



## Bcl-xL overexpression decreases GILZ levels and inhibits glucocorticoid-induced activation of caspase-8 and caspase-3 in mouse thymocytes

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### ABSTRACT

Glucocorticoids promote thymocyte apoptosis and modulate transcription of numerous regulators of thymic apoptosis. Among these, glucocorticoid-induced leucine zipper (GILZ) is strongly upregulated in the thymus. We have previously demonstrated that GILZ decreases Bcl-xL expression, activates caspase-8 and caspase-3, and augments apoptosis in mice thymocytes. To better understand the causal links between glucocorticoids, GILZ, Bcl-xL, caspase-8, and caspase-3, we analyzed the thymocytes of Bcl-xL-overexpressing transgenic mice with or without glucocorticoid stimulation *in vitro*. Overexpression of Bcl-xL inhibited the glucocorticoid-induced up-regulation of GILZ in murine thymocytes as well as the glucocorticoid-dependent activation of caspase-8 and caspase-3. By contrast, no appreciable change in caspase-9 activation was observed upon Bcl-xL overexpression. Thus, these experiments highlighted a novel thymocyte apoptotic pathway in which Bcl-xL overexpression inhibited the glucocorticoid-induced activation of caspase-8 and caspase-3, but not caspase-9, as well as the accumulation of GILZ protein. These findings, together with our previous results showing that caspase-8 protects GILZ from proteasomal degradation, suggest the presence of a glucocorticoid-induced apoptosis self-amplification loop in which GILZ decreases Bcl-xL expression with a subsequent activation of caspase-8 and caspase-3; caspase-8 activation then enhances the stability and accumulation of GILZ and ensures the unimpeded and irreversible progression of apoptosis. By contrast, inappropriate increases in Bcl-xL levels could have catastrophic effects on thymic apoptosis as it would shut down caspase-8/3 activation, diminish the expression of GILZ, and impair the fine control necessary for thymic generation of a healthy immune repertoire.

### Credit author statement

IM, SA and LC designed and performed the experiments and acquired and interpreted the data; AML, EA and CR contributed their knowledge of the thymus and pharmacology and helped critically review and revise the manuscript; TVS and TT reviewed and edited the manuscript; DVD conceived and designed the study, supervised the research, acquired funds and wrote the original draft.

### 1. Introduction

Apoptosis is a central process in thymus physiology, because about

90% of thymocytes die, leaving only 10% to complete their maturation and migrate out towards peripheral lymphoid organs to mount a functional immune response [1,2]. Two different thymic processes direct the death of thymocytes by apoptosis: (1) negative selection, by which about 10% of the putative autoreactive thymocytes die before leaving the thymus [3,4], and (2) death by neglect, by which 80% of thymocytes with insufficient affinity for self-antigens die because they are not selected for, either positively or negatively [5].

Classically, apoptosis is described as occurring through two different energy-dependent molecular pathways—the extrinsic and the intrinsic pathways; however, there is considerable evidence suggesting that these pathways can be linked and influence each other. The extrinsic pathway

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involves stimulation of cell surface death receptors, such as Fas, by their ligands; this step is followed by the recruitment of adaptor molecules, such as Fas-associated protein with death domain (FADD), that, in turn, recruit and activate the initiator caspase, caspase-8. The intrinsic pathway is set by the dynamic balance between anti- and pro-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family, followed by the release of cytochrome *c* from mitochondria and the activation of caspase-9. The two pathways converge on the activation of the terminal/executioner caspase, caspase-3, and consequent apoptosis [6].

Bcl-2 family members [7] play a key role in regulating thymocyte apoptosis via the intrinsic pathway by promoting a balance between pro-apoptotic members, such as Bcl-2-associated X protein (Bax), Bcl-2 antagonist/killer (Bak), Bim, P53-upregulated modulator of apoptosis (PUMA), Noxa, Bcl-2-associated agonist of cell death (Bad), Bcl-2-interacting killer (Bik), Harakiri (Hrk), Bcl-2 modifying factor (Bmf), and BH3-interacting domain death agonist (Bid), and anti-apoptotic members such as Bcl-2, Bcl-xL, myeloid cell leukemia 1 (Mcl-1), and Bclw [8]. When pro-apoptotic proteins are not neutralized by anti-apoptotic members, a caspase-9/-3 cascade is activated resulting in apoptosis [9]. The anti-apoptotic Bcl-2 family member Bcl-xL seems to be a central player in death by neglect since Bcl-xL is upregulated when DN thymocytes become DP cells. Furthermore, Bcl-xL deficiency drives an increase in DP, but not SP thymocyte apoptosis [10,11]. One positive regulator of Bcl-xL expression is the nuclear orphan receptor ROR $\gamma$ t which contributes to a prolongation of DP thymocyte life-span [12]. Negative regulators of Bcl-xL expression are glucocorticoids (GCs) that promote thymocyte apoptosis [13,14].

