a calcium and PI-3K dependent pathway (Van den Brandt J, 2004). So, we first tested the change of Bcl-xl expression in rat peripheral T cells by Western-Blot (Figure 18). We could see Bcl-xl expression was upregulated after JJ316 stimulation. Its expression in control and Dex treatment group was too low to be detected. Next, we tested the influence of calcium inhibitor (Cyclosporine A) and PI3k inhibitor (LY294002) on Dex induced rat peripheral T cell apoptosis. As we had expected, CsA and LY294002 could prevent the rescue effect of JJ316 although LY294002 didn't prevent it completely. This indicates that calcium and PI3K was indispensable for the signaling of JJ316 (Figure 17).

JJ316 could rescue GC induced rat peripherial T cell apoptosis

CsA could prevent the rescue effect of JJ316 on GC induced peripheral T Cell apoptosis

PI3K inhibitor could prevent the rescue effect of JJ316 on GC induced peripheral T cell apoptosis
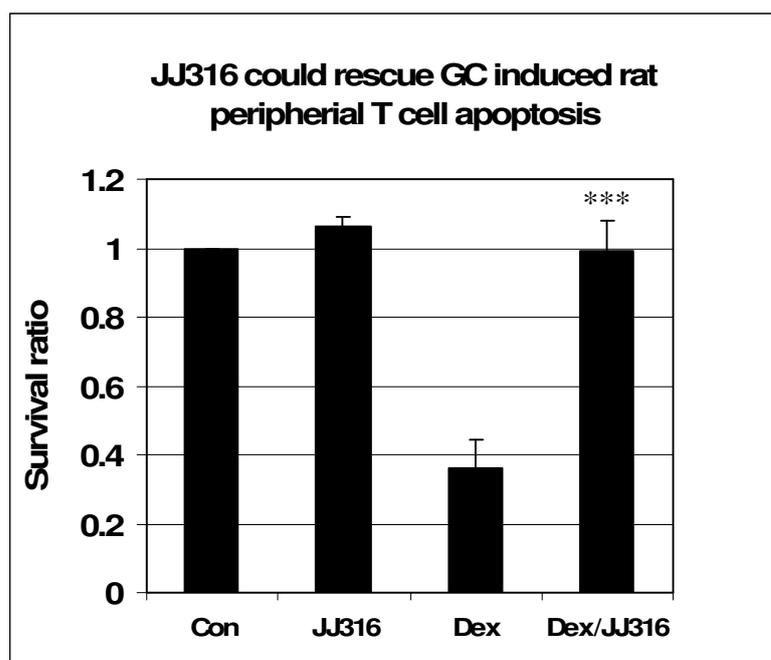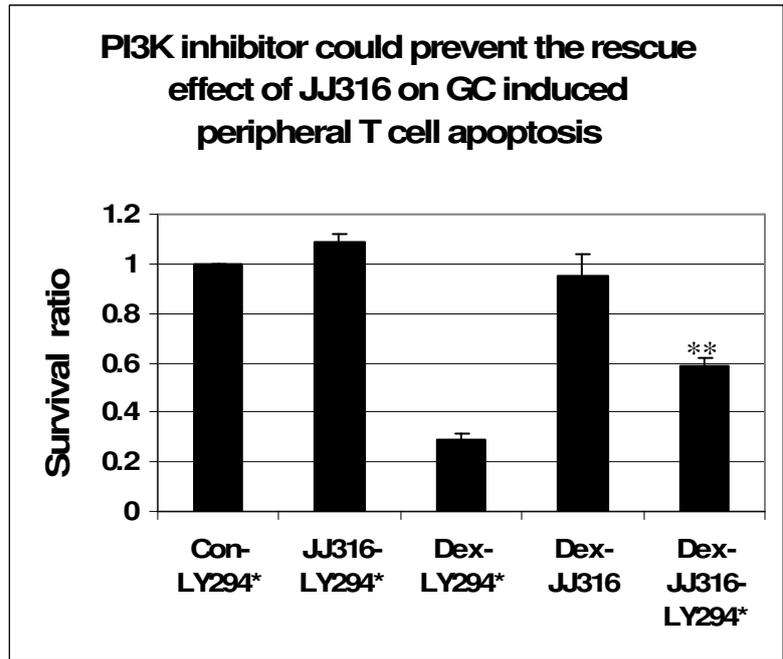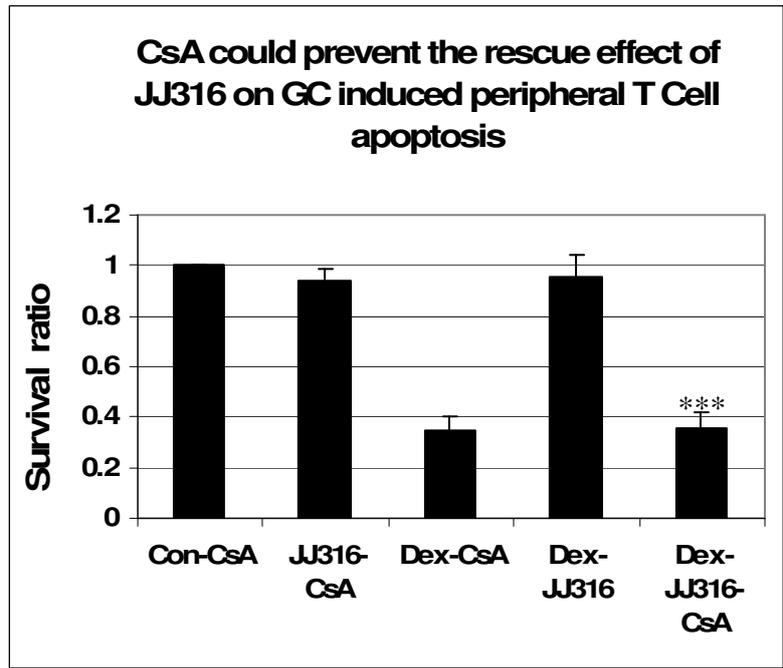
Figure17. CD28 superagonist, JJ316 rescues rat peripheral T cells from GC induced apoptosis via calcium and PI3k.

A. Lewis rat peripheral T cells were treated with 10^{-8}M Dex for 20 hours in the presence or absence of CD28 superagonist, JJ316 (5μg/ml). The cell survival ratio was checked by flow cytometry. B and C. Lewis rat peripheral T cells were treated with 10^{-8}M Dex for 20 hours in the presence of JJ316 and CsA(0.1ng/ml) or LY294002(30μmol). Cell survival ratio was checked by flow cytometry. The error bar is the SEM for three independent experiments. *Student's T* test was used in this assay. Statistical significance is only depicted for the comparison of Dex+JJ316 treatments with the Dex+JJ316 +Inhibitor treated cells.

Con   JJ316   Dex   Dex/JJ316
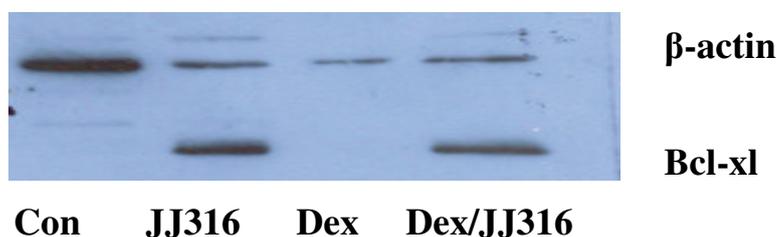
Figure18.   The expression of Bcl-xl in peripheral T cell is upregulated upon JJ316 stimulation

Rat peripheral T cells were treated with 10^{-8} Dex for 20hours in the presence or absence of 5μg/ml JJ316. At the end of culture, cells were collected and the total proteins were extracted. After measuring the protein concentration, 50μg protein were separated on a 15% SDS-PAGE gel and the expression of Bcl-xl was checked by Western-blot. Equal loading was confirmed by β-actin blot. One representive result was shown here.

