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TITLE PAGE

Title: Pharmacological modulation of caspase-8 in thymus-related medical conditions

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RUNNING TITLE PAGE

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Beta-estradiol.17-valerate (E2); Gamma-tocopheryl quinine (Gamma-TQ); protein kinase C (PKC); Cyclophosphamide (CY); Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK); receptor-induced protein (RIP); Lipopolysaccharide (LPS); NO-synthase (mNOS); dibutyltin (DBTC); tributyltin (TBTC); 5-azacytidine (5AzC); 3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1).

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Abstract

The thymus is a lymphoid organ that governs the development of a diverse T cell repertoire capable of defence against non-self antigens and avoiding autoimmunity. However, the thymus can also succumb to different diseases. Hypertrophic diseases such as thymomas are typically associated with impairment of negative selection, which leads to autoimmune disease, or disruption of positive selection, which results in immunodeficiency. Hypotrophic diseases of the thymus can manifest during acute infections, cancer, allogeneic bone marrow transplantation, or with aging. This condition leads to decreased immune function and can be treated by either replacing lost thymic tissue or by preventing thymic tissue death. Studies have demonstrated the critical role of caspase-8 in regulating apoptosis in the thymus. In this review, we discuss how pharmacological activation and inhibition of caspase-8 activation can be used to treat hypertrophic and hypotrophic diseases of the thymus, respectively, to improve its function.

INTRODUCTION

Thymus physiology. The thymus is an ancient lymphoid organ that was present in both jawed and jawless vertebrates as a "thymoid" region (Hirano et al., 2013). Composed of thymocytes of hematopoietic origin and thymic epithelial cells (TECs) of nonhematopoietic origin, the thymus is the primary organ responsible for *de novo* generation of a diverse repertoire of immunocompetent T cells capable of conferring defence against non-self antigens (Delfino et al., 2011a). T cell development depends on interactions between antigen-MHC complexes on the surface of medullary TECs (mTECs) and the T-cell receptor (TCR) complex on the surface of thymocytes. In recent years, several novel molecules have been identified as critical modulators of thymic physiology. For example, studies have demonstrated that, in addition to interacting physically with thymocytes, TECs produce soluble factors such as thymic stromal lymphopoietin (TSLP). Polymorphisms in the gene encoding this cytokine have been associated with the development of multiple allergic disorders in humans, indicating that TSLP is a critical regulator of Th2 cytokine-associated inflammatory disease (Siracusa et al., 2011).

Other molecules involved in thymocyte differentiation have also been discovered and become the focus of much research. During T cell development, thymocytes survive by positive selection and are exported to the periphery if their affinity with TECs is weak. One important player in this interaction is Themis, a protein that increases the affinity threshold for activation, thus enabling positive selection of T cells with a naïve phenotype in response to low affinity self-antigens (Fu et al., 2013). Undergoing correct TCR rearrangement is also critical for thymocyte development. This maturation step occurs via a multistep process involving a concert of control factors such as intergenic control region 1 (IGCR1) (Guo et al., 2011) and cohesin (Seitan et al., 2011). These processes lead to the differentiation of thymocytes through various stages characterized by the expression of the CD4 and CD8 co-receptors, namely double negative (DN, $CD4^-CD8^-$) to double positive (DP, $CD4^+CD8^+$) to single positive (SP) $CD4^+$ or $CD8^+$ T cells. The

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positively selected mature T cell subsets can be further distinguished. For instance, $CD4^+$ T regulatory (Treg) cells expressing the transcription factor Foxp3 play a key role in limiting the inflammatory response in many organs, particularly in the intestine where they are derived from thymic (Cebula et al., 2013) and extrathymic (Lathrop et al., 2011; Arpaia et al., 2013) regions. Interestingly, different T cell subpopulations develop in the thymus under the guidance of signature transcription factors that induce rapid epigenetic remodelling. For instance, promyelocytic leukaemia zinc finger (PLZF), a BTB-zinc finger transcription factor that directs the innate-like effector program of natural killer T cells, has been associated with cullin 3 (CUL3), an E3 ubiquitin ligase previously shown to use BTB domain-containing proteins as adaptors for substrate binding (Mathew et al., 2012). Approximately 85-90% of thymocytes that do not undergo positive selection die by apoptosis in a process called negative selection (deletion of autoreactive thymocytes) or neglect (neither positively nor negatively selected thymocytes)(figure 1). Additionally, thymocyte apoptosis can be triggered by exogenous molecules such as glucocorticoids, which can lead to atrophy (Pozzesi et al., 2013).

Caspase-8 in thymus function. One important molecule critical to apoptosis in the thymus is caspase-8. Considered an initiator caspase, this pro-apoptotic member of the caspase family is activated upon stimulation by a death receptor (e.g., Fas), recruitment of the adaptor molecule FADD, and recruitment and subsequent processing of pro-caspase-8 via the extrinsic pathway. In the thymus, this cysteine protease mediates (1) TCR-triggered, (2) death receptor-mediated, and (3) glucocorticoid-induced apoptosis. Caspase-8 is active in medullary, semi-mature heat-stable $antigen^{hi}$ (HSA^{hi}) SP thymocytes as a consequence of strong TCR stimulation. The death receptors, Fas, FADD, Faim (Huo et al., 2009), and FLIP, are involved upstream of caspase-8 activation, while Bid and HDAC7 are activated downstream. Finally, caspase-8 has been linked to the protein Gilz in mediating glucocortocoid-induced thymocyte apoptosis (Pozzesi et al., 2013).

