

## Review

# Glucocorticoids in T cell apoptosis and function

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**Abstract.** Glucocorticoids (GCs) are a class of steroid hormones which regulate a variety of essential biological functions. The profound anti-inflammatory and immunosuppressive activity of synthetic GCs, combined with their power to induce lymphocyte apoptosis place them among the most commonly prescribed drugs worldwide. Endogenous GCs also exert a wide range of immunomodulatory activities, including the control of T cell homeostasis. Most, if not all of these effects are mediated through the glucocorticoid receptor, a member

of the nuclear receptor superfamily. However, the signaling pathways and their cell type specificity remain poorly defined. In this review, we summarize our present knowledge on GC action, the mechanisms employed to induce apoptosis and the currently discussed models of how they may participate in thymocyte development. Although our knowledge in this field has substantially increased during recent years, we are still far from a comprehensive picture of the role that GCs play in T lymphocytes.

**Key words.** Apoptosis; glucocorticoid; T cell development; caspase; Bcl-2 family; transgenesis; gene targeting.

## Introduction

T cells form a major branch of the acquired immune system [1]. They derive their name from the thymus, where they develop to immunocompetent T lymphocytes through a series of lineage and selection steps. This ensures that all cells which lack a functional T cell receptor (TCR) or bear a TCR with useless or dangerous specificity are deleted [2]. In the periphery, mature T cells undergo clonal expansion upon encountering their cognate antigen. Once the invading pathogen is cleared, the superfluous lymphocytes are removed in a process known as activation-induced cell death (AICD) [3]. Thus, both during thymocyte development and T cell-mediated immune responses, it is essential that cells are deleted in a controlled manner to prevent immunopathologies such as

autoimmunity and cancer. This is achieved by a process called apoptosis [4] which, among other things, involves a series of proteolytic events that result in characteristic morphological alterations and ultimately lead to cell death.

Glucocorticoids (GCs) are a class of steroid hormones which display potent immunomodulatory activities including the ability to induce T lymphocyte apoptosis [5]. Although this was one of the first recognized forms of programmed cell death, it is still poorly understood [6]. GCs exert most, if not all, of their effects through binding to the glucocorticoid receptor (GR), a ligand-activated transcription factor [7]. While GR-mediated gene activation is clearly an essential component of the apoptotic pathway [8], the downstream events are debatable. Although GC-induced cell death does not directly proceed via one of the two classical apoptotic pathways, Bcl-2 proteins and caspases still appear to

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be involved in this process [5, 9, 10]. Furthermore, the physiological role of GC-induced T cell apoptosis is also not fully resolved. GCs have been hypothesized to direct positive and negative selection in the thymus [11], to limit AICD during the contraction phase of an adaptive immune response [12] and to induce generalized thymocyte apoptosis after polyclonal T cell activation [13]. This has been assigned to effects of both adrenally derived hormones as well as GCs synthesized within the thymus itself. Thus, not only the mechanism but also the physiological function of GC-induced apoptosis requires further investigation. Owing to their ability to mediate apoptosis in leukemia, lymphoma and myeloid cells, synthetic GCs are among the most widely prescribed drugs for the treatment of hematological malignancies [10, 14]. Consequently, a better understanding of GC-induced apoptosis is not only of theoretical interest but is also urgently required to improve the suitability of these drugs for clinical therapy. In this review we try to provide a broad picture of how GCs contribute to the development and apoptosis of thymocytes and mature T cells and the molecular mechanisms, signaling pathways and enzymatic systems that may be utilized during their action. Furthermore, we summarize how the generation and analysis of transgenic, knock-out and knock-in mice has contributed to our present knowledge about these processes [15]. We hope this provides the basis for a better understanding of this important class of steroid hormones and their role in T cell immunology.

## The molecular and physiological basis of GC action

### The glucocorticoid receptor

The GR belongs to the nuclear receptor superfamily which are characterized by their common arrangement of functionally distinct domains mediating transactivation, DNA binding, nuclear localization, dimerization and ligand recognition [16]. In the absence of hormone, the GR is found within a multimeric complex of heat shock proteins and immunophilins in the cytoplasm [17] (fig. 1). GCs such as cortisol in humans and corticosterone in rodents diffuse passively into the cell, where they bind to the GR [7]. Following dissociation from the heat shock protein complex, the GR translocates into the nucleus. There it recognizes imperfect palindromic sequences, so-called glucocorticoid response elements (GREs), present in the promoter and enhancer regions of a variety of genes [18]. The GR can drive transcription from these response elements by using its surfaces as platforms for the docking of transcriptional coactivators that are capable of altering the local chromatin or recruiting and stabilizing the transcription machinery [19].