4.9 The GC-induced Jurkat-GR, Wehi7.1 and TALL-1 death signaling pathways are similar to GC-induced thymocyte signaling

As mentioned above GC is widely used in the clinical treatment of hematological malignancy although the exact mechanism is not fully understood. For example, synthetic GC derivatives such as prednisonlone are part of the CHOP therapy used to treat patients with Non-Hodgkin lymphomas. Here, we tried to investigate the death signal transducing pathways in neoplastic cells. We chose three T-ALL cell lines as our targets for investigation. The first cell line is Jurkat E6.1. Because Jurkat E6.1 doesn't express GR, we used its derivative Jurkat.GR, which had been transduced with GR using a

lentivirus. The second cell line is Wehi7.1, a mouse TALL cell line. The last TALL cell line is TALL-1, a human T leukemia cell line. We first compared their sensitivity to GC-induced apoptosis. Compared with the other two TALL cell lines, Jurkat GR is more sensitive to GCs induced apoptosis since 1×10^{-7}M Dex could induce the apoptosis of Jurkat GR efficiently within 24 hours (Figure 19A). In contrast, the GC concentration had to be increased to 1×10^{-5}M to get the similar apoptosis rate for Wehi7.1 (Figure 19B). As for the TALL-1 cell line, 24 hours Dex treatment (1×10^{-5}M) is not long enough to induced apoptosis. Apoptosis was only observed after 72h Dex treatment (Figure 19C). We wondered what might be the reason that led to this difference. Since GC induced apoptosis is mediated by GR and the sustained GR expression is necessary for the induction of apoptosis, we did GR Western-blot to compare the GR expression (Figure 19D). We found GR expression of Jurkat.GR is similar to thymocyte or even higher. In contrast, in Wehi7.1, GR expression is much lower than in the other cell type. This could explain the lower sensitivity of Wehi7.1 to GCs. As for TALL-1, GR expression is comparable to Jurkat.GR and thymocytes. Then, the question arose, how to explain the low sensitivity of TALL-1 cells to Dex. Given that Bcl-2 family members play an important role in apoptosis, we compared that expression of Bcl-2 and Bcl-xl which are both anti-apoptotic members of Bcl-2 family. We found the Bcl-2 was only expressed in TALL-1 and thymocytes (Figure19D). In TALL-1, the expression was a little higher than it in thymocytes. Thus, Bcl-2 could partially explain the retarded apoptosis of TALL-1. In addition, Wehi7.1 highly expresses Bcl-xl. Together with the lower expression of GR, it is easy to understand why it is relatively insensitive to GC induced apoptosis.

Next, we want to know whether GC-induced TALL apoptosis shares some characteristics with thymocytes or peripheral T cells. We treated TALL cells with Dex in the presence or absence of different caspase inhibitors. After apoptosis induction, cell viability was measured by flow cytometry. We found that all of the TALL cell lines are similar to thymocytes since caspase-3 inhibitor, caspase-8 inhibitor, caspase-9 inhibitor and pan caspase inhibitor could prevent Dex induced apoptosis very efficiently (Figure 19). This indicates that TALL cell lines share a similar apoptosis signaling pathways as thymocytes. Because cycloheximide, cathepsin B inhibitor and lactacystin are toxic to Jurkat.GR at

lower concentration (data not shown), we didn't assess their effects on Dex induced TALL apoptosis.

A

Effect of different caspase inhibitors on Dex induced Jurkat GR apoptosis

B

Effect of different caspase inhibitors on Dex induced Wehi7.1 apoptosis

C

Effect of different caspase inhibitors on Dex induced TALL-1 apoptosis

D

β-tublin

Bcl-2

GR

Bcl-xl

TALL-1   Wehi7.1   Jurkat.GR   Thymocyte
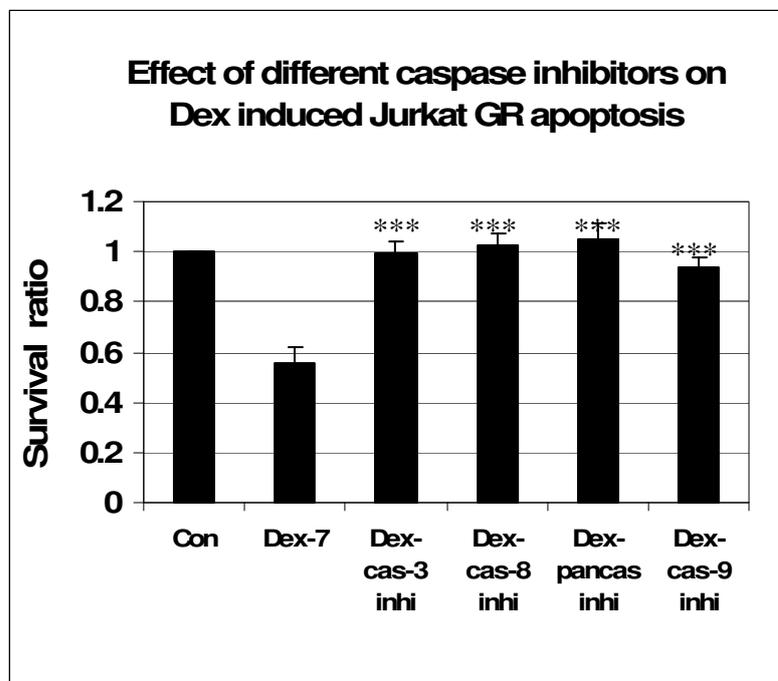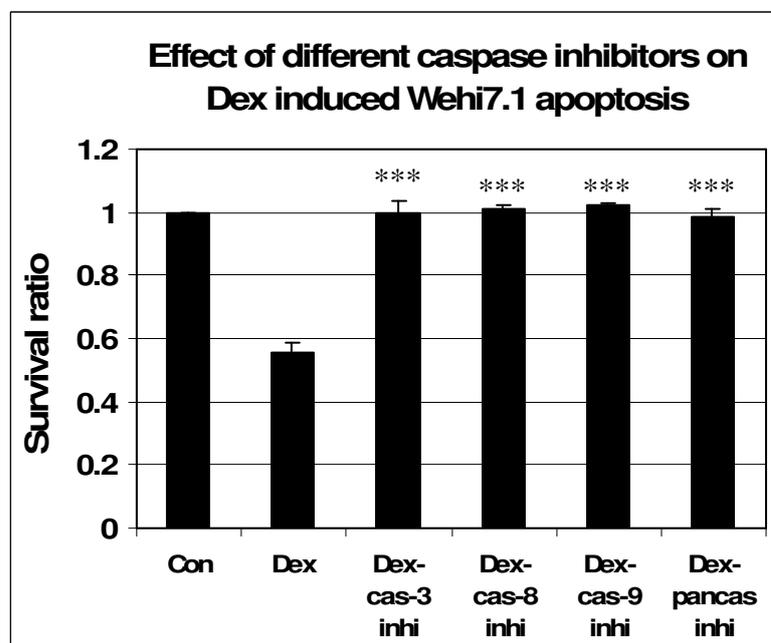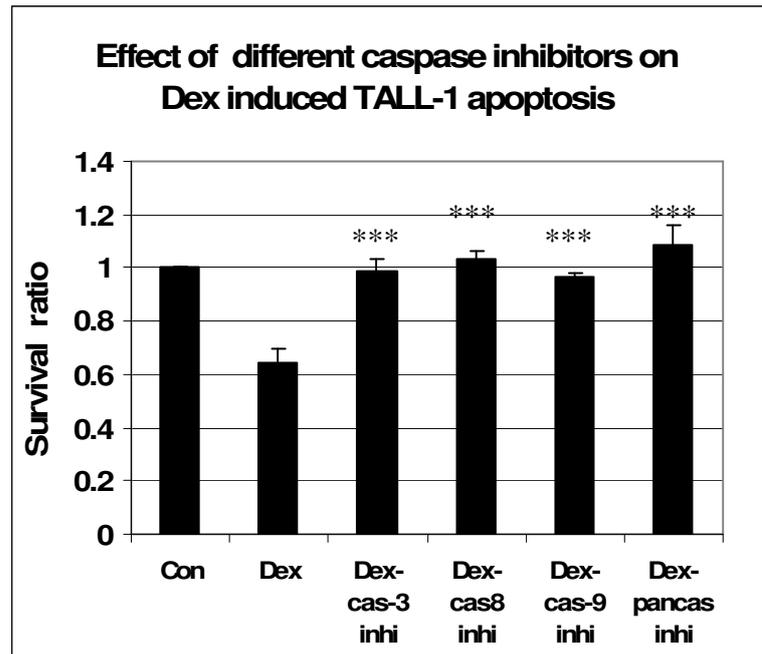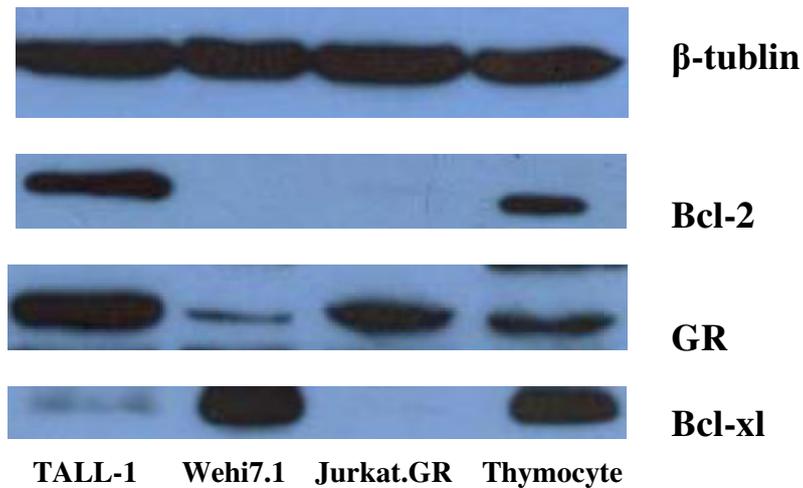
Figure19. The death signal transducing pathway of TALL cell lines is similar to thymocytes.