The importance of GCs in the thymus is highlighted by their local production in both thymic epithelial cells and thymocytes, and their possible involvement in regulation of thymocyte differentiation and age-related involution [15]. In the thymus, GCs decrease Bcl-xL expression by activating signal transducer and activator of transcription 5B (STAT-5B) and recruiting it to the Bcl-xL promoter [16]. GCs decrease Bcl-xL expression also through the transcription of glucocorticoid-induced leucine zipper (GILZ), a protein upregulated by GCs in the thymus [17, 18]. However, in the latter case, Bcl-xL expression is not related to the intrinsic pathway since caspase-9 is not involved in the apoptotic process, but could rather be involved in a different pathway since a GILZ-dependent activation of caspase-8 is associated with the decrease of Bcl-xL [17].

Is there any cause-effect relationship between the GCs/GILZ-dependent decrease of Bcl-xL and the activation of caspase-8 or is this just an incidental association? The study presented herein addresses this pivotal mechanistic question, and involves an analysis of GC-induced caspase activation in thymocytes derived from transgenic mice overexpressing Bcl-xL [19], which results in a suppression of GC-induced thymocyte apoptosis. Our results indicate that a novel non-intrinsic, non-extrinsic apoptotic pathway operates in GC-stimulated thymocytes; Bcl-xL suppresses the activation of caspase-8 but not caspase-9 via this pathway, and in doing so, precipitates a decrease in the expression of GILZ.

## 2. Materials and methods

### 2.1. Animals

*Lck<sup>fl</sup>-bcl-xL* transgenic mice bred on the C57BL6/J background constitutively overexpressing Bcl-xL within all thymocyte subsets were a generous gift of Dr. Craig Thompson [19]. They were housed in an isolated colony and provided with laboratory chow and acidified (pH 2.4) water *ad libitum*. The protocol was approved by the Italian Ministry of health.

### 2.2. Cell harvesting and cell culture

Female wild type (WT) or *Lck<sup>fl</sup>-bcl-xL* transgenic (TG) mice of 4–8 weeks of age were sacrificed by cervical dislocation, thymi were excised, thymocytes were reduced to single cell suspension and counted with a hemocytometer. Single cell suspensions were cultured at a concentration

of  $3 \times 10^6$  in Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% fetal calf serum (FCS), 100 U/mL penicillin/streptomycin, 0.1 mM non-essential amino acids,  $5.5 \times 10^2$   $\mu$ M  $\beta$ -mercaptoethanol ( $\beta$ -ME; GIBCO Invitrogen, San Giuliano Milanese, Italy) in flat-bottomed, 96-well plates (Costar, Cambridge, MA, USA) with or without  $10^{-7}$  M dexamethasone (DEX).

### 2.3. Western blot analysis

Protein extracts were obtained using RIPA buffer supplemented with protease (Sigma-Aldrich) and phosphatase (Thermo Fisher Scientific) inhibitor cocktail. Total proteins were separated via 12% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Primary antibodies used were anti-human mouse GILZ (CMFKG15, eBioscience), anti-caspase-8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase-3, anti-caspase-9 (Cell Signaling Technologies, Beverly, MA, USA), anti-Bcl-xL (R&D Systems, Minneapolis, MN), anti-tubulin monoclonal antibody (Sigma, St. Louis, MO, USA). Secondary antibodies were horseradish peroxidase-labeled goat anti-rabbit or anti-mouse antibodies (Pierce, Rockford, IL, USA).