Although many genes are known to be upregulated in response to GCs, including some important to the activation, function and apoptosis of immune cells, such as Bcl-x<sub>L</sub> [20], IκB [21], GILZ [22] and GITR [23], there is scant evidence that they are directly upregulated through GREs. Genes which have been shown to be positively regulated through well-defined GREs *in vivo* include tyrosine amino transferase [24] and phosphoenolpyruvate carboxykinase [25]. In contrast to these DNA binding-

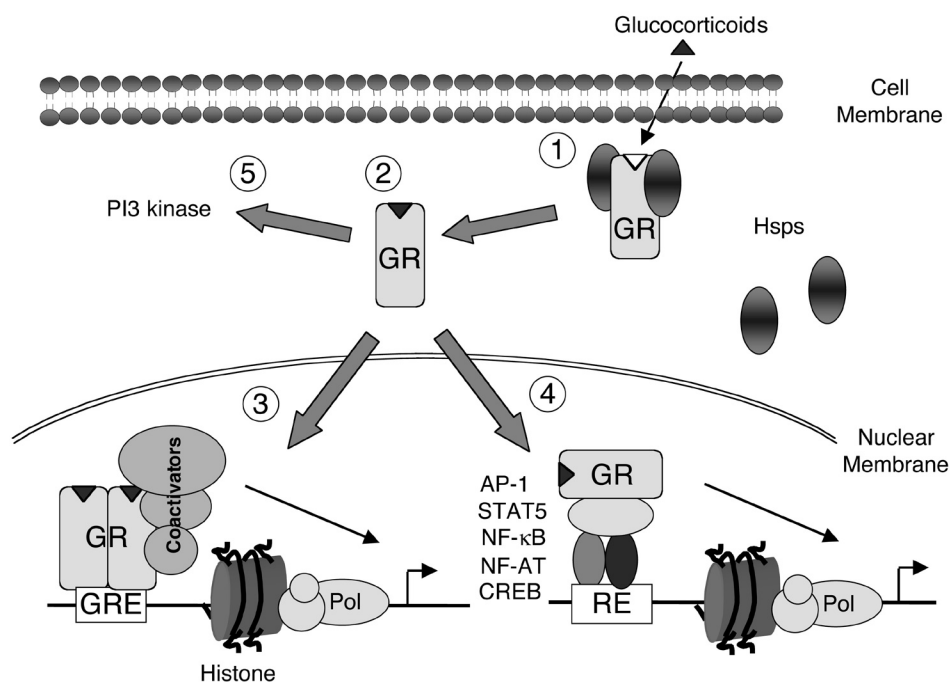


Figure 1. Molecular modes of GR action. GCs passively diffuse into the cell and bind to the GR (1). This results in the dissociation of the heat shock protein complex (Hsps) (2) and translocation of the ligand-bound GR into the nucleus. There the GR modulates transcription either by binding to DNA (3) or via interaction with other transcription factors (4). Non-genomic mechanisms of GR action include interference with cytosolic signaling molecules (5).

dependent activities, the GR also regulates gene expression by interfering with other transcription factors such as NF- $\kappa$ B [26], AP-1 [27], NF-AT [28], CREB [29] and Stat5 [30]. Intriguingly, the GR can control gene expression in this way, mostly in a transrepressing manner, without binding to DNA itself (fig. 1). This was most convincingly demonstrated by introducing a point mutation into the GR which prevents it from both dimerizing and binding DNA [8]. When present in the germline of knock-in mice (GRdim), the mutated GR was unable to mediate transcription from GREs but its ability to regulate transcription from AP-1-, NF- $\kappa$ B- and Stat5-driven promoters was not compromised [31–34]. In addition to its established effects on transcription, evidence is growing that the GR can also play a role in cytosolic signaling pathways, including the activation of PI3 kinase [35].

It has also been recently recognized that multiple N-terminal isoforms are generated from the GR gene by translational mechanisms [36]. Importantly, these isoforms appear to regulate different sets of genes, suggesting that they are not functionally equivalent. Given the tissue-specific distribution of the various isoforms, it will be intriguing to see whether they are differentially involved in immunoregulatory functions in the same way that the use of alternative promoters of the GR has been shown to correlate with the sensitivity of T lymphocytes to apoptosis [37, 38]. Taken together, differential transcription and translation events give rise to a plethora of GR species which control cellular processes by several different mechanisms [39]. This in turn forms the basis for the variety of activities that GCs exert in the immune system.

### Control of GC secretion and synthesis

The primary site of GC synthesis is the adrenal gland, which was first described in the 16th century by the Italian anatomist Eustachi [40]. However, only in 1855 did Addison realize the importance of this organ for survival. In the first half of the 20th century, scientists finally discovered the importance of cortical hormones which, in the 1940s, were shown not only to be involved in the stress response but also to exert potent anti-inflammatory activity [41].

GC synthesis is under the control of the hypothalamus-pituitary-adrenal (HPA) axis, which controls hormone levels in the serum [42]. This mechanism involves the release of corticotrophin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) in response to stress and elevated cytokine levels which results in GC secretion from the adrenal gland. GCs ultimately limit the activity of the HPA axis at the level of the hypothalamus and the pituitary, thus establishing a negative feedback loop. Superimposed on that, GC production also follows a circadian rhythm, characterized in humans by high levels in the morning and low levels in the evening

[43]. GCs have also been hypothesized to be produced in other tissues but the evidence for this remains scarce (see below). Collectively, GC action is controlled on multiple levels by the neuroendocrine as well as the immune system itself.