A. Jurkat-GR cells are treated with Dex (1×10^{-7}M) in the presence or absence of pancaspase inhibitor (10μM), caspase-3 inhibitor (10μM), caspase-8 inhibitor (10μM) and caspase-9 inhibitor (10μM) for 24hours. The survival ratio was determined by Annexin V-PI staining. Cells were cultured in 48 well plates at 5×10^{4} cells per well(300μl medium). B. Wehi7.1 cells are treated with Dex (1×10^{-5}M) in the presence or absence of pancaspase inhibitor (10μM), caspase-3 (10μM), caspase-8 inhibitor (10μM) and caspase-9

inhibitor(10μM) for 24hours. The survival ratio is determined by Annexin V-PI staining. Cells were cultured in 48 well plates at 5×10^4 cells per well(300μl medium). C.TALL-1 cell is induced apoptosis(1×10^{-5}M) in the presence or absence of pancaspase inhibitor (10μM), caspase-3 inhibitor (10μM), caspase-8 inhibitor (10μM) and caspase-9 inhibitor (10μM) for 72hours. The survival ratio is determined by Annexin V-PI staining. Cells were cultured in 48 well plates at 5×10^4 cells per well(300μl medium) D.The expression level of GR, Bcl-2 and Bcl-xl in the TALL cell lines was compared with those of thymocytes by Western-blot . β-tublin is used as loading control. One representative result out of three is shown here. The error bar is the SEM for three independent experiments. ANOVA was used for statistical analysis as depicted in Methods in this assay. Statistical significance is only depicted for the comparison of caspase inhibitor treatments with the Dex treated cells.

5.   Discussion

5.1 The initiation phase of GC induced thymocytes and peripheral T cells apoptosis was equal requirement for gene expression

Glucocorticoids have been used for the treatment of hematological malignancy for many years although the exact mechanism is not fully understood. Because of the GC resistance of some kinds of leukemia and the side effects of GCs, it's absolutely necessary to further clarify the mechanism of GC induced apoptosis. It will be of great help for the clinical application of GCs if this aim were achieved. Here, we try to clarify the signal transduction pathways of GC induced thymocyte and peripheral T cell apoptosis since both of them are main targets of GCs.

We found several differences between the signal transducing pathways of GC induced thymocytes and peripheral T cells apoptosis and maybe these difference could be used for the design of new drugs in the future.

First, the initiation phase of GC induced thymocytes and peripheral T cells apoptosis appear to be identical since both of them require the GR and gene expression. RU486 completely prevented apoptosis induced by GCs which indicates apoptosis is mediated by GR. We know that GR acts as a transcription factor and regulates gene expression. Whether transactivation or transrepression is responsible for apoptosis is still controversial. Helmberg et al. (Helmberg, 1995) reported induction of apoptosis by GCs in GR-deficient Jurkat cells after stable transfection with the transactivation-defective GR-LS7 mutant, arguing against a requirement for transactivation. GR dim mice, on the other hand, carry a functionally similar GR mutation, yet are deficient in GC-induced thymocyte apoptosis, suggesting that transactivation is necessary (Reichardt et al. 1998). In our experiments, the protein synthesis inhibitor, cycloheximide, could rescue both thymocytes and peripheral T cells from apoptosis. It seems that transactivation is indispensable although we can't exclude the possibility that transrepression was also

involved in this process. How to compromise this contradiction? In 1989 and 1990, E.B. Thompson's group (Antakly T, 1989; Gomi M, 1990) has reported and implicated GR upregulation in CCRF-CEM leukemia and certain myeloma cells during GC-induced cell death. By using DNA chip analyses, Schmidt (1994) found GR itself was one of the few genes upregulated in both proliferating and arrested CCRF-CEM cells. This finding was remarkable because GC downregulates GR expression in tissues which do not undergo apoptosis. It seemed that sustained GR expression might cause cell apoptosis or cell cycle arrest. By combining these concepts, it was proposed that the continuous repression of various metabolic pathways caused by GR upregulation (or the lack of its downregulation) might contribute to cell cycle arrest and ultimately lead to cell death (R. Kofler, 2000). Cell death induction might critically depend upon GR auto-upregulation with its persistent repressive activity of the GR, which ultimately leads to a metabolic disaster. This could resolve the controversy of whether gene transactivation or transrepression was involved in GC induced apoptosis. Most probably, the GR is one of the few genes which is transactivated after GC treatment. How the differential response of GR expression to GCs (induction in tissues sensitive to GC-induced apoptosis, repression in those that are not) is mediated is not known. Mauro Brunetti (1995) reported that cycloheximide accelerated GC induced human peripheral T lymphocytes apoptosis instead of rescue. The problem of these experiments was that the concentration at which cycloheximide was used was much higher than ours. Maybe at that concentration, cycloheximide was toxic to peripheral T cells although we haven't tested.