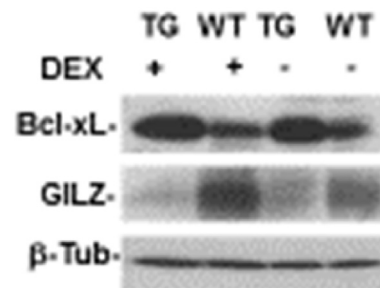
## 3. Results

### 3.1. Bcl-xL overexpression is associated with a decrease in GILZ expression levels

Proteins were extracted from thymocytes of wild type (WT) or Bcl-xL transgenic (TG) mice and cultured with or without GCs. After 3 h, cells were harvested and proteins were extracted and probed in Western blot experiments. As shown in Fig. 1, the expected increased expression of Bcl-xL was confirmed both in untreated or dexamethasone (DEX)-treated TG compared to WT thymocytes. Since, we have previously demonstrated that GC-induced expression of GILZ in the thymus decreases the expression of Bcl-xL [17], we wondered if also Bcl-xL could affect the expression of GILZ, establishing a reciprocal relationship between the two molecules. To dissect this point, GILZ protein was detected in Bcl-xL TG thymocytes untreated or treated with DEX and compared with WT thymocytes. As expected, DEX increased the expression of GILZ in WT thymocytes but, surprisingly, the expression of GILZ in TG transgenic thymocytes was decreased irrespective of their culturing with or without DEX (Fig. 1). Thus, Bcl-xL overexpression decreased the expression of the GC-induced protein GILZ.

### 3.2. Bcl-xL overexpression inhibits GC-induced activation of caspase-8 and caspase-3

To dissect the effect of Bcl-xL overexpression on GC-induced



**Fig. 1. Bcl-xL overexpression is associated with a decrease in GILZ expression levels.** Western blot analysis of proteins extracted from thymocytes of wild type (WT) or Bcl-xL transgenic (TG) mice ( $n = 3$ ). Bands indicate the expression of Bcl-xL (Bcl-xL) (upper lane), GILZ (GILZ) (middle lane) or  $\beta$ -Tubulin ( $\beta$ -Tub) (lower lane) in WT (WT) or TG (TG) thymocytes that were either untreated (-) or treated (+) with dexamethasone (DEX) at  $10^3$  mM.

activation of caspases, the above-mentioned thymocyte lysates were probed to detect activation of caspase-3, caspase-8, and caspase-9. As shown in Fig. 2A, caspase-3 was not activated in untreated WT and TG thymocytes. The addition of DEX induced the activation of caspase-3 (as evidenced by the appearance of its cleaved form) in control thymocytes; however, DEX treatment did not induce activation of caspase-3 in Bcl-xL TG thymocytes, as expected by the known suppression of apoptosis in TG thymocytes [19]. These results were not surprising since, canonically, Bcl-xL inhibits the caspase-9-dependent intrinsic apoptosis pathway, which in turn activates caspase-3 in a common end with the extrinsic caspase-8-dependent pathway. To further dissect the contribution of either the intrinsic or the extrinsic pathway to the inhibition of caspase-3 activation, the same WT and TG thymocyte lysates were probed with anti-caspase-8 or -caspase-9 monoclonal antibodies. As shown in Fig. 2B, whereas DEX was able to activate caspase-8 in WT thymocytes, the DEX-dependent activation of caspase-8 was completely abolished in Bcl-xL TG thymocytes. Surprisingly, this was not the case when caspase-9 activation was probed: caspase-9 activation was not inhibited by DEX in Bcl-xL transgenic thymocytes, suggesting that in thymocytes, Bcl-xL inhibits caspase-3 activation through inhibition of caspase-8 but not caspase-9 activation. Taken together, these results suggest that Bcl-xL overexpression inhibits GC-induced activation of caspase-8 and caspase-3 through a novel non-intrinsic/non-extrinsic thymocyte apoptotic pathway.

#### 4. Discussion

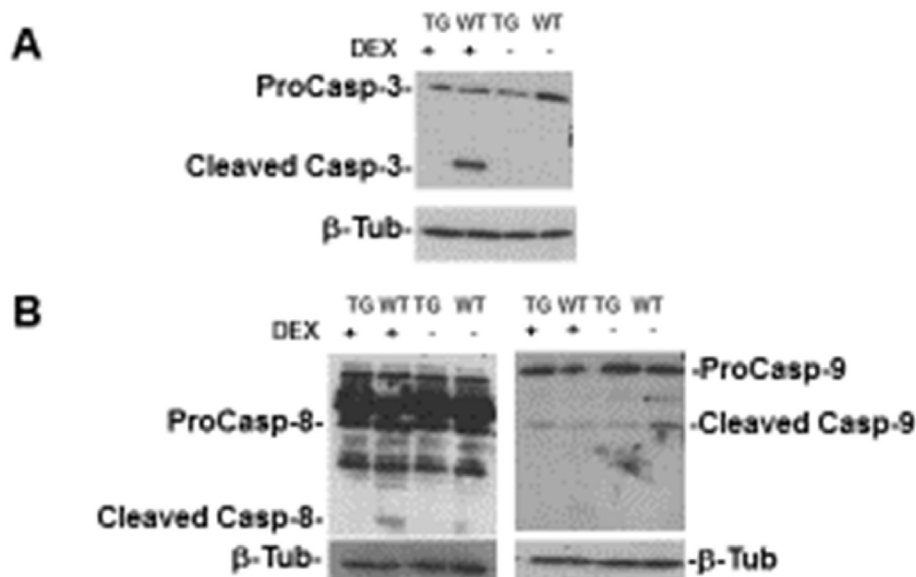
In the thymus, the organ where T lymphocytes complete their differentiation, the intensive proliferation of differentiating cells is well balanced by their apoptotic death, thus allowing a stable organ size. Apoptosis (especially the process of death by neglect) is not only a quantitative process resulting in an 80% decrease in thymocyte numbers, but also a qualitative process, since it eliminates, through negative selection, 5–10% of autoreactive thymocytes that would otherwise give rise to autoimmune diseases [20]. GCs are strong inducers of apoptosis in the thymus and, consequently, of immunodepression during stress, aging, and also infections, since it has been shown that an acute *Trypanosoma cruzi* infection results in thymic atrophy [21,22]. Although the exact mechanistic role of GCs in death by neglect is currently unclear, their repression of Bcl-xL expression, a signature of death by neglect, indicates a high probability of their involvement in the process. One GC-induced