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THYMUS PATHOLOGY

Impairment of any molecules or processes involved in thymic physiology and thymocyte differentiation can lead to the development of disease. The thymus is the site of proliferative diseases (thymomas) as well as immunodeficiency and autoimmunity. This organ is also affected by extrathymic diseases, such as acute infections, cancers, or allogeneic bone marrow transplantation, all of which provoke thymic atrophy and disrupt the immune response (figure 2).

Thymomas are indolent, malignant tumours with a propensity for spreading locally. Thymomas are found in 15% of patients with myasthenia gravis, 50% of those with pure red cell aplasia (PRCA), and 10% of those with adult-onset hypogammaglobulinemia (Venna et al., 2011; Detterbeck and Zeeshan, 2013; Seton et al., 2013). Malignant thymomas are also found in 10% of Good's syndrome cases (Masuda, 2011). With similar NK and B cell deficiency, one of these cases strongly resembled the phenotype of an Ikaros-null knockout mouse (Ng et al., 2011). A recent report has also demonstrated thymoma development with acquired autoimmune polyglandular syndrome type 1 (APS1), a monogenic syndrome of pleomorphic autoimmunity characterized by hypoparathyroidism, hypoadrenalism, and candidiasis. APS1 arises from defects in the gene encoding auto-immune regulator (AIRE), which regulates the expression of tissue-specific self-antigens by mTECs. In the absence of AIRE, many tissue-specific self-antigens are not expressed in the thymus, resulting in multi-organ autoimmunity due to faulty negative selection of autoreactive T cells (Cheng et al., 2010).

Role of caspase-8 pathway in thymic diseases. Resistance to chemotherapy is a common challenge with various cancers, and dissecting the mechanisms leading to this resistance can lead to improved therapies. One mechanism of resistance involves the inhibition of caspase-8 (Lee et al., 2012) activation to block apoptosis of cancer cells (figure 3). In the example reported in the figure,

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stimulation of epidermal growth factor receptor, EGFR, blocks caspase-8 activation (fig. 3, left panel), whereas inhibition of EGFR by a chemotherapeutic agent (fig. 3, right panel) re-activates the caspase-8 pathway, leading to cancer cell death. The murine thymoma EL-4 cell line is resistant to Fas ligand (FasL)-induced apoptosis due to constitutive expression of the caspase-8 inhibitor c-FLIP. Forced expression of Fas rendered EL-4 cells highly susceptible to FasL-induced cell death, demonstrating that apoptosis is dependent on the expression of c-FLIP relative to cell surface-expressed Fas (Kataoka et al., 2002).

Recent evidence indicates that the long form of c-FLIP (c-FLIP(L)) is required for proliferation and effector T cell development. c-FLIP(L) transgenic mice exhibit splenomegaly, lymphoadenopathy, multi-organ infiltration, high titers of auto-antibodies, and proliferative glomerulonephritis with immune complex deposition in a strain-dependent manner. The development of autoimmunity requires $CD4^+$ T cells and may result from impaired thymic selection. At the molecular level, c-FLIP(L) overexpression inhibits ZAP-70 activation and signalling required for thymic selection. Thus, c-FLIP(L) has been identified as a susceptibility factor under the influence of epistatic modifiers in the development of autoimmunity (Qiao et al., 2010).

Ian4, a mitochondrial outer membrane protein with GTP-binding activity, is normally present in thymocytes, T cells, and B cells. A mutation in the rat Ian4 gene results in severe T cell lymphopenia that is associated with the pathogenesis of autoimmune diabetes. Lack of Ian4 in T cells causes mitochondrial dysfunction, increased mitochondrial levels of stress-inducible chaperonins and a leucine-rich protein, and spontaneous apoptosis of T cells. T cell activation and caspase-8 inhibition both prevented apoptosis, whereas transfection of T cells with Ian4-specific small interfering RNA induced apoptosis. These data suggest that Ian4-dependent pro-apoptotic

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events are active in thymocytes and may be responsible for autoimmune diabetes (Pandarpurkar et al., 2003).

In mouse thymic lymphoma 3SB cells expressing wild-type p53, ionizing radiation (IR) and UV light are potent triggers of caspase-3-dependent apoptosis. Although cytochrome c is released from mitochondria, caspase-9 activation is not observed following UV exposure. Instead UV light triggers the extrinsic apoptotic pathway and caspase-8 activation, which leads to Bid-mediated cytochrome c release from mitochondria. Thus, the post-mitochondrial apoptotic (intrinsic) pathway downstream of cytochrome c release cannot mediate the apoptosome function in UV-induced apoptosis in thymic 3SB cells (Okamoto et al., 2010).