5.2 The proteasome plays an important role in GC induced thymocyte apoptosis but not in peripheral T cell

Second, the proteasome plays an important role in GC induced thymocyte apoptosis but not in peripheral T cells since the proteasome inhibitor, Lactacystin, could only rescue thymocytes from apoptosis. We know that the proteasome is a multicatalytic protease complex located in both the cytoplasm and nucleus that degrades proteins targeted for destruction by polyubiquitination (GoldbergAL, 1992; Rechsteiner M, 1993; Ciechanover A, 1994).Compared with caspases, much less is known about the role of the proteasome in

GC induced apoptosis. Our result is consistent with the work of Grimm et al (Grimm LM, 1996). They demonstrated that proteasome inhibitors block corticosteroid-induced cleavage of poly-ADP ribose polymerase (PARP), a downstream caspase target, and apoptosis in thymocytes. These findings raised the possibility that the proteasome may either degrade regulatory proteins that normally inhibit the apoptotic pathway, or may proteolytically activate proteins that promote cell death. In corticosteroid-induced apoptosis of thymocytes, proteasome activity appears to be involved at a step preceding mitochondrial changes and caspase activation. Beyette et al (1998) have shown that thymocytes are rich in proteasome and a chymotryptic component of proteasome activity decreases following dexamethasone treatment. Moreover, proteasome inhibitors prevented disruption of the mitochondrial transmembrane potential and also prevented exposure of phosphatidylserine and nuclear DNA fragmentation (Hirsch T, 1998). Pharmacologic stabilization of the mitochondrial permeability transition pore, or inhibition of caspase, did not prevent the activation of proteasome (Hirsch T, 1998; Dallaporta B, 2000). In summary, the multicatalytic proteasome appears to play an important role in the decision of a lymphoid cell to die following corticosteroid treatment. It is interesting that lactacystin only rescued thymocyte and leukemia (lymphoma) but not peripheral T cells from GC induced apoptosis. This indicates that the proteasome doesn't participate in GC induced peripheral T cell apoptosis or at least it is not indispensable although the proteasome plays an important role in GC induced thymocyte apoptosis. This also suggests that thymocytes and leukemia cell share some common pathway in GC induced apoptosis. The meaning of this difference is still waiting to be clarified. In addition, proteasome activity works upstream of mitochondrial dysfunction since proteasome inhibitor could prevent the loss of mitochondrial transmembrane potential ($\Delta\Psi$m) in thymocyte after GC treatment whereas stabilization of the $\Delta\Psi$m using the permeability transition pore inhibitor bongkrekic acid does not prevent the activation of proteasome(Tamara,1998).

5.3 Lysosomal proteases participate in GC induced thymocytes apoptosis but not peripheral T cells

Next, lysosomes, which together with late endosomes constitute the acidic vacuolar apparatus, are the main cellular compartment for intracellular degradation and contain a wide spectrum of hydrolytic enzymes including cathepsin B, cathepsin D and cathepsin L. In the classic apoptosis-necrosis paradigm, lysosomes were solely considered involved in necrotic and autophagic cell death. The lysosomal proteases were believed to take care only of nonspecific protein degradation within the lysosome. In recent years, however, it has become evident that the role of lysosomes in cell death is far more sophisticated. One of the first studies reporting an active role for lysosomal proteases in cell death was based on the cloning of ''regression selected genes'' in rat prostate and mammary glands after hormone ablation. Increased amounts of the lysosomal enzyme cathepsin B were found in the basal aspect of cells in regressing tissue, indicating that cathepsin B is required for the local degradation of the basement membrane, which is one of the earliest morphologically recognizable events of active cell death(Guenette RS, 1994). Active participation of lysosomal proteases has since then been observed in cell death induced by several stimuli. For example, cathepsin D was found to be released from lysosome in oxidative stress induced apoptosis of several cell types (Roberg K, 1998, 1999). The activated cathepsin could promote the release of cytochrome c from mitochondria and in turn activate caspase. Thus, a positive feedback loop was formed (Zhao M, 2003).

Guicciardi (Guicciardi ME, 2000, 2001) found that cathepsin B contributes to TNF-α-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. The release of activated cathepsin B was mediated by caspase-8. Deletion of the cathepsin B gene resulted in diminished liver injury and enhanced survival after treatment in vivo with TNF-α and an adenovirus construct expressing the IκBα suppressor (Guicciardi ME, 2001). Cathepsin B could act not only as initiator protease, but also act as execution protease in apoptosis. In TNF-α induced WEHI-S fibro-sarcoma cells apoptosis, Lasse

Foghsgaard (2001) found that cathepsin B acted as a dominant executioner protease. All the studies indicate that lysosomal cathepsin could act as mediators of apoptosis.

Studies with the synthetic lysosomotropic detergent MSDH had indicated that the key factor in determining the type of cell death is the magnitude of lysosomal permeabilization and the amount of proteolytic enzymes released into the cytosol (Li W, 2000). When MSDH was applied at low concentrations, resulting in partial lysosomal rupture, activation of pro-caspase-3-like proteases and apoptosis followed after some hours. Early during apoptosis, but clearly secondary to lysosomal destabilization, the mitochondrial transmembrane potential declined. At high concentrations, MSDH caused extensive lysosomal rupture and necrosis. It is suggested that lysosomal proteases, if released into the cytosol, may cause apoptosis directly by pro-caspase activation and/or indirectly by mitochondrial attack with ensuing discharge of pro-apoptotic factors. Several mechanisms to achieve the translocation of a balanced amount of lysosomal proteases to the cytoplasm, without risking a complete breakdown of the organelle and induction of necrotic cell death have been proposed. One theory involves accumulation of the lysosomotropic detergent sphingosine in the lysosomes, which could facilitate the release of lysosomal enzymes into the cytoplasm (Kagedal K, 2001). Another possible mechanism is the generation of reactive oxygen species, which also can induce lysosomal leakage. Indeed, experimental evidence suggests that reactive oxygen species–induced lysosomal permeabilization usually precedes mitochondrial dysfunction (Roberg K, 1998,1999)thereby creating a feedback loop in which mitochondrial-reactive oxygen species can lead to more lysosomal permeabilization (Zhao M, 2003). Recently, it has been described that heat shock protein 70, which antagonizes the apoptogenic effects of AIF, promotes cell survival by inhibiting lysosomal membrane permeabilization (Nylandsted J, 2004).

 The cysteine protease cathepsin B and L and the aspartatic protease cathepsin D are the most abundant lysosomal proteases. Cathepsin B and D are most stable at physiologic, cytoplasmatic pH and seem to have the most prominent role in apoptotic and necrotic like PCD (Guicciardi ME, 2004. Leist M, 2001). Cathepsin B has been shown to

translocate to the nucleus and thereby contribute to bile salt-induced apoptosis (Roberts, L.R, 1997). Indeed, cathepsin B can act as an effector protease, downstream of caspase in certain cell types (Foghsgaard L,2001; Jones B,1998), and is capable of executing cell death independent of the apoptotic machinery in WEHI-S fibrosarcoma and non–small cell lung cancer (NSCLC) cells (Broker LE, 2004; Foghsgaard L, 2001). Other reports have, however, showed that lysosomal proteases rather promote cell death more indirectly by triggering mitochondrial dysfunction and subsequent release of mitochondrial proteins (Boya P, 2003; Guicciardi ME, 2000; Johansson AC, 2003; Roberg K, 2002). This may occur via the Bcl-2 family protein Bid (Stoka, 2001; Heinrich M, 2004; Werneburg N, 2004), which is cleaved and translocated to the mitochondria after lysosomal disruption by lysosomotropic agents (Cirman T, 2004). In addition, cathepsin D can trigger activation of Bax, leading to selective release of AIF from the mitochondria and apoptosis in T lymphocytes (Bidere N, 2003). Finally, lysosomal proteases have been reported to directly cleave and activate caspases, thereby confirming that lysosomal permeabilization often is an early event in the apoptotic cascade (Ishizaki Y, 1998; Schotte P, 1998; Vancompernolle K, 1998).