### Animal models investigating GR function in T cells

#### Loss-of-function mutations

During the last decade, a considerable number of genetically modified mouse strains have been generated to study the role of the GR in various physiological settings and anatomical locations [15], including the development and function of T lymphocytes (table 1). The first approach of this kind was described in 1995 when Ashwell and colleagues expressed an antisense fragment to the GR under the control of the proximal Ick promoter [44]. GR mRNA and protein expression were reduced by around half, resulting in a lower thymic cellularity and a partial resistance to GC-induced thymocyte apoptosis. Intriguingly, analysis of a second strain of transgenic mice which was generated by Okret and colleagues using a largely similar construct arrived at exactly the opposite conclusion [45]. In particular, the total thymocyte number in this experiment was found to be elevated instead of diminished.

Analysis of knock-out mice is generally considered more reliable than using antisense transgenic animals since deletion should be complete and unspecific effects reduced. GR knock-out mice were first reported by Schütz and colleagues in 1995 [46]. The initial analysis of these mutants revealed that they die perinatally due to lung atelectasis. It took several years before the thymic phenotype of these mice was investigated using fetal mice and bone marrow chimeras. Thymocytes derived from these knock-out mice were found to be completely resistant to GC-induced apoptosis but otherwise no significant differences in cell number, subtype distribution and thymic selection were observed [47, 48]. Muglia and colleagues described another strain of GR-deficient mice, confirming the lack of any role for the GR in the thymus beyond inducing apoptosis [13]. Recently, two strains of T cell-specific GR knock-out mice were generated by independent laboratories. Like GR-deficient mice, these mutants turned out to be resistant to GC-induced apoptosis while having normal thymic cellularity and subtype distribution [12, 49]. As mentioned earlier, the GR modulates transcription through two principal modes of action. These can be dissected using the previously described GRdim mice which are impaired in their ability to transactivate genes from GRE-containing promoters [8]. While no effect on T cell development was detected, thymocytes from these knock-in mice were refractory to GC-induced cell death *in vitro* (table 1). This confirmed that the DNA-binding

Table 1. Transgenesis and gene-targeting experiments addressing GR function in T lymphocytes.

Strain	Type of mutation	T cell phenotype	Ref.
GRTKO	T cell-specific GR antisense transgenic mouse	reduced thymocyte number, lower sensitivity to GC-induced apoptosis, altered T cell development and function	44, 123, 124, 130
lck <sup>Pr</sup> -asGR	T cell-specific GR antisense transgenic mouse	elevated number of thymocytes, in particular DP and CD4 single-positive cells	45
GR <sup>hypo</sup> (GRKO)	GR knock-out mouse (hypomorphic allele)	unaltered thymocyte number and subtype distribution, thymocytes refractory to GC-induced apoptosis, normal T cell function	46, 48
GRN	GR knock-out mouse (exon 3 deleted)	thymic cellularity and subtype distribution unaltered	131
GRKO	GR knock-out mouse (exon 2 deleted)	unaltered thymic cellularity and subtype distribution, thymocytes refractory to GC-induced apoptosis, mice resistant to $\alpha$ CD3-induced apoptosis <i>in vivo</i>	13
TGRKO	T cell-specific GR knock-out mouse (exon 2 deleted)	thymocyte number and subtype distribution unaltered, high mortality after $\alpha$ CD3-induced polyclonal T cell activation	49
GR <sup>lckcre</sup>	T cell-specific GR knock-out mouse (exon 3 deleted)	no phenotypic characterization published, GCs are unable to counteract AICD	12
GR <sup>dim</sup>	GR knock-in mouse carrying the point mutation A458T	thymocyte number and subtype distribution unaltered, thymocytes refractory to GC-induced apoptosis, repression of cytokine expression in thymocytes and splenic T cells normal, GCs unable to counteract AICD	8, 33, 12
YGR	ubiquitous GR overexpression (increased gene dosage)	increased sensitivity towards GC-induced thymocyte apoptosis	50
lck <sup>Pr</sup> -sGR	T cell-specific GR overexpression	GC-induced thymocyte apoptosis increased, reduced thymocyte and peripheral T cell number	45
hCD2-GR	conditional T cell-specific GR overexpression	reduced thymic cellularity after induction of GR overexpression, even in adrenalectomized animals	51

activity of the GR is dispensable for T cell development but essential for thymocyte apoptosis. Collectively, the analysis of all five GR mutant mouse strains has consistently failed to demonstrate a role for the receptor in T cell development and thymic selection which contrasts with the observations made in antisense transgenic mice (table 1). In conclusion, GCs, at least when acting via the classical GR, seem unlikely to be involved in these processes while their role in inducing thymocyte apoptosis is beyond question.

### Gain-of-function mutations

Two mouse strains displaying increased GR signaling have been described in recent years (table 1). The first approach aimed at doubling the GR gene dosage by introducing a large DNA fragment covering the whole genomic locus into the germline of mice [50]. In accordance with the increased GR expression, transgenic thymocytes were more sensitive to GC-induced apoptosis than control cells. This indicates that apoptosis critically depends on the amount of GR protein in the cell. In the second transgenic mouse line, GR overexpression was targeted to developing thymocytes by expressing the GR under the control of the proximal lck promoter [45]. Overexpression was accompanied by increased sensitivity to GC-induced apoptosis, lower recovery of thymocytes and reduced numbers of peripheral T cells. The same group recently reported the generation of another mouse strain characterized by conditional GR expression

in thymocytes and T cells [51]. Upon induction of the GR, thymic cellularity was reduced, both in the presence and absence of adrenally derived GCs, although no influence on subtype distribution could be demonstrated (table 1). This was interpreted as a proof for GCs of thymic origin, a conclusion which is not supported by our own studies [unpublished data].