Thymic graft-versus-host disease (tGVHD) can contribute to profound T cell deficiency and repertoire restriction after allogeneic BM transplantation (allo-BMT). However, the cellular mechanism underlying tGVHD and the interactions between donor alloreactive T cells and thymic tissues remain poorly defined. Using clinically relevant murine allo-BMT models, studies have shown that low numbers of donor alloreactive T cells, which caused mild non-lethal systemic GVHD, were sufficient to damage the thymus, delay T lineage reconstitution, and compromise donor peripheral T cell function. Radiation in BMT conditioning regimens can upregulate the expression of Fas and death receptor 5 (DR5) on thymic stromal cells (especially the epithelium), and decrease expression of the anti-apoptotic regulator of cellular caspase-8-like inhibitory protein. tGVHD can be mediated by interaction of donor alloreactive T cells with FasL and TNF-related apoptosis-inducing ligand (TRAIL) but not TNF or perforin, thereby disrupting thymic stromal cells, cytoarchitecture, and function (Na et al., 2010).

THYMUS PHARMACOLOGY

The thymus has been the target of numerous therapeutics designed to treat the aforementioned disease conditions. For example, a therapy that targets the thymus can be an

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important cause of immunosuppression. Antibodies against thymocytes (anti-thymocyte globulin, ATG) are raised in rabbits (rATG) or horses (hATG). rATG is used in combination with cyclophosphamide as part of a non-myeloablative transplant regimen (Burt et al., 2013) following autologous hematopoietic stem-cell transplantation (HSCT) in patients with systemic sclerosis. This antibody is also used with fludarabine for reduced-intensity conditioning in HLA-matched HSCT in patients with chronic granulomatous disease (Gungor et al., 2013). Interestingly, the response and survival of aplastic anaemia patients elicited by hATG is far superior over rATG treatment (European and Marrow Transplant Group, 2011).

Pharmacological and toxicological induction of caspase-8-dependent thymic apoptosis

Targeting the caspase-8 pathway can be an important element in treating thymomas and rendering chemotherapy-resistant thymomas more responsive to chemotherapeutic agents. Therefore, many compounds have been developed for these purposes (table 1).

Immunomodulators

Glucocorticoids (GC) are well-established inducers of apoptosis in the thymus. Caspase-8 has been implicated in this process (Pozzesi et al., 2013) along with glucocorticoid-induced leucine zipper (Gilz), which is upregulated by GCs in thymocytes. Recently, Gilz has been demonstrated to be involved in regulating T helper cell differentiation (Cannarile et al., 2009) and controlling malignant transformation through inhibition of Ras-driven tumourigenesis (Ayroldi and Riccardi, 2009; Ayroldi et al., 2012). In the thymus, GC-induced expression of Gilz is strictly linked to caspase-8 expression and activation, suggesting a feedback loop between Gilz and caspase-8 that begins with GC-mediated *Gilz* transcription. Once expressed, Gilz induces caspase-8 activation, which in turn promotes Gilz maintenance through its sumoylation and subsequent inhibition of ubiquitination/proteasomal degradation (Delfino et al., 2011b). Thus, Gilz protein levels are

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dependent on both GC-dependent regulation of *Gilz* transcription and caspase-8 activation (Pozzesi et al., 2013).

Cannabinoids are known to interact with CB1 and CB2 receptors expressed in the nervous and immune systems, respectively. These compounds mediate a wide range of effects, including anti-inflammatory ones. Studies have shown that JWH-015, a synthetic CB2-selective agonist, can trigger thymocyte apoptosis *in vitro*. JWH-015 induced cross-talk between the extrinsic and intrinsic pathways of apoptosis by involving caspase-8, -9, and -3, thereby leading to loss of mitochondrial membrane potential. Finally, JWH-015 administration *in vivo* caused thymic atrophy. Taken together, data from this study suggest that CB2-selective agonists, devoid of any psychotropic effect, may serve as novel anti-inflammatory/immunosuppressive agents (Lombard et al., 2007).

Terfenadine. The treatment of rat thymocytes with terfenadine (an anti-allergic drug) resulted in a dramatic increase in DNA fragmentation. Terfenadine stimulated caspase-8, -9, and -3-like activities in a time-dependent manner in thymocytes. The active forms of caspase-3 and -9 were detected in extracts from terfenadine-treated cells by immunoblotting using specific antibodies to caspases; however, active caspase-8 was not detected in this fraction, suggesting that terfenadine induces apoptosis in rat thymocytes via a mitochondrial pathway (Enomoto et al., 2004).

Heat-labile enterotoxin (EtxB). The B subunit of *Escherichia coli* EtxB is a potent immunomodulatory molecule capable of treating and preventing autoimmune disease. The finding that $CD8^+$ SP thymocytes from transgenic mice expressing a dominant-negative form of IkB alpha were markedly less susceptible to EtxB-induced apoptosis than cells from wild-type mice indicate that NFkB is important in the induction of apoptosis. Further investigation revealed that caspase-8 is active concomitant with caspase-3, consistent with death-receptor-mediated signalling. However,

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experiments using *lpr/lpr* and *p55 Tnfr*$^{-/-}$ mice eliminate the involvement of Fas and the p55 TNF receptor, respectively, in this process (Salmond et al., 2002).

Chemotherapeutic agents

Gamma-tocopheryl quinine (Gamma-TQ), an oxidative metabolite of gamma-tocopherol, is a potentially powerful chemotherapeutic agent since this molecule exerts powerful cytotoxic effects, induces apoptosis, and escapes drug resistance in human acute lymphoblastic leukaemia and promyelocytc leukaemia cells. The apoptotic potential of gamma-TQ was studied *in vivo* in murine thymoma cells grown in ascites. Gamma-TQ induced apoptosis in a dose- and time-dependent manner in all cell types examined. However, HL-60 and thymoma cells exhibited greater sensitivity, possibly owing to activation of caspase-8, caspase-9, Bid, and mitochondrial cytochrome c release (Calviello et al., 2003).