The findings mentioned above aroused our interest to investigate the role of lysosomal cathepsins in GC induced thymocyte and peripheral T cells apoptosis. Here, we report for the first time that cathepsin B is involved in GC induced thymocyte apoptosis. After GC treatment, activated caspase-9 leads to the release of cathepsin B from lysosome. Then, the released cathepsin B could activate caspase-8 and caspase-3. Although the mechanism that caspase-9 could cause the permeabilization of lysosome is still unknown, a similar phenomenon was reported in TNF-α–mediated hepatocyte apoptosis in which activated caspase-8 could induce the release of cathepsin B and the released cathepsin B in turn accelerated the release of cytochrome c from mitochondria. Maybe this is also true in our apoptosis model although we haven't tested it. Our results also showed that cathepsin B activity was an early event after GC treatment since cathepsin B activity could be observed as early as 30 minutes after GCs treatment. Thus, cathepsin B activation is an early step of GCs-induced apoptosis, initiating a series of events which rapidly lead to thymocyte death. Whether cathepsin B could act as effector protease in this apoptosis

model is still not known. But, at least, it won't be the only executioner since inhibition of cathespin B activity could only partially rescue thymocyte from apoptosis.

The position of cathepsins in death signaling pathways of cathepsins triggered apoptosis is still controversial. Some experiments provided direct evidence that cathepsins could process caspases (Ishizaki Y, 1998; Schotte P, 1998; Vancompernolle K, 1998) whereas other experiments showed the major human apoptosis relevant caspases are poor direct targets for lysosomal extracts or a number of cysteine cathepsins. Stoka (2001) present a striking new explanation for apoptosis activation by cathepsins. They demonstrate that the pro-apoptotic Bcl-2 family member Bid is cleaved at the peptide loop joining the p15 and p7 subunits by lysosomal proteases in an in vitro system combining mitochondria and cytosol. Thus, very much in analogy to the cleavage by caspase-8, a Bid species is created that can potently trigger cytochrome c release from mitochondria. The authors go further in their experiments by demonstrating that Bid cleavage is in fact the major event leading to cathepsin-mediated cytochrome c release, since cytosol from Bid deficient mice was hardly functional in their assay. In GC induced thymocyte apoptosis, cathepsin B was activated by caspase-9 and most probably activated cathepsin B cleaves Bid and accelerates the release of cytochrome c. Thus, a positive feedback is formed. Whether cathepsin B could activate caspase-8 and caspase-3 directly is unknown in this case. Vancompernolle (Vancompernolle, 1998) reported that cathepsin B can't activate caspase-3 directly. Since caspase-9 could also activate caspase-8 and caspase-3, it's possible that cathepsin B activates caspase-8 and caspase-3 through increasing the activation of caspase-9. Someone may argue that in our experimental system high concentration of caspase inhibitor (100μM) may cross react with cathepsin B so the results are unreliable. According to the literature (Schotte.P, 1999), the pan-caspase inhibitor (Z-VAD-FMK) could cross react with cathepsin B and no one has reported caspase-9 inhibitor (Z-LEHD-FMK) could also cross react with it. On the other hand, caspase-8 and caspase-3 inhibitor can't inhibit the activation of cathepsin B (data not shown). This could also prove that caspase-9 inhibitor won't react with cathepsin B. If not, caspase-8 and caspase-3 inhibitor should also prevent cathepsin B activity. Since pan-caspase inhibitor and caspase-3 inhibitor could inhibit cathepsin B to some extent

(Vancompernolle,1998), it is possible that the rescue effect of these two caspase inhibitors on GC induced thymocytes apoptosis partially arise from the inhibition of cathepsin B.

As for the cathepsin B activity of peripheral T cells, there was no difference between the control and the GC treatment. Flow cytometry showed that cathepsin B inhibitor didn't prevent GC induced peripheral T cell apoptosis. All these data told us cathepsin B was not involved in GC induced peripheral T cells apoptosis although it played an important role in antithymocyte globulins induced human peripheral T cell apoptosis (Marie-Ce,2003). This difference also tells us the apoptotic signaling pathway is stimulus specific.

5.4 Calpain is not involved in GC induced thymocytes and peripheral T cell apoptosis

Calpain has been implicated in the cleavage and targeting of Bax to the mitochondria. Recently, calpain has also been reported to cleave Bid, resulting in a cleavage product that induces cytochrome c release from mitochondria (A.Mandic,2002, M. Chen,2002).Calpain mediated Bid-cleavage has been shown to be important in cisplatin-induced apoptosis (A.Mandic,2002) and in ischemia/reperfusion injury (M. Chen,2002). Importantly, calpain activation seems to be downstream of a rise in Ca^{2+} concentration in both cisplatin and ischemia/reperfusion-induced apoptosis.

In 1997, calpain was reported to regulate GCs induced thymocytes apoptosis (Margaret K. 1997). Several calpain inhibitors, including the Ca^{2+} binding site inhibitor, PD150606, could rescue GC induced thymocytes apoptosis. In contrast, we didn't get the similar results in our experiments although we tried a wide range of PD150606 concentration. PD150606 rescued neither thymocyte nor peripheral T cells apoptosis. We compared our experimental protocol with theirs and there wasn't any obvious difference. So, the role of calpain in GCs induced thymocyte and peripheral T cell apoptosis is still controversial.

5.5 Caspase plays an important role in GC induced thymocyte apoptosis but not peripheral T cell apoptosis

Two methods are usually used to study the role of caspase family members in apoptosis. One is gene knock out and the other is the application of specific caspase inhibitors. We choose the latter since it is not easy to get the caspase knock out mice. From the previous gene knock out experiments, we have known GC induced thymocytes apoptosis was Apaf-1(Cecconi, F, 1998; Yoshida, 1998) and caspase-9 dependent (Hakem R, 1998; Kuida K, 1998). Our inhibitor assay was consistent with these reports since caspase-9 inhibitor could rescue thymocyte from GC induced apoptosis. Moreover, caspase-8 and caspase-3 were also involved in GC induced thymocyte apoptosis. Both caspase-8 and caspase-3 inhibitor could rescue GC induced thymocyte apoptosis although the rescue was not complete. The result of flow cytometry and Western-blot further confirmed the role of caspase-8 and caspase-3 since they were activated as early as 2 hours after GC treatment. Using gene knock out mice (Li P, 1997. Kuida K, 1996.), Li P and Kuida K reported caspase-3 was not essential for GC induced apoptosis. This didn't contradict with our results because caspase-3 inhibitor only partially rescued GC treated thymocyte and the involvement of caspase-3 does not mean it was indispensable. It is possible that some caspase-3 independent mechanism exists and the new executioner has not been found out. In other words, several parallel signal transducing pathways may work at the same time. One investigation suggested that caspase-6, instead of caspase-3(Miyashita T. 1998) acted as the executioner. Our caspase-6 inhibitor assay showed it can't prevent GC induced apoptosis. Most probably, this difference arises from the different experimental system. In order to delineate the sequence of caspase activation, we did caspase-3 and caspase-8 activation assay in the presence of relevant caspase inhibitor. We found caspase-9 inhibitor could inhibit the activation of both caspase-8 and caspase-3 and caspase-8 inhibitor could inhibit caspase-3 activation. This indicates that caspase-9 works upstream of caspase-8 and caspase-3, and caspase-8 works upstream of caspase-3. Obviously, in GC treated thymocytes, death signal are transduced along the mitochondrial pathway. This conclusion is consistent with previous reports. The new