### Apoptosis

#### The concept of apoptosis and its function in the immune system

Apoptosis is defined by a series of molecular and morphological events involving the loss of mitochondrial membrane potential, chromatin condensation, DNA fragmentation, membrane blebbing and the generation of apoptotic vesicles (fig. 2). Mediated by executioner caspases, this process culminates in the orchestrated disassembly and phagocytosis of the dying cell [52].

Apoptosis is essential for the development and maintenance of the immune system [4]. It may be initiated because the developing lymphocytes fail to express a productively rearranged antigen receptor or alternatively because they produce a receptor displaying too weak or too strong of an affinity for MHC/self-peptide [1]. These selection steps ensure proper recognition of a broad range of foreign antigens and help to achieve tolerance and prevent autoimmune diseases. Apoptosis is not only

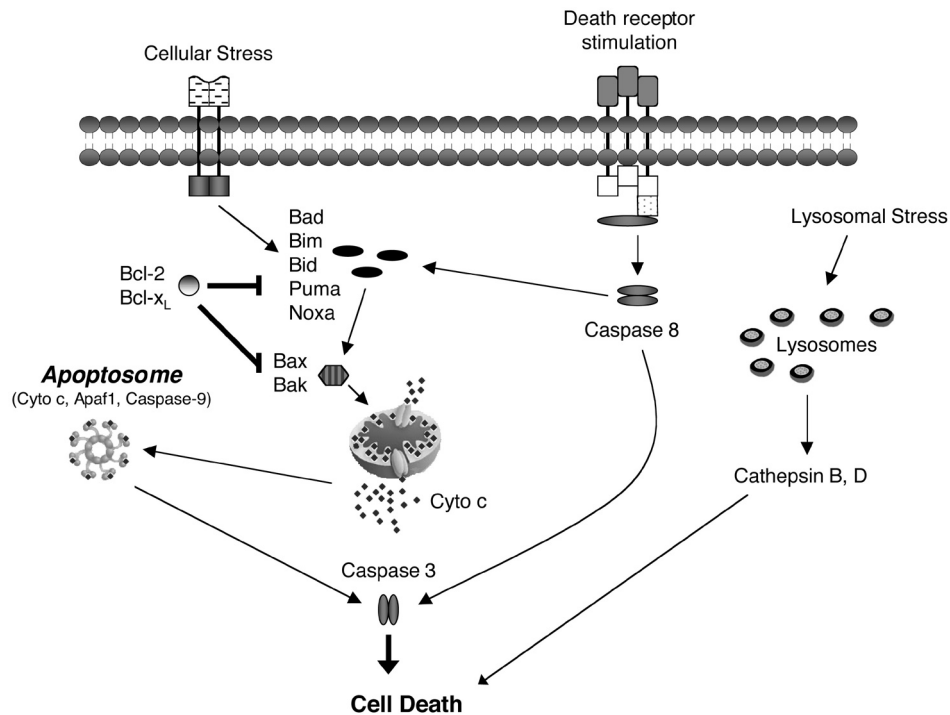


Figure 2. The major pathways of lymphocyte apoptosis. The 'intrinsic' pathway involves the activation of 'BH3-only' molecules (Bad, Bim etc.) which in turn activate the 'multidomain' proteins Bax and Bak. This leads to the formation of the 'apoptosome' and activation of caspase-3, a process which is counteracted by the anti-apoptotic proteins Bcl-2 and Bcl-x<sub>L</sub>. The 'extrinsic' pathway is initiated by oligomerization of death receptors followed by caspase-8 activation and also converges on caspase-3. An alternative pathway induced by lysosomal stress involves the release of cathepsins.

critical to the establishment of the immune system but also plays an important role in the termination of adaptive immune responses [3]. During the contraction phase, clonally expanded antigen-specific lymphocytes are rapidly removed by AICD, a process induced by TCR triggering and cytokine withdrawal. This serves to prevent uncontrolled proliferation of activated lymphocytes and thereby avoids immunopathology and autoimmune disorders.

### The pathways of apoptosis

Lymphocytes are known to undergo two distinct apoptotic pathways which have been well characterized in recent years [4] (fig. 2). One is initiated by cellular stress or high-affinity ligation of antigen receptors during negative selection of autoreactive lymphocytes. This so-called 'intrinsic' pathway is regulated by the interplay of pro- and anti-apoptotic members of the Bcl-2 family at the level of the mitochondria [53]. The second mechanism, also known as the 'extrinsic' apoptotic pathway, is activated by ligation of death receptors and occurs, for example, during AICD [3]. These two pathways share common downstream components such as certain caspases, a family of aspartate-specific proteases.