Etoposide. This anti-cancer drug induces apoptosis and, surprisingly, CD95-independent processing of caspase-8 in Jurkat cells. Consistent with this, thymocytes from CD95-deficient *lpr/lpr* mice readily undergo apoptosis in response to etoposide, indicating that CD95 is not required for etoposide-mediated caspase-8 processing and apoptosis (Boesen-de Cock et al., 1998).

1,4-benzothyazine (1,4-B) derivatives exert numerous effects *in vivo* and *in vitro*, including neurotoxicity and antitumor cytotoxicity. Results indicate that several 1,4-B analogues can induce both thymocyte apoptosis *in vitro* and thymus cell loss *in vivo*. Moreover, *in vitro* experiments have shown that 1,4-B-induced apoptosis is associated with different biochemical events, including phosphatidylcholine-specific phospholipase C activation, acidic sphingomyelinase activation and ceramide generation, loss of mitochondrial membrane potential and cytochrome c release, and caspase-8, -9, and -3 activation (Marchetti et al., 2002). Removal of the alcohol group by dehydration of olefin or transformation into ether increases the apoptotic activity of 1,4-B. Analogs

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of this compound activate caspase-8 and their structural properties correlate with those required for inducing apoptosis (Fringuelli et al., 2003).

Curcumin. Tumours induce thymic atrophy to evade the cellular immune response. Severe thymic hypocellularity, along with decreased thymic integrity, has been observed in tumour patients. Studies have shown that tumour-induced oxidative stress plays a critical role in thymic atrophy. This effect resulted from perturbation of nuclear factor kappa B (NFkB) activity by increasing cytosolic IkB alpha retention, which inhibited NFkB nuclear translocation in thymic T cells. These cells are vulnerable to tumour-secreted TNF-alpha, which mediates apoptosis by activating TNF receptor-associated protein, death domain-associated, Fas-associated protein death domain, and caspase-8. Curcumin prevents tumour-induced thymic atrophy by restoring NFkB activity. Thus, these results suggest that, unlike many other anticancer agents, curcumin is not only devoid of immunosuppressive effects, but can in fact restore immunity in tumour patients (Bhattacharyya et al., 2007b).

Cyclophosphamide (CY), a DNA-damaging drug, is widely used to treat haematological malignancies and autoimmune disorders. However, the molecular mechanism of how apoptosis is induced by this drug remains largely unknown. Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK) did not inhibit thymocyte and splenocyte depletion after CY treatment in mice. Caspase-8 and receptor-induced protein (RIP) were dispensable for 4-OOH-CY-mediated apoptosis, while overexpression of Bcl-2 was partially protective. These results strongly indicate that oxidative damage-induced nuclear translocation of AIF and EndoG in 4-OOH-CY-treated cells may represent an alternative death pathway in the absence of caspase activity (Strauss et al., 2008).

5-azacytidine (5AzC) is a cytidine analogue that causes DNA damage, which results in apoptosis, and DNA hypomethylation, which restores normal cell growth and differentiation. Treatment with 5azC increased the frequency of TUNEL-positive thymocytes and level of cleaved

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caspase-3 protein, two features of apoptosis. 5AzC-induced apoptosis was even observed in the thymus of mice deficient in p53, a critical factor in the intrinsic apoptotic pathway. Moreover, mice harbouring a mutation in the gene encoding Fas exhibited enhanced apoptosis. Following 5AzC treatment, the level of cleaved caspase-8 increased with cleavage of its target protein, Bid. Moreover, the level of TRAIL protein, which induces apoptosis through caspase-8 cleavage, increased dramatically in the thymus of 5AzC-treated animals. In conclusion, 5AzC-induced apoptosis of thymocytes *in vivo* is mediated by the extrinsic pathway and TRAIL activation (Tochitani et al., 2011).

Enzymes and enzyme inhibitors

YO-2. Treatment of rat thymocytes with YO-2, a novel inhibitor of plasmin, results in increased DNA fragmentation and, therefore, increased thymocyte apoptosis. Plasmin inhibitory activity may play an important role in YO-2-induced apoptosis. Furthermore, stimulation of caspase-8, -9, and -3-like activities has been observed in thymocytes treated with YO-2 (Lee et al., 2002).

Indoleamine 2,3-dioxygenase (IDO) is a tryptophan-catabolizing enzyme with regulatory effects on T cells resulting from tryptophan depletion in specific tissue microenvironments. Tryptophan metabolites in the kynurenine pathway, such as 3-hydroxyanthranilic and quinolinic acids, induce selective apoptosis of murine thymocytes *in vitro*. Relatively low concentrations of kynurenines can induce T cell apoptosis that does not require Fas/Fas ligand interactions and is associated with caspase-8 activation and mitochondrial cytochrome c release. When administered *in vivo*, these two kynurenines can deplete specific thymocyte subsets similar to dexamethasone (Fallarino et al., 2002).