finding is that cathepsin B could regulate the activation of caspase activity in thymocytes .We found in the presence of cathepsin B inhibitor, caspase-8 and caspase-3 activation were suppressed whereas in the presence of caspase-9 inhibitor, cathepsin B activity was completely deleted. This suggested that the activated cathepsin B could activate caspase-8 and /or caspase-3 although the mechanism is still unknown. Thus, two parallel caspase-3 activation pathways work at the same time. Caspase-3 is only sufficiently activated at initiating apoptosis when the two pathways act in concert. The activation of cathepsin B needs caspase-9. Then, we arrived to the questions mentioned above. How caspase-9 could act on lysosomes and how activated cathepsin B regulated capase-8 and /or caspase-3 activation? By now, there is no literature which explained the mechanism of this phenomenon. More work should be done to solve this problem.

As to peripheral T cells, no caspase inhibitor prevented GC induced apoptosis although we have tried nearly all the caspase inhibitors at several different working concentration. It seems that GC induced peripheral T cells apoptosis is caspase independent. It was reported that caspase-3 was activated during GC induced peripheral T cell apoptosis (Hadassah Sade, 2003). We don't think their results are reliable because they only found the expression of caspase-3 was decreased and they didn't found the caspase-3 cleaved band. Further more, they didn't investigate the role of other caspases. Usually caspase-3 works downstream in the caspase cascade. Some initiator caspase, either caspase-9 or caspase-8, should be activated before caspase-3 activation. Our flow cytometry results clearly showed that in peripheral T cells both caspase-8 and caspase-3 were intact after GC treatment and no caspase inhibitor could rescue peripheral T cell from apoptosis. The results of caspase-3 Western-blot corroborated the result of flow cytometry. Thus, our results are more persuasive.

5.6 The role of Bcl-2 family members in GC induced thymocyte and peripheral T cell apoptosis

The pro-apoptotic members of the Bcl-2 family are categorized into two groups based on their structure, i.e., multi-domain members and BH3- only members. Proteins in these two categories are also functionally distinct. Multi-domain members such as Bax and Bak act as a gateway for a variety of apoptotic signals, since cells from Bax/Bak-double knockout mice are totally resistant to numerous apoptotic stimuli. (Lindsten, 2000; Wei, 2001b). Bax and Bak are functionally redundant in many types of cells, although functional differences between them have been suggested in certain types of cells (Putcha et al., 2002). BH3-only proteins seem to function as death signal sensors (Puthalakath and Strasser, 2002). Most BH3-only proteins (including Bid, Bim, Bmf, and Bad) are localized outside the mitochondria in living cells. After apoptotic stimulations, these proteins are modified by several different mechanisms and translocated to the mitochondria, leading to increased permeability of the outer mitochondrial membrane.

The change of Bcl-2 family members was only observed in GC treated thymocytes but not peripheral T cells. Bax and Bcl-xl underwent translocation from cytosol to mitochondria in thymocyte after Dex treatment. This was consistent with the previous report (Hsu, 1997). The difference is that we only find the partial translocation of Bax whereas in the previous report Bax translocated at large scale. Maybe the reason is that we used lower concentration of Dex to induce apoptosis so the translocation is not so evident. In another report, Amotz Nechushtan (Nechushtan, 2001) found that Bax underwent translocation and multimerization after Dex treatment. But most of the Bax clusters existed outside the mitochondria, only a very small percentage of Bax clusters inserted in the mitochondria membrane. It seemed that a small percentage of translocated Bax was enough to induce the release of cytochrome c and other mitochondrial interspace proteins. Bcl-xl is present in both soluble and membrane bound forms under normal conditions. Bcl-xl could prevent the multimerization and translocation of Bax when cell undergoes apoptosis. But how Bcl-xl interact with Bax is still not known.

5.7 Glucocorticoid could influence the homestasis of murine thymus

The sensitivity of different thymus cell populations to GC is different. DP thymocyte is more sensitive than CD4 or CD8 single positive thymocyte. At physiological GC concentration, single positive thymocytes are insensitive to GC. This could be attributed to the costimulation signal of CD28. When single positive thymocytes were treated with GC in vitro, they were also sensitive to GC. Whereas apoptosis was prevented when thymocytes were cocultured with B7.1/7.2 high expressing cells (wagner, 1996). It was hypothesized that CD28 costimulating signal rescued single positive thymocytes from apoptosis. Our group proved this hypothesis using the CD28 superagonist JJ316 (Van den Brandt J, 2004). We found that in single positive thymocytes this protection depends on the regulation of Bcl-xl expression. Also, calcium and PI3k signaling were critical since CsA and LY294002 could contravene this protection. In this contest, we tested this hypothesis also on peripheral T cells. As we have expected, JJ316 rescued peripheral T cells apoptosis induced by GC. Bcl-xl expression was increased in peripheral T cells treated with GCs. How does CD28 signaling regulate Bcl-xl expression is not known. Similar to thymocytes, the CD28 signaling pathway involved calcium and PI3K since CsA and LY294002 could contravene the protection effect of JJ316.

5.8 TALL cell lines share similar signaling pathways as thymocytes in GC induced apoptosis

GCs have been used for the treatment of hematological malignancy including leukemia and lymphoma for many years. Unfortunately, until now, the exact mechanism of GC induced leukemia cell apoptosis is still not clarified. Because some hematological malignancy could resist GC and GC could also kill healthy peripheral T cells, it is necessary to find out the difference of signaling pathways between leukemia cell and peripheral T cells. Thus, new GC application strategy and /or new GC derivatives could

be used to avoid the side effects and to improve its efficacy. To realize this aim, we first compared the GR expression in the TALL cell lines and thymocytes by western-blot. The GR expression is very low in Wehi7.1. In contrast, Jurkat GR and TALL-1 cells express GR at nearly the same level as thymocytes or even higher. Next, we compared the expression of Bcl-2 and Bcl-xl. Compared with thymocytes, Bcl-xl is highly expressed in Wehi7.1 and Bcl-2 is highly expressed in TALL-1 whereas none of them is expressed in Jurkat GR. Since Bcl-2 and Bcl-xl are anti-apoptotic members of Bcl-2 family, together with the low GR expression in Wehi7.1, this could explain why Jurkat GR cells are more sensitive to GC induced apoptosis than Wehi 7.1 and TALL-1. Lower GC concentrations (10^{-7}M) and a shorter treatment time with GCs (24h) are sufficient for apoptosis induction of Jurkat GR, whereas higher Dex concentration (10^{-5}M) and/or a longer treatment time (72h) are needed for Wehi7.1 and TALL-1. Thus, the regulation of GR and anti-apoptotic Bcl-2 family member expression is one of the mechanisms underlying the observation that TALL cell lines are relatively resistant to GC treatment. Last, we compared the signal transduction pathways of TALL cell lines with that of thymocytes and peripheral T cells and we found the signal transducing pathway of GCs induced TALL cell apoptosis was similar to thymocytes. Caspase inhibitors could efficiently rescue TALL from GC induced apoptosis as they did in thymocytes, which indicates that GC induced TALL apoptosis was caspase dependent. We also analysed the role of proteasome and cathepsin B in GCs induced TALL apoptosis. Unfortunately, the proteasome inhibitor, lactacystin, and the cathepsin B inhibitor are toxic to TALL cell line at very low concentrations, which made it impossible to compare. All in all, our data showed that the signaling pathways of GC induced TALL apoptosis resembles thymocytes. This is also consistent with previous reports.