Initiation of the 'intrinsic' pathway involves the release of apoptotic mediators from the mitochondria following disruption of its outer membrane [54]. This is tightly regulated by the balance between pro- and anti-apoptotic Bcl-2 family members (fig. 2). Pro-apoptotic factors of the so-called 'BH3-only' family such as Bim, Bid, Bad, Puma and Noxa serve as conduits of apoptotic stimuli by

activating the 'multi-domain' family members Bax and Bak [55, 56]. This process is opposed by anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-x<sub>L</sub> which bind and neutralize their pro-apoptotic counterparts [53, 57–60]. Following formation of pores in the outer mitochondrial membrane by Bax and Bak, cytochrome c is released, resulting in the formation of the so-called 'apoptosome' (fig. 2). This multimeric complex containing caspase-9 and Apaf1 activates caspase-3 which finally leads to apoptosis [52].

The 'extrinsic' apoptotic pathway is initiated by the ligation, oligomerization and subsequent activation of death receptors as typified by Fas [61] and results in the activation of caspase-8 (fig. 2). Depending on the cell type, subsequent activation of caspase-3 occurs either directly or requires an additional amplification loop involving cleavage of the pro-apoptotic factor Bid and destabilization of the mitochondrial membrane. However, irrespective of which biochemical events are involved, the 'intrinsic' and the 'extrinsic' pathway both converge on the activation of caspase-3, the point of no return in most cell death cascades (fig. 2).

Although the two aforementioned pathways are considered to explain most forms of apoptosis, evidence for alternative pathways is accumulating [62, 63]. One postulated mechanism involves the destabilization of lysosomal membranes and subsequent release of cathepsins B and D [64] (fig. 2). This results not only in generalized proteolysis but also causes disruption of the outer mitochondrial membrane and caspase-3 activation [65–68]. A second unconventional pathway involves the release

of death-inducing factors from the mitochondria such as AIF and endonuclease G, which are capable of initiating cell death independently of caspases [63].

### The mechanism of GC-induced apoptosis

**Initiation of GC-induced apoptosis.** The current model implies that GC-induced apoptosis requires the presence of a functional GR and in particular its transactivating function [8,13, 15, 47, 50]. Although most data suggest that initiation of cell death by GCs is linked to *de novo* gene expression, it is noteworthy that some events may also involve so-called non-genomic actions of the GR (fig. 3).

During the last couple of years several approaches to identify GC-induced genes have been undertaken, but only a few convincing candidates were identified. Genes described to be up- or downregulated during GC-induced apoptosis include *c-myc* [69], *tdag8* [70, 71], *dig2* [72], *Bim* [73, 74] and *PUMA* [75]. Furthermore, large-scale expression analysis of GC-regulated genes has only been reported in cell lines so far. Both Tonko *et al.* [76] and Medh *et al.* [77] have studied GC-induced gene regulation in leukemic lymphoid CEM cells, whereas Wang *et al.* [73] have performed a large-scale analysis examining the two murine lymphoma cell lines S49.A2 and WEHI 7.2 [73]. The most promising gene identified in these screens is the pro-apoptotic protein *Bim*, but its function in this process still requires further investigation (see below).

### The role of Bcl-2 proteins in GC-induced apoptosis

A plethora of experiments have implicated pro- and anti-apoptotic Bcl-2 family members in GC-induced apoptosis. Mice reconstituted with fetal liver cells from Bcl-2 transgenic mice [78, 79] as well as Bcl-2-overexpressing

murine lymphoma cells [80, 81] are protected from GC-induced cell death, indicating that the disruption of mitochondrial integrity is essential to GC-induced apoptosis [82]. Studies carried out in murine T hybridoma cells suggest that Bcl-2 overexpression also blocks the release of both cytochrome *c* and AIF in response to GCs [83]. The importance of Bcl-2 is further underscored by the analysis of Bcl-2-deficient mice which display fulminant lymphoid apoptosis *in vivo* and enhanced cell death of thymocytes *in vitro* after GC treatment [84]. Collectively, these data suggest that the level of Bcl-2 determines the sensitivity to GCs. Since Bcl-2 is known to localize and act in mitochondria as well as the endoplasmic reticulum, both organelles might play an important role in GC-induced apoptosis [58].

The role of Bcl- $x_L$  in this process is less clear. Bcl- $x_L$  becomes redistributed to the mitochondria following GC treatment which can be prevented by inhibitors of translation [85, 86]. Overexpression of Bcl- $x_L$  in a T hybridoma cell line can block GC-induced apoptosis [80] and sustained Bcl- $x_L$  expression was found to be a prerequisite for the resistance of single-positive thymocytes to GC-induced apoptosis *in vivo* [87]. Thus, Bcl- $x_L$  contributes to determining the sensitivity of lymphocytes to GC-induced apoptosis although this might be limited to certain developmental stages of T lymphocytes. Since Bcl- $x_L$ -deficient mice die around embryonic day 13 [88] and neither mice reconstituted with fetal liver cells nor tissue-specific knock-out mice have been described, further analysis of Bcl- $x_L$  function in GC-induced apoptosis *in vivo* is not possible at this point.

The pro-apoptotic members of the Bcl-2 family have also been implicated in GC-induced apoptosis. The role of the 'multidomain' members Bax and Bak has been assessed by gene targeting [89–92]. While thymocytes from individual knock-out mice showed no difference in their sensitivity to GC-induced apoptosis, in the absence of both molecules, thymocytes were completely resistant. This suggests that Bax and Bak are involved in mediating GC effects but at the same time fulfill redundant roles. As to how GCs affect Bax and Bak, nothing is known so far. Bax is partially redistributed from the cytosol to the membrane fraction upon GC treatment [85, 86]. However, given the redundancy of Bax and Bak, GCs, possibly through 'BH3-only' proteins such as Bim, are more likely to induce a conformational change of these pro-apoptotic proteins which then leads to destabilization of the mitochondrial membrane.