Beta-estradiol.17-valerate (E2) induces thymic apoptosis and decreases thymic cellularity. Interestingly, however, the extent of thymic atrophy in *lpr/lpr* (Fas negative) and *gld/gld* (FasL

negative) mice was significantly less than that seen in wild-type mice. A caspase-8 inhibitor blocked E2-induced apoptosis of thymocytes *in vitro,* suggesting that E2 may induce apoptosis by activating a death receptor rather than the mitochondrial pathway. E2 treatment decreased the expansion and proliferation of peripheral Vbeta3+ T cells in response to the bacterial superantigen SEA, thereby suggesting increased induction of apoptosis in these cells (Do et al., 2002).

Toxic agents and contaminants

Nickel(II) exposure produces multiple effects on the immune system, including thymic involution. Studies on a murine T cell hybridoma cell line, KMls 8.3.5.1, demonstrate that this effect is due possibly to induction of caspase-8-dependent apoptosis. Co-incubation of nickel(II) with caspase inhibitors markedly abrogated this apoptotic effect, with Z-IETD.FMK, an inhibitor of caspase-8, nearly as effective as less selective caspase inhibitors (Kim et al., 2002).

Lipopolysaccharide (LPS), the endotoxin of Gram negative bacteria, can elicit a wide variety of pathophysiological effects, including endotoxin shock, tissue injury, and lethality in humans and animals. Lymphocytes exposed to *Carassius auratus* LPS exhibited a significant increase in intracellular reactive oxygen species, loss of mitochondrial transmembrane potential, depletion of ATP production, downregulation of Bcl-2 expression, upregulation of Bax and mitochondrial NO-synthase (mNOS) expression, and selective activation of caspase-9 rather than caspase-8. This mechanism differs from LPS-induced apoptosis in mammalian macrophage/thymocytes, which occurs via the TNF-alpha-mediated death receptor pathway (Xiang et al., 2008).

3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), a contaminant in our daily diet, induces apoptosis in cultured lymphocytes. Trp-P-1 treatment induced DNA fragmentation and morphological changes in the thymus. Moreover, this compound activated caspase-8 and -3, leading to cleavage of poly(ADP-ribose) polymerase 1 h after injection. Nevertheless, Trp-P-1

upregulated the anti-apoptotic factors Bcl-2 and Bcl-X_L and downregulated the pro-apoptotic factor Bax in mitochondria 1 hr after injection, indicating that Trp-P-1 can also trigger anti-apoptosis. Therefore, Trp-P-1 activates both pro- and anti-apoptotic signals *in vivo* in the immune system, particularly in the thymus, with apoptotic signals overcoming the survival ones (Hashimoto et al., 2002).

Sympathomimetic agents

Methamphetamine has been demonstrated to promote apoptosis by activating caspase-9 but not caspase-8 in rat thymocytes (Fujikawa et al., 2007).

Agents causing thymus cell death

Geldanamycin, a compound that binds heat shock protein 90 (HSP90), modulates various cellular activities. For instance, when administered along with TPA, an activator of protein kinase C (PKC), geldanamycin induced apoptosis in thymocytes by reducing the mitochondrial transmembrane potential. These findings suggest that HSP90 modulates thymocyte apoptosis in concert with PKC by destabilizing Lck in a caspase-8 and -3-dependent manner (Ohta et al., 2007).

Organotin compounds are known to cause thymic atrophy along with a deficiency in cell-mediated immunity. Cell death was demonstrated to be a contributing factor in the induction of thymic atrophy following exposure to dibutyltin (DBTC) and tributyltin (TBTC). In an *in vivo* study, reversible thymic atrophy was induced in rats by a single intraperitoneal administration of DBTC or TBTC; the magnitude of this effect over a 4-day treatment period differed between the two agents. TBTC exposure induced apoptosis and caused a marked increase in caspase-8, -9, and -3 activity, while DBTC exposure induced necrosis without any significant change in caspase activity. Therefore, these results indicate that increased cell death induced by organotin compounds likely contributed to the thymic atrophy observed in the rats (Tomiyama et al., 2009).

DISCUSSION

In this review, we dissected the pharmacological modulation of caspase-8 in the thymus from the perspective of treating diseases by inducing caspase-8-dependent apoptosis. Despite its ubiquitous expression throughout the body, which raises the possibility of widespread side effects, modulation of caspase-8 activation may be selectively important for immunity because its genetic absence results in immunodeficiency disease (Pozzesi et al., 2013). Furthermore, targeting caspase-8 activation could be effective in treating and preventing thymic atrophy, which is the hallmark of extrathymic medical conditions such as acute infections (Farias-de-Oliveira et al., 2013), cancer (Bhattacharyya et al., 2007a; Bhattacharyya et al., 2007b), or thymic GVHD (Na et al., 2010), and aging (Aspinall et al., 2010). The current trend in research focuses on discovering regenerative therapies (Boehm and Swann, 2013); however, preventing thymic atrophy by inhibiting caspase-8-dependent apoptosis could be an effective strategy for improving and prolonging thymus function.

Authorship Contribution.

Editing: Pozzesi

Wrote or contributed to the writing of the manuscript: Fierabracci, Martelli, Liberati, Ayroldi, Riccardi, Delfino

References

Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ and Rudensky AY (2013) Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* **504**:451-455.

Aspinall R, Pitts D, Lapenna A and Mitchell W (2010) Immunity in the elderly: the role of the thymus. *Journal of comparative pathology* **142 Suppl 1**:S111-115.