5.9 Possible players in GC induced peripheral T cell apoptosis

Compared with thymocytes, we have less knowledge about the signaling pathways of GC induced peripheral T cell apoptosis. We only know that it was GR mediated and gene transactivation dependent. Caspases, the proteasome and cathepsin B were not involved in this process. Usually, cell undergoes apoptosis along two different pathways. The death

receptor pathway and the mitochondria pathway (Figure1, copied from Stephanie Greenstein, 2002). Although the death receptor pathway has been clarified, there are still some gaps in the mitochondria pathways. Obviously, thymocytes undergo GC induced apoptosis along the mitochondria pathways. The release of cytochrome c, the activation of caspase-9, caspase-8 and caspase-3 proved this notion. But none of them was involved in GC induced peripheral T cell apoptosis. So, neither the death receptor pathway nor the classical mitochondrial pathway was responsible for GC induced peripheral T cell apoptosis. Then, how was the death signal transduced? By now, this signaling network hasn't be delineated. Few players were identified and too many gaps exist. In recent years, some caspase independent apoptosis pathways have been reported. Two mitochondrial proteins, AIF and Endo G played an important role in some apoptotic model.

Apoptosis-inducing factor (AIF) is a phylogenetically ancient mitochondrial intermembrane flavoprotein endowed with the unique capacity to induce caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation. AIF was first characterized by Kroemer (Susin, 1999). The idea that AIF can induce caspase-independent death is based on several pieces of evidence. The mitochondria-nuclear translocation of AIF is in a caspase independent fashion, at least in some examples of apoptosis. Similarly, the translocation of AIF can be observed in vitro in cells in which there is no caspase activation, owing to knockout of Apaf-1, caspase-9 or caspase-3 (Susin, 2000). This AIF translocation also occurs in vivo in mice lacking Apaf-1, which fail to activate caspases (Cecconi, 1998; Yoshida, 1998). In contrast, there is crosstalk between AIF and the caspase cascade at several levels. When caspase activation occurs early during apoptosis, for instance in CD95-triggered cell death, the release of AIF is secondary to activation of caspase-8 (Susin, 1997). Similarly, in etoposide-induced apoptosis, the activation of caspase-2 occurs upstream of MMP and presumably upstream of the release of AIF (Lassus, 2002; Robertson, 2002). This suggests that, at least in some cases, AIF can be required for the cytochrome-c-dependent caspase activation cascade. However, in other examples of cell death, mitochondria release AIF

well after cytochrome c (Cregan, 2002), which underlines the idea that different modes of MMP can operate in apoptosis.

In GCs induced peripheral T cells apoptosis, AIF may work in a caspase independent fashion since no caspase activation was observed and caspase inhibitor can't prevent apoptosis.

Eukaryotic EndoG is a divalent cation-dependent endonuclease described in chicken erythrocytes in 1987 (Ruiz-Carrillo, 1987). EndoG is encoded by a nuclear gene and is mainly associated with functions in DNA repair or mitochondrial DNA (mtDNA) duplication. Other than its function in mtDNA replication, a new role in the apoptotic process has been assigned to EndoG. Two elegant studies disclosed EndoG's 'double life', as it is the case for other apoptogenic proteins (i.e, AIF, cytochrome c, and Omi/HtrA2) (Li, L.Y, 2001; Parrish, J, 2001). These works demonstrated that upon apoptotic stimulus (including tBid, Bax, calcium and chemotherapeutic drugs), EndoG translocates from mitochondria to nucleus, where extensively degrades nuclear DNA into oligonucleosomal fragments, similar to those generated by the caspase effector CAD (caspase-activated DNase).

In 1992(Ana M) reported that IL-2 and IL-4 could selectively rescue Th cell subsets from GC induced apoptosis which indicated the cross talk between GC signaling pathway and the cytokine signaling pathway. The interesting thing is that the Th subset could only be rescued by their own growth factor. IL-2 could only rescue Th1 subset and IL-4 could rescue Th2 subset. This further tells us the cell type specificity of GC signaling pathway. Probably the rescue was mediated by protein kinases because the protein kinase inhibitor H7 could block the action of IL-2 and IL-4. IL-7 could rescue GC induced human activated T lymphocytes by maintaining the Bcl-2 expression (Trinidad, 1995) suggesting the role of Bcl-2 family members in GC induced peripheral T cell apoptosis. Hadasssah (2003) found IL-7 could inhibit dexamethasone induced peripheral T cell apoptosis by preventing the loss of Bcl-xl and IAP-2. The regulation of Bcl-xl and IAP-2 occurs along different pathways. The former is PI3k independent and the latter is PI3K dependent.

This is different from our result. In our work, CD28 signaling increased the expression of Bcl-xl via PI3K. How to compromise this contradiction is still a problem. More work should be done to solve this problem.

6   Summary

Glucocorticoids (GCs) are small lipophilic compounds that mediate a plethora of biological effects by binding to the intracellular glucocorticoid receptor (GR) which, in turn, translocates to the nucleus and directly or indirectly regulates gene transcription. GCs remain the cornerstone in the treatment for a number of hematological malignancies, including leukemia, lymphoma and myeloma. Extensive literature suggests that the efficacy of GCs stems from their ability to mediate apoptosis. Despite the enormous strides made in our understanding of regulated cell death, the exact mechanism by which GCs cause apoptosis is still unknown. The data obtained so far provide strong evidence that gene transactivation by the GR underlies the initiation phase of GC-induced thymocyte apoptosis. Furthermore, the multicatalytic proteasome, several members of the Bcl-2 family, changes in calcium flux as well as caspases have been identified as important players in the execution phase of GC-mediated cell death. However, the exact sequence of events in this process still remains elusive.

A major problem of the current discussion arises from the fact that different cell types, such as thymocytes, peripheral T cells and lymphoma cells are compared without acknowledging their different characteristics and gene expression profiles. Although it is generally assumed that GCs induce apoptosis via a conserved mechanism, this is not supported by any data. In other words, it is possible that thymocytes, peripheral T cells and lymphoma cells may undergo cell death along different pathways. We therefore wondered whether a unique signal transduction pathway is engaged by GCs to initiate and execute cell death in all types of T lymphocytes or whether distinct pathways exist. Therefore, we compared the role of the proteasome, various caspases, the lysosomal compartment and other factors in GC-induced apoptosis of murine thymocytes and peripheral T cells as well as T-ALL lymphoma cells. Our findings show that the initiation phase of GC-induced apoptosis is similar irrespective of the differentiation state of the cell. Apoptosis in both thymocytes and peripheral T cells is mediated by the GR and

depends on gene transcription. In contrast, the execution phase significantly differs between thymocyte and peripheral T cells in its requirement for a number of signal transduction components. Whilst in thymocytes, the proteasome, caspases 3, 8 and 9 as well as cathepsin B play an important role in GC-induced apoptosis, these factors are dispensable for the induction of cell death in peripheral T cells. In contrast, changes in the expression and intracellular location of Bcl-2 family members do not appear to contribute to GC-induced apoptosis in either cell type. Importantly, our observation that GC treatment of thymocytes leads to an activation of the lysosomal protease cathepsin B and that this is an essential step in the induction of cell death by GCs, is the first indication that a lysosomal amplification loop is involved in this process.