The current model of the pro-apoptotic 'BH3-only' proteins implies that individual family members are responsible for mediating the response to different apoptotic stimuli [53, 93]. Bid, for example, is known for its involvement in Fas-mediated apoptosis in certain cells [94]. In keeping with this notion, GC-induced thymocyte apoptosis was unaffected in Bid-deficient mice, excluding

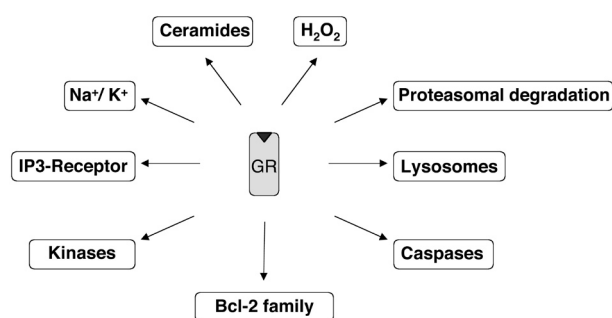


Figure 3. Cellular processes involved in GC-induced apoptosis. Bcl-2 family: transcription of *Bim* and *Puma* is upregulated; caspases: caspase-3, -8 and -9 are activated. lysosomes: cathepsin B is released. proteasomal degradation: c-IAP1 and XIAP are degraded at the protein level;  $H_2O_2$  levels are increased; ceramides are produced, and  $Na^+/K^+$  levels altered. The IP3 receptor is engaged. Kinases: PKC, Raf and 14-3-3 proteins interact with the GR.

an essential role in GC-mediated cell death [95]. The role of Bad, which is posttranscriptionally regulated by Akt/PKB-dependent phosphorylation, has been addressed in T cell-specific transgenic mice. Overexpression of Bad sensitized thymocytes to GC-induced apoptosis [96]. However, this may simply reflect its antagonistic effect on the death-repressing activity of Bcl-x<sub>L</sub> and thus Bad is not involved in this process under normal circumstances. Unfortunately, the role of Bad in GC-induced apoptosis has neither been investigated in the recently published Bad-deficient mice nor in a strain of knock-in mice in which BAD can not be phosphorylated due to three engineered point mutations [97, 98].

As outlined above, Bim was identified in one of the large-scale screenings as being upregulated during GC-induced apoptosis in T lymphoma cell lines [73]. In keeping with this observation, reducing Bim levels by RNAi in pre-B ALL cell lines rendered them partially resistant to GC-induced apoptosis [74]. Furthermore, GC-induced apoptosis in Bim-deficient mice was partially impaired compared to wild-type animals [78, 79]. In this context, it is interesting that PUMA was also shown to be upregulated in response to GC treatment [75]. In addition, analysis of two distinct strains of PUMA knock-out mice by independent laboratories has revealed a partial resistance of thymocytes to GC-induced apoptosis [99, 100]. In contrast, Noxa knock-out mice do not appear to be compromised in this process. Taken together, Bim and PUMA are the most promising candidates for pro-apoptotic 'BH3-only' proteins induced by GCs during the initiation of apoptosis.

#### **The role of caspases in GC-induced apoptosis**

The involvement of caspases in GC-induced apoptosis is continually debated. Pharmacological inhibition of caspases can be achieved by application of chemically modified small-peptide derivatives but their specificity for individual family members is sometimes variable [101]. Taking such an approach, thymocytes were found to be refractory to GC treatment in the presence of the pan-caspase inhibitor z-VAD-fmk [82, 102]. Moreover, caspase-3 and -8 were proposed to mediate GC-induced apoptosis but whether this holds true for all cell types needs to be demonstrated [103]. In particular, our own unpublished results have revealed that the role of caspases in this process is cell type specific. In summary, experiments based on the pharmacological interference with caspase activity suggest some role for these enzymes in GC-induced thymocyte apoptosis.

In contrast, analysis of knock-out mice deficient in individual caspases led to contradictory conclusions. First, thymocytes and mature T cells derived from caspase-1- and caspase-2-deficient mice, as well as from mice with a T lymphocyte-specific deletion of caspase-8, show no differences from wild-type mice in their responsive-

ness to GC treatment [104–106]. In contrast, apoptosis in response to GCs was found to be reduced in both caspase-9- and Apaf-1-deficient mice [107, 108]. Since these animals suffer from severe developmental defects, the impaired apoptosis could also result from unspecific effects. This notion is supported by the analysis of animals reconstituted with fetal liver cells from Apaf-1- or caspase-9-deficient mice. Thymocytes and mature T cells from these mice undergo apoptosis in response to a large range of GC concentrations, suggesting that the intrinsic lack of caspase-9 and Apaf-1 in T lymphocytes does not impair GC-induced apoptosis [79]. Interestingly, thymocytes from caspase-3-deficient mice, which die within the first month after birth, show normal responsiveness to GC-induced apoptosis [109]. This is in sharp contrast to the pharmacological data which are in support of an important role for caspase-3 in this process [103]. However, other family members conceivably compensate for its lack under these circumstances.