Ayroldi E, Cannarile L, Migliorati G, Nocentini G, Delfino DV and Riccardi C (2012) Mechanisms of the anti-inflammatory effects of glucocorticoids: genomic and nongenomic interference with MAPK signaling pathways. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **26**:4805-4820.

Ayroldi E and Riccardi C (2009) Glucocorticoid-induced leucine zipper (GILZ): a new important mediator of glucocorticoid action. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23**:3649-3658.

Bhattacharyya S, Mandal D, Saha B, Sen GS, Das T and Sa G (2007a) Curcumin prevents tumor-induced T cell apoptosis through Stat-5a-mediated Bcl-2 induction. *The Journal of biological chemistry* **282**:15954-15964.

Bhattacharyya S, Mandal D, Sen GS, Pal S, Banerjee S, Lahiry L, Finke JH, Tannenbaum CS, Das T and Sa G (2007b) Tumor-induced oxidative stress perturbs nuclear factor-kappaB activity-augmenting tumor necrosis factor-alpha-mediated T-cell death: protection by curcumin. *Cancer research* **67**:362-370.

Boehm T and Swann JB (2013) Thymus involution and regeneration: two sides of the same coin? *Nature reviews Immunology* **13**:831-838.

Boesen-de Cock JG, de Vries E, Williams GT and Borst J (1998) The anti-cancer drug etoposide can induce caspase-8 processing and apoptosis in the absence of CD95 receptor-ligand interaction. *Apoptosis : an international journal on programmed cell death* **3**:17-25.

Burt RK, Oliveira MC, Shah SJ, Moraes DA, Simoes B, Gheorghiade M, Schroeder J, Ruderman E, Farge D, Chai ZJ, Marjanovic Z, Jain S, Morgan A, Milanetti F, Han X, Jovanovic B, Helenowski IB and Voltarelli J (2013) Cardiac involvement and treatment-related mortality after non-myeloablative haemopoietic stem-cell transplantation with unselected autologous peripheral blood for patients with systemic sclerosis: a retrospective analysis. *Lancet* **381**:1116-1124.

Calviello G, Di Nicuolo F, Piccioni E, Marcocci ME, Serini S, Maggiano N, Jones KH, Cornwell DG and Palozza P (2003) gamma-Tocopheryl quinone induces apoptosis in cancer cells via caspase-9 activation and cytochrome c release. *Carcinogenesis* **24**:427-433.

Cannarile L, Cuzzocrea S, Santucci L, Agostini M, Mazzon E, Esposito E, Muia C, Coppo M, Di Paola R and Riccardi C (2009) Glucocorticoid-induced leucine zipper is protective in Th1-mediated models of colitis. *Gastroenterology* **136**:530-541.

Cebula A, Seweryn M, Rempala GA, Pabla SS, McIndoe RA, Denning TL, Bry L, Kraj P, Kisielow P and Ignatowicz L (2013) Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota. *Nature* **497**:258-262.

Cheng MH, Fan U, Grewal N, Barnes M, Mehta A, Taylor S, Husebye ES, Murphy EJ and Anderson MS (2010) Acquired autoimmune polyglandular syndrome, thymoma, and an AIRE defect. *The New England journal of medicine* **362**:764-766.

Delfino DV, Pozzesi N, Pierangeli S, Ayroldi E and Fierabracci A (2011a) Manipulating thymic apoptosis for future therapy of autoimmune diseases. *Current pharmaceutical design* **17**:3108-3119.

Delfino DV, Spinicelli S, Pozzesi N, Pierangeli S, Velardi E, Bruscoli S, Martelli MP, Pettirossi V, Falchi L, Kang TB and Riccardi C (2011b) Glucocorticoid-induced activation of caspase-8 protects the glucocorticoid-induced protein Gilz from proteasomal degradation and induces its binding to SUMO-1 in murine thymocytes. *Cell death and differentiation* **18**:183-190.

Detterbeck FC and Zeeshan A (2013) Thymoma: current diagnosis and treatment. *Chinese medical journal* **126**:2186-2191.

Do Y, Ryu S, Nagarkatti M and Nagarkatti PS (2002) Role of death receptor pathway in estradiol-induced T-cell apoptosis in vivo. *Toxicological sciences : an official journal of the Society of Toxicology* **70**:63-72.

Enomoto R, Komai T, Yoshida Y, Sugahara C, Kawaguchi E, Okazaki K, Kinoshita H, Komatsu H, Konishi Y and Lee E (2004) Terfenadine induces thymocyte apoptosis via mitochondrial pathway. *European journal of pharmacology* **496**:11-21.

European B and Marrow Transplant Group SAAWP (2011) Rabbit ATG for aplastic anaemia treatment: a backward step? *Lancet* **378**:1831-1833.

Fallarino F, Grohmann U, Vacca C, Bianchi R, Orabona C, Spreca A, Fioretti MC and Puccetti P (2002) T cell apoptosis by tryptophan catabolism. *Cell death and differentiation* **9**:1069-1077.

Farias-de-Oliveira DA, Villa-Verde DM, Nunes Panzenhagen PH, Silva dos Santos D, Berbert LR, Savino W and de Meis J (2013) Caspase-8 and caspase-9 mediate thymocyte apoptosis in Trypanosoma cruzi acutely infected mice. *Journal of leukocyte biology* **93**:227-234.