mediator of Bcl-xL decrease is GILZ that promotes thymocyte apoptosis by both decreasing Bcl-xL expression and increasing activation of caspase-8 and -3 but not -9 activation [17].

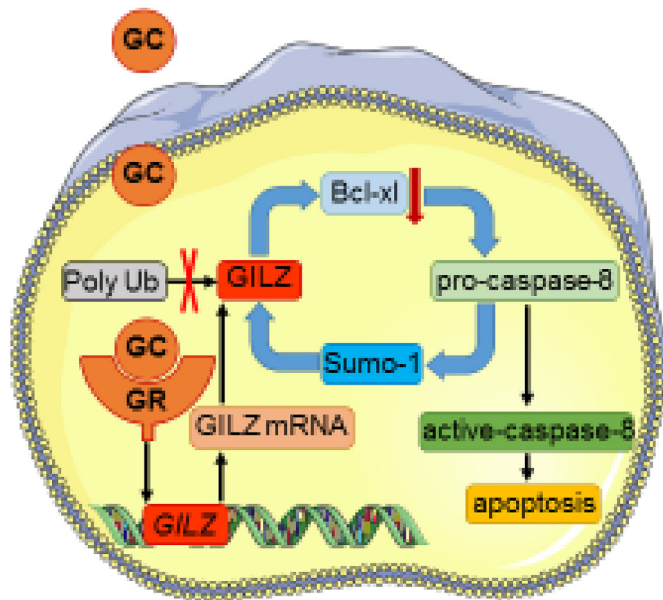
In order to test if these two processes are causally linked, we analyzed the GC-induced activation of caspases in thymocytes derived from Bcl-xL overexpressing transgenic mice [19]. Our results indicated that increase of Bcl-xL inhibited activation of caspase-8 and caspase-3, but not that of caspase-9, suggesting the presence of a new GC-dependent apoptotic pathway that (a) links Bcl-xL with caspase-8, and (b) is distinct from the well-known intrinsic (Bcl-xL-caspase-9) apoptotic pathway. We have demonstrated that GCs promote thymocyte apoptosis through upregulating the transcription of GILZ that in turn decreases the expression of Bcl-xL and induces the activation of caspase-8 and caspase-3. An additional connection exists between GILZ and caspase-8: GILZ activates caspase-8, and activated caspase-8 induces the binding of Sumo-1 to GILZ. This binding increases the life-span of GILZ, since Sumo-1 inhibits the conjugation of a poly-ubiquitin chain to GILZ and the consequent proteasomal degradation of GILZ [23].

Thus, we suggest that GCs, GILZ, Bcl-xL, and caspase-8 are linked in a loop in which GCs induce the production of GILZ, and GILZ decreases Bcl-xL expression, that in turn induces the activation of caspase-8; activated caspase-8 then sumoylates GILZ thus increasing GILZ's stability and half-life with the result of an amplification of this apoptotic process (Fig. 3). We do not know what regulates this loop. However, a balance between ubiquitination (that degrades GILZ) and sumoylation (that protects GILZ from proteasomal degradation) may be responsible for limiting the positive loop, but additional experiments are required to analyze such a mechanism. The proposed loop raises the question of how Bcl-xL can inhibit the activation of caspase-8. The molecular mechanism underlying the Bcl-xL-mediated inhibition of caspase-8 activation is currently undefined and future studies may illuminate it. However, since it has been shown that Bcl-xL and caspase-8 interact in a complex together with Bap31 and a Ced-4-like protein—an integral protein of the endoplasmic reticulum—it is possible that Bcl-xL regulates the activation of caspase-8 through the mediation of BAP31 and a Ced-4-like adaptor [24,25] in the thymocyte endoplasmic reticulum.