#### **Other mediators of GC-induced apoptosis**

There is growing evidence that lysosomes may play an important role in both caspase-dependent and -independent forms of apoptosis [67]. We have recently shown that activation of cathepsin B precedes caspase activation during GC-induced cell death, suggesting an important role for this cellular compartment [unpublished data].

Several studies have implicated the proteasome complex in GC-induced thymocyte apoptosis [110]. Its pharmacological inhibition prevents disruption of the mitochondrial membrane potential and nuclear fragmentation in thymocytes. Furthermore, the apoptosis inhibitory proteins c-IAP1 and XIAP have been shown to be degraded by the proteasome following GC treatment [111]. Although good evidence exists for an important role for proteasomal degradation, our unpublished results suggest that this is limited to certain cell types rather than being part of a ubiquitous pathway of GC-induced apoptosis.

Hydrogen peroxide, which can act as a second messenger in cell signaling, is synthesized at modest levels following the ligation of cell surface receptors and in response to various apoptotic stimuli [112]. Evidence that this pathway can be initiated by GCs comes from the observation that treatment of thymocytes with GCs leads to an increase in the intracellular level of hydrogen peroxide [110]. This precedes the loss in mitochondrial membrane potential, cytochrome c release and caspase-3 activation. However, in our hands, attempts to repeat this experiment failed [unpublished data].

Additional events that have been hypothesized to be involved in GC-induced apoptosis include ceramide production [113], changes in the intracellular levels of sodium and potassium ions [114], activation of PI3K [35] and the inositol trisphosphate receptor [115], as well as interaction of the GR with signaling proteins such as

protein kinase C, Raf and members of the 14-3-3 family [116]. Importantly, some of these events have been shown to occur in the cytosol independent of *de novo* gene expression [35] but their relative contribution to GR-mediated apoptosis remains elusive. For further information on the non-classical effects of GCs, the reader is directed to a review by Distelhorst [9].

### The cell type specificity of GC-induced thymocyte apoptosis

It was recognized more than 20 years ago that thymocytes differ in their sensitivity to GC treatment dependent on their developmental stage [117]. When administered at pharmacological doses, CD4+CD8+ double-positive (DP) thymocytes undergo cell death *in vivo* whereas single-positive (SP) thymocytes are resistant to GCs (fig. 4). We have recently proposed a model involving CD28-mediated protection of SP thymocytes to explain this difference [87]. Such a specific role for CD28 in mature thymocytes is also suggested by the finding that its expression in humans and rats increases during the course of thymocyte development [118] and that its principal ligands B7-1 and B7-2 are exclusively expressed by thymic medullary cells where the mature T cells reside [119].

Although refractory to GC-induced cell death *in vivo*, SP thymocytes become sensitive when in culture. However, enforced CD28 signaling in the absence of TCR engage-

ment restores their resistance to GC treatment, presumably by upregulating Bcl-x<sub>L</sub> [87]. This model is consistent with the previous finding that anti-apoptotic proteins such as Bcl-2 and Bcl-X<sub>L</sub> are developmentally regulated during thymocyte development [120–122]. Similarly, SP thymocytes in CD28 knock-out mice show increased sensitivity to GC-administration. This suggests that CD28 signaling is responsible for conferring resistance to GCs *in vivo*. The differential sensitivity of immature DP and mature SP thymocytes to apoptosis may form the basis for the postulated role GCs play in positive and negative selection [11] (see below). If this model holds true, DP thymocytes need to be sensitive to GCs during selection but loose responsiveness to the same stimulus upon maturation. This could be achieved by the translocation of selected SP thymocytes from the cortical region to the medulla, where they would come into close contact to B7-expressing epithelial cells. Subsequent ligation of CD28 on these cortical SP thymocytes would then afford them protection from GCs.

### Glucocorticoids and T cell development

#### GCs and thymic selection

An important checkpoint of T cell development occurs at the DP stage where interaction of the TCR with MHC/self-peptides on thymic epithelial and stromal cells initiates selection processes that lead to death by neglect, positive or negative selection [1]. At this stage, the majority of DP thymocytes die due to their inefficient ability to interact with self-ligands in the context of MHC. According to the avidity model of selection, DP cells with TCRs that interact with moderate affinity to self-ligands will be positively selected and further differentiate into mature functional T cells [2]. High-affinity interaction with self-MHC, which would be equivalent to autoreactivity in the periphery, destines DP cells to apoptosis and thus results in negative selection.

Most models of positive and negative selection postulate that the strength of TCR signaling determines thymocyte fate. In the 'mutual antagonism' model, this is assigned to an interplay between GC- and TCR-induced apoptosis [11]. Alone, signaling through the TCR or GR induces apoptosis, but when both receptors are coordinately stimulated they oppose each other (fig. 4). Thus, thymocytes expressing a TCR with high affinity for MHC/self-peptide undergo negative selection since GR signaling is not sufficient to overcome the strong signal originating from the TCR. In contrast, thymocytes with low-avidity TCRs undergo GC-induced cell death, since in this case the TCR signal cannot override GR signaling. Finally, in thymocytes with moderate-avidity TCRs, the two signals neutralize each other and thereby rescue the cells from apoptosis.