Fringuelli R, Schiaffella F, Utrilla Navarro MP, Milanese L, Santini C, Rapucci M, Marchetti C and Riccardi C (2003) 1,4-benzothiazine analogues and apoptosis: structure-activity relationship. *Bioorganic & medicinal chemistry* **11**:3245-3254.

Fu G, Casas J, Rigaud S, Rybakin V, Lambolez F, Brzostek J, Hoerter JA, Paster W, Acuto O, Cheroutre H, Sauer K and Gascoigne NR (2013) Themis sets the signal threshold for positive and negative selection in T-cell development. *Nature* **504**:441-445.

Fujikawa DG, Shinmei SS, Zhao S and Aviles ER, Jr. (2007) Caspase-dependent programmed cell death pathways are not activated in generalized seizure-induced neuronal death. *Brain research* **1135**:206-218.

Gungor T, Teira P, Slatter M, Stussi G, Stepensky P, Moshous D, Vermont C, Ahmad I, Shaw PJ, da Cunha JM, Schlegel PG, Hough R, Fasth A, Kentouche K, Gruhn B, Fernandes JF, Lachance S, Bredius R, Resnick IB, Belohradsky BH, Gennery A, Fischer A, Gaspar HB, Schanz U, Seger R, Rentsch K, Veys P, Haddad E, Albert MH, Hassan M, on behalf of the Inborn Errors Working Party of the European Society for B and Marrow T (2013) Reduced-intensity conditioning and HLA-matched haemopoietic stem-cell transplantation in patients with chronic granulomatous disease: a prospective multicentre study. *Lancet*.

Guo C, Yoon HS, Franklin A, Jain S, Ebert A, Cheng HL, Hansen E, Despo O, Bossen C, Vettermann C, Bates JG, Richards N, Myers D, Patel H, Gallagher M, Schlissel MS, Murre C, Busslinger M, Giallourakis CC and Alt FW (2011) CTCF-binding elements mediate control of V(D)J recombination. *Nature* **477**:424-430.

Hashimoto T, Furuyashiki T, Sano T, Kihara K, Fukuda I, Ito W, Park P, Kanazawa K, Danno G and Ashida H (2002) Apoptosis in the thymus after intraperitoneal injection of rats with Trp-P-1. *Environmental and molecular mutagenesis* **40**:175-183.

Hirano M, Guo P, McCurley N, Schorpp M, Das S, Boehm T and Cooper MD (2013) Evolutionary implications of a third lymphocyte lineage in lampreys. *Nature* **501**:435-438.

Huo J, Xu S, Guo K, Zeng Q and Lam KP (2009) Genetic deletion of faim reveals its role in modulating c-FLIP expression during CD95-mediated apoptosis of lymphocytes and hepatocytes. *Cell death and differentiation* **16**:1062-1070.

Kataoka T, Ito M, Budd RC, Tschopp J and Nagai K (2002) Expression level of c-FLIP versus Fas determines susceptibility to Fas ligand-induced cell death in murine thymoma EL-4 cells. *Experimental cell research* **273**:256-264.

Kim K, Lee SH, Seo YR, Perkins SN and Kasprzak KS (2002) Nickel(II)-induced apoptosis in murine T cell hybridoma cells is associated with increased fas ligand expression. *Toxicology and applied pharmacology* **185**:41-47.

Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, Peterson DA, Stappenbeck TS and Hsieh CS (2011) Peripheral education of the immune system by colonic commensal microbiota. *Nature* **478**:250-254.

Lee E, Enomoto R, Takemura K, Tsuda Y and Okada Y (2002) A selective plasmin inhibitor, trans-aminomethylcyclohexanecarbonyl-L-(O-picolyl)tyrosine-octylamide (YO-2), induces thymocyte apoptosis. *Biochemical pharmacology* **63**:1315-1323.

Lee MJ, Ye AS, Gardino AK, Heijink AM, Sorger PK, MacBeath G and Yaffe MB (2012) Sequential application of anticancer drugs enhances cell death by rewiring apoptotic signaling networks. *Cell* **149**:780-794.

Lombard C, Nagarkatti M and Nagarkatti P (2007) CB2 cannabinoid receptor agonist, JWH-015, triggers apoptosis in immune cells: potential role for CB2-selective ligands as immunosuppressive agents. *Clinical immunology* **122**:259-270.

Marchetti C, Ulisse S, Bruscoli S, Russo FP, Migliorati G, Schiaffella F, Cifone MG, Riccardi C and Fringuelli R (2002) Induction of apoptosis by 1,4-benzothiazine analogs in mouse thymocytes. *The Journal of pharmacology and experimental therapeutics* **300**:1053-1062.

Masuda S (2011) More on NK-cell and B-cell deficiency with a thymic mass. *The New England journal of medicine* **364**:1979-1980; author reply 1980-1971.

Mathew R, Seiler MP, Scanlon ST, Mao AP, Constantinides MG, Bertozzi-Villa C, Singer JD and Bendelac A (2012) BTB-ZF factors recruit the E3 ligase cullin 3 to regulate lymphoid effector programs. *Nature* **491**:618-621.