In conclusion, our study suggests the existence of a novel non-intrinsic/non-extrinsic thymic apoptotic pathway that links GCs, GILZ, Bcl-xL, and caspase-8 in an auto-amplifying loop. Future studies are needed to clarify molecular and functional aspects of this novel pathway.



**Fig. 2. Bcl-xL overexpression** inhibits GC-induced activation of caspase-8 and caspase-3. Western blot analysis of proteins extracted from thymocytes of wild type (WT) or Bcl-xL transgenic (TG) mice ( $n = 3$ ). (A) Bands indicate the expression of procaspase-3 (ProCasp-3) and activated caspase-3 (Cleaved Casp-3) or  $\beta$ -Tubulin ( $\beta$ -Tub) (lower lane) in WT (WT) or TG (TG) thymocytes either untreated (-) or treated (+) with dexamethasone (DEX). (B) Bands indicate the expression of procaspase-8 (ProCasp-8) and activated caspase-8 (Cleaved Casp-8) (left panel), procaspase-9 (ProCasp-9) and activated caspase-9 (Cleaved Casp-9) (right panel) or  $\beta$ -Tubulin ( $\beta$ -Tub) (lower lanes) of both panels) in WT (WT) or TG (TG) thymocytes either untreated (-) or treated (+) with dexamethasone (DEX).



**Fig. 3. Hypothesis of a novel GILZ-Bcl-xL-caspase-8 apoptotic loop.** Glucocorticoids (GC) penetrate the thymocyte membrane to bind the glucocorticoid receptor (GR). The GC/GR complexes translocate to the nucleus and stimulate the transcription of *GILZ*. *GILZ* mRNA is then translated into its protein. Once produced, GILZ protein (GILZ) decreases the expression of Bcl-xL that, in turn, results in the activation of caspase-8 via an unknown mechanism. Activated caspase-8 promotes a caspase-3-dependent apoptosis and, at the same time, induces the binding of Sumo-1 to GILZ. This post-translational modification inhibits the binding of GILZ to a poly-ubiquitin chain and prevents the proteasomal degradation of GILZ, thus prolonging GILZ's half-life in a self-amplifying loop.

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### Contribution to the field statement

Thymus is the primary immune organ in which T lymphocytes, which play central roles in our immune responses, develop and mature. Inside the thymus, the “good” T lymphocytes complete their maturation and are exported to the peripheral lymphoid organs to defend our body against external or internal dangers. At the same time, glucocorticoids induce programmed cell death (apoptosis) mechanisms within the thymus that ensure that “bad/unwanted” T lymphocytes are eliminated and do not lead to autoimmune diseases. Although previous research has shown that glucocorticoids, a glucocorticoid-induced protein GILZ, and an anti-apoptotic protein called Bcl-xL are all involved in regulating the activity of a family of enzymes (called caspases) that are crucial for controlling thymic apoptosis, the precise molecular pathways through which this process is controlled was not fully defined. Our work has uncovered a novel apoptosis pathway through which Bcl-xL controls activation of caspase-8 rather than that of caspase-9, the caspase that usually participates in the Bcl-xL pathway. Better understanding of the pathways controlling apoptosis in the thymus may enable (a) more selective control of thymic apoptosis and the immune response in the periphery, and (b) development of new strategies for treatment of caspase-8-dependent disorders, and immune-deficiencies.

### Declaration of competing interest

The authors declare that the submitted work was not carried out in the presence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest.

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### List of abbreviations:

GC	Glucocorticoid
GILZ	Glucocorticoid-induced leucine zipper
Dex	Dexamethasone
GR	Glucocorticoid Receptor
GRE	Glucocorticoid-responsive element
SUMO	Small ubiquitin-like modifier
FADD	Fas-associated protein with death domain
Bax	Bcl-2-associated X protein
Bak	Bcl-2 antagonist/killer
PUMA	P53-Upregulated Modulator of Apoptosis
Bad	Bcl-2-associated agonist of cell death
Hrk	Harakiri
Bik	Bcl-2-interacting killer
Bmf	Bcl-2 modifying factor
Bid	BH3 interacting domain death agonist
Mcl-1	Myeloid cell leukemia 1

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