Analysis of GC-induced apoptosis in several T-ALL cell lines further indicates that the signaling pathway induced by GCs in thymocytes but not in peripheral T cells is shared by all lymphoma cell-types analyzed. Given the therapeutic importance of high-dose GC-therapy for the treatment of hematological malignancies, this finding could potentially form a basis for new anti-cancer strategies in the future, which specifically target tumor cells whilst leaving peripheral T cells of patients untouched.

7.   Zusammenfassung

Glucocordicoide sind kleine lipophile Verbindungen, die viele biologische Effekte verursachen, wenn sie an den intrazellulären Glukokortikoidrezeptor (GR) binden. Dieser wandert wiederum in den Nucleus, um dort direkt oder indirekt die Transkription der Gene zu regulieren. Glukokortikoide sind der Grundstein in der Behandlung für eine Anzahl von hämatologischen bösartigen Erkrankungen, wie Leukämie, Lymphome und Myelome. In der Literatur wird beschrieben, dass Glukokortikoide über die Vermittlung von Apoptose wirken.die Wirkung. Trotz der enormen Fortschritte im Verständnis des regulierten Zelltodes, ist der genaue Mechanismus, den Glukokortikoide bei der Apoptose vermitteln, unbekannt. Die Daten, die bis jetzt erzielt wurden, deuten stark darauf hin, dass Gentransaktivierung durch den GR für den Beginn der durch Glukokortikoide verursachten Thymozytenapoptose verantwortlich ist. Außerdem wurde gezeigt, dass das multikatalytische Proteasom, einige Mitglieder der BCL2-Familie, Änderungen im Kalziumfluss sowie Caspasen eine wichtige Rolle in der Durchführungsphase des durch Glukokortikoide vermittelten Zelltodes spielen Jedoch ist die genaue Reihenfolge dieses Prozesses bisher nicht bekannt.

Ein Hauptschwierigkeit der gegenwärtigen Diskussion entsteht aus der Tatsache, dass unterschiedliche Zellarten, wie Thymozyten, reife T-Zellen und Lymphomzellen verglichen werden, ohne ihre unterschiedlichen Eigenschaften und Genexpressionsprofile zu beachten. Obwohl angenommen wird, dass Glukokortikoide Apoptose über einen konservierten Mechanismus, wird dies nicht durch irgendwelche Daten unterstützt. In anderen Worten, es ist möglich, dass Apoptose in Thymozyten, reifen T-Zellen und Lymphomzellen über unterschiedliche Signalwege vermittelt wid. Wir fragten uns daher, ob ein einzelner durch Glukokoritkoide eingeleiteter Signaltransduktionsweg dafür verantwortlich ist, dass Apoptose in allen T-Lamphozytenarten eingeleitet wird, oder ob noch andere Signalwege existieren. Daher verglichen wir die Rolle des Proteasomes, verschiedener Caspasen, des lysosomalen Kompartements und anderer Faktoren in der durch Glukokortikoide induzierten Apoptose in Mausthymozyten und pepripheren T-

Zellen sowie T-ALL Lymphomzellen. Unsere Entdeckungen zeigen, dass die Anfangsphase der durch Glukokortikoide induzierten Apoptose unabhängig von der Differenzierungsstadien der Zelle ist. Apoptose wird sowohl in Thymozyten als auch in reifen T-Zellen durch den GR vermittelt und ist von der Gentranskription abhängig. Im Gegensatz dazu unterscheidet sich die Durchführungsphase erheblich in ihren Anforderungen für eine Anzahl von Signaltransduktionskomponenten zwischen Thymozyten und peripheren T-Zellen. Während in Thymozyten das Proteasom, die Caspasen 3, 8 und 9 sowie Cathepsin B eine wichtige Rolle in durch Glukokortikoide induzierten Zelltod spielen, sind diese Faktoren für die Induktion des Zell-Todes in peripheren T- Zellen entbehrlich. Im Gegensatz dazu scheinen Änderungen in der Expression und intrazellulären Lokalisation von Mitgliedern der Bcl-2 Familie nicht zum durch Glukokortikoide induzierten Zelltod beitzutragen, egal um welchen Zelltyp es sich handelt. Wir haben beobachtet, dass eine Behandlung von Thymozyten mit Glukokortikoiden zu einer Aktivierung der lysosomalen Protease Cathepsin B führt. Dies ist ein essentieller Schritt zur Einleitung von Apoptose durch Glukortikoide und zeigt zum ersten Mal, dass der lysosomale Amplifikationsloop in diesen Prozess involviert ist.

Die Analyse des durch Glukokortikoide induzierten Zelltodes in verschiedenen T-ALL Zelllinien deutet darauf hin, dass die durch Glukokortikoide induzierten Signalwege in Thymozyten und allen Lymphonzelllinien aber nicht in peripheren T Zellen übereinstimmen. Da die hoch-dosierte Glukokortikoidbehandlung eine wichtige Rolle in der Behandlung von hematologischen bösartigen Erkrankungen spielt, können unsere Beobachtungen eine Grundlage für eine neue Anti-Krebs-Stragie bilden, die darauf ausgelegt ist, spezifisch Tumorzellen zu eliminieren aber reife T-Zellen unberührt lassen.

8.   Reference

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9. Abbreviation

AIF	**apoptosis inducing factor**
ACTH	**adrenocorticotrophic hormone**
ACTR	**activator of thyroid hormone and retinoid receptors**
AP-1	**apoptotic protease-activating factor**
APS	**Ammonium Persulfate**
BSS	**balanced salt solution**
BSA	**bovine serum albumin**
CAD	**caspase-activated deoxyribonuclease**
CBG	**corticosteroid-binding globulin**
CBP	**CREB binding protein**
Cdk2	**cyclin dependent kinases**
CHOP	**Cyclophosphamide, Adriamycin, Vincristine, Prednisone**
CREB	**cAMP response element binding protein**
CsA	**cyclosporine A**
cyt c	**cytochrome c**
Dex	**dexamethasone**
DP	**double positive**
ECL	**enhanced chemo luminescence**
EndoG	**Endonuclease G**
ER	**endoplasmic reticulum**
FACS	**fluorescence activated cell sorting**
FCS	**fetal calf serum**
GC	**glucocorticoid**
GCs	**glucocorticoids**
GR	**glucocorticoid receptor**
GRE	**glucocorticoid responsive elements**
GRIP1	**glucocorticoid receptor interacting protein 1**
HAT	**histone acetytransferase**

Hsp	**heat shock protein**
IAP	**inhibitor of apoptosis**
IP	**intra peritoneal**
IP3R	**inositol trisphosphate receptor**
PAGE	**polyacrylamide gel electrophoresis**
PARP	**poly-ADP ribose polymerase**
PI3K	**phosphatidylinositol 3-kinase**
PKC	**protein kinase C**
PTP	**permeability transition pore**
PVDF	**Polyvinylidene fluoride**
rCD28 Ab	**recombinant anti-CD28 antibody**
SSC	**standard serum complement**
SP	**single positive**
SRC-1	**steroid receptor coactivator- 1**
T-ALL	**human acute T cell leukemia cell line**
TEMED	**N,N,N´,N´-Tetraethylendiamin**
TGS	**Tris-glycin-SDS protein electrophoresis buffer**
TIF2	**transcriptional intermediary factor 2**
Z-DEVD-FMK	**Z-D(OMe)-E(OMe)-V-D(OMe)-FMK**

10.   *Curriculum Vitae*

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11.   Publication

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