Na IK, Lu SX, Yim NL, Goldberg GL, Tsai J, Rao U, Smith OM, King CG, Suh D, Hirschhorn-Cymerman D, Palomba L, Penack O, Holland AM, Jenq RR, Ghosh A, Tran H, Merghoub T, Liu C, Sempowski GD, Ventevogel M, Beauchemin N and van den Brink MR (2010) The cytolytic molecules Fas ligand and TRAIL are required for murine thymic graft-versus-host disease. *The Journal of clinical investigation* **120**:343-356.

Ng S, Fanta C, Okam M and Bhatt AS (2011) NK-cell and B-cell deficiency with a thymic mass. *The New England journal of medicine* **364**:586-588.

Ohta K, Okoshi R, Wakabayashi M, Ishikawa A, Sato Y and Kizaki H (2007) Geldanamycin, a heat-shock protein 90-binding agent, induces thymocyte apoptosis through destabilization of Lck in presence of 12-O-tetradecanoylphorbol 13-acetate. *Biomedical research* **28**:33-42.

Okamoto M, Koga S and Tatsuka M (2010) Differential regulation of caspase-9 by ionizing radiation- and UV-induced apoptotic pathways in thymic cells. *Mutation research* **688**:78-87.

Pandarpurkar M, Wilson-Fritch L, Corvera S, Markholst H, Hornum L, Greiner DL, Mordes JP, Rossini AA and Bortell R (2003) Ian4 is required for mitochondrial integrity and T cell survival. *Proceedings of the National Academy of Sciences of the United States of America* **100**:10382-10387.

Pozzesi N, Fierabracci A, Liberati AM, Martelli MP, Ayroldi E, Riccardi C and Delfino DV (2013) Role of caspase-8 in thymus function. *Cell death and differentiation*.

Qiao G, Li Z, Minto AW, Shia J, Yang L, Bao L, Tschopp J, Gao JX, Wang J, Quigg RJ and Zhang J (2010) Altered thymic selection by overexpressing cellular FLICE inhibitory protein in T cells causes lupus-like syndrome in a BALB/c but not C57BL/6 strain. *Cell death and differentiation* **17**:522-533.

Salmond RJ, Pitman RS, Jimi E, Soriani M, Hirst TR, Ghosh S, Rincon M and Williams NA (2002) CD8+ T cell apoptosis induced by Escherichia coli heat-labile enterotoxin B subunit occurs via a novel pathway involving NF-kappaB-dependent caspase activation. *European journal of immunology* **32**:1737-1747.

Seitan VC, Hao B, Tachibana-Konwalski K, Lavagnolli T, Mira-Bontenbal H, Brown KE, Teng G, Carroll T, Terry A, Horan K, Marks H, Adams DJ, Schatz DG, Aragon L, Fisher AG, Krangel MS, Nasmyth K and Merkenschlager M (2011) A role for cohesin in T-cell-receptor rearrangement and thymocyte differentiation. *Nature* **476**:467-471.

Seton M, Wu CC and Louissaint A, Jr. (2013) Case records of the Massachusetts General Hospital. Case 26-2013. A 46-year-old woman with muscle pain and swelling. *The New England journal of medicine* **369**:764-773.

Siracusa MC, Saenz SA, Hill DA, Kim BS, Headley MB, Doering TA, Wherry EJ, Jessup HK, Siegel LA, Kambayashi T, Dudek EC, Kubo M, Cianferoni A, Spergel JM, Ziegler SF, Comeau MR and Artis D (2011) TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. *Nature* **477**:229-233.

Strauss G, Westhoff MA, Fischer-Posovszky P, Fulda S, Schanbacher M, Eckhoff SM, Stahnke K, Vahsen N, Kroemer G and Debatin KM (2008) 4-hydroperoxy-cyclophosphamide mediates caspase-independent T-cell apoptosis involving oxidative stress-induced nuclear relocation of mitochondrial apoptogenic factors AIF and EndoG. *Cell death and differentiation* **15**:332-343.

Tochitani T, Kanemitsu H, Yamauchi H, Uchida K and Nakayama H (2011) 5-azacytidine, a chemotherapeutic drug, induces TRAIL-mediated apoptosis in mouse thymocytes in vivo. *Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie* **63**:237-242.

Tomiyama K, Yamaguchi A, Kuriyama T and Arakawa Y (2009) Analysis of mechanisms of cell death of T-lymphocytes induced by organotin agents. *Journal of immunotoxicology* **6**:184-193.

Venna N, Gonzalez RG and Zukerberg LR (2011) Case records of the Massachusetts General Hospital. Case 39-2011. A woman in her 90s with unilateral ptosis. *The New England journal of medicine* **365**:2413-2422.

Xiang LX, Peng B, Dong WR, Yang ZF and Shao JZ (2008) Lipopolysaccharide induces apoptosis in Carassius auratus lymphocytes, a possible role in pathogenesis of bacterial infection in fish. *Developmental and comparative immunology* **32**:992-1001.

Footnotes:

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FIGURE LEGENDS

Figure 1: Thymic microenvironment and T-cell differentiation. The development of thymocytes into mature T cells takes place within the thymus. There are three basic steps in this process. 1) A few $CD4^-CD8^-$ DN cells (orange, left) originating from the bone marrow proliferate and differentiate into $CD4^+CD8^+$ DP cells inside niches of thymic epithelial cells (TECs). During this process, T-cell receptor (TCR) rearrangement takes place through the involvement of IGCR1 and cohesin. 2) The large majority (80