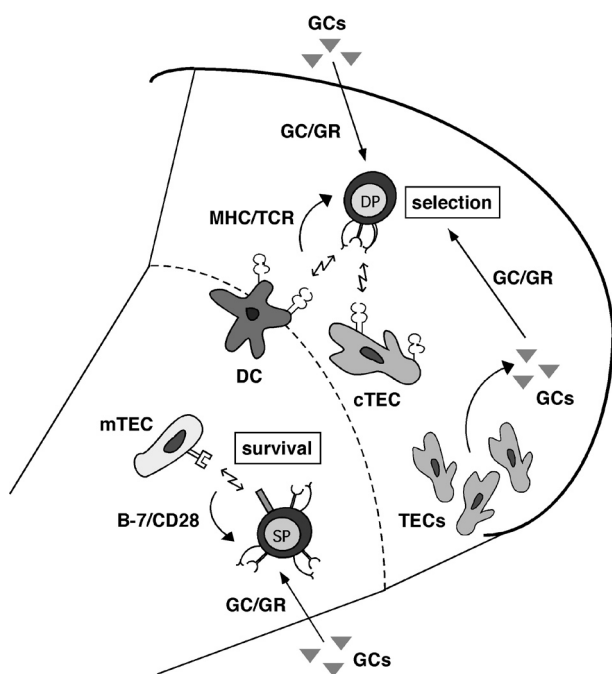


Figure 4. The role of GCs in thymocyte development. At the double-positive (DP) stage developing thymocytes undergo positive and negative selection which is controlled by an interplay between GR- and T cell receptor (TCR)-derived signals. Mature single-positive (SP) thymocytes are protected from GC-induced apoptosis by CD28 signaling. cTEC, cortical thymic epithelial cell; DC, dendritic cells; mTEC, medullary thymic epithelial cell.



Since the 'mutual antagonism' model was proposed, arguments both in favour and against it have been put forward. Analysis of mice expressing an antisense GR in the thymus were found to possess a T cell repertoire which is shifted toward cells with lower affinity for MHC/self-peptide and thus have a reduced number of potentially autoreactive cells [44, 123, 124]. In contrast, analysis of several strains of GR knock-out mice failed to provide any support for the 'mutual antagonism' model [13, 47–49]. However, most of these strains have not been analyzed for more subtle effects on the antigen receptor repertoire. Thus, the idea that GC-induced apoptosis participates in thymic selection remains attractive, although it awaits further support by more refined approaches.

### The role of thymus-derived GCs

There has been a long-standing debate as to whether the thymus synthesizes GCs itself or whether this is an exclusive characteristic of the adrenal gland [125] (fig. 4). The presence of the necessary biosynthetic machinery was demonstrated in the thymus [126]. In agreement with this finding, thymic epithelial cells (TECs) were shown to convert GC precursors to deoxycorticosterone *in vitro* [127, 128]. In addition, TECs also make metabolites which are able to activate GR-dependent transcription [127]. In fetal thymus organ culture, inhibition of GC synthesis by metyrapone turned out to impact on apoptosis, suggesting that steroid hormones might indeed be produced in the thymus [126]. However, another group could show that metyrapone impairs thymocyte development regardless of the presence or absence of a functional GR, indicating that this reagent inhibits thymocyte development in an unspecific fashion [48]. Recently, Okret and colleagues reported a new transgenic mouse model in which overexpression of the GR in thymocytes was controlled by the tetracycline-inducible system [51]. Increased expression of the GR in mice lacking adrenally derived GCs caused a slight reduction in thymic cellularity while subtype composition was unaltered. This observation was assigned to increased apoptosis following induction of GR overexpression. The finding that application of the GR antagonist RU486 was able to increase thymic cellularity even in adrenalectomized mice was taken as additional evidence of a role for intrathymically-synthesized GCs. However, our own unpublished results argue against extra-adrenal GC synthesis. Thymocyte numbers in transgenic rats overexpressing a mutant GR with increased ligand affinity are dramatically reduced while adrenalectomy restored cellularity within 3 weeks after surgery [unpublished data]. Thus, on the basis of our own findings, a major role for thymic GCs in T cell apoptosis and development is unlikely. However, more subtle influences on thymocyte selection, such as those suggested by the mutual antagonism model still remain possible.

### Concluding remarks

Since the cloning of the GR [129] and the observation that GCs induce the death of T cells [6], we have come a long way in understanding the mechanisms underlying the actions of these hormones in T cell apoptosis and development. However, we are still far from having a coherent picture of either the physiological role or the molecular mechanisms of GCs in these processes. For example, we have limited knowledge of which genes are essential for T cell apoptosis. The issue of thymus-derived GCs and the involvement of these hormones in thymocyte selection also remains debatable. Transgenesis and gene targeting have contributed enormously to our current understanding of GC action but new animal models carrying more refined mutations and transgenes are required to adequately address these questions. This is not only essential to extend our theoretical knowledge but is also of clinical relevance, since GCs are still among the most widely prescribed drugs for the treatment of hematological malignancies owing to their ability to mediate apoptosis in leukemia, lymphoma and myeloma cells [10, 14]. Thus, despite the long history of research on GC-induced apoptosis, it will continue to be a fertile and fascinating ground for basic and applied biomedical studies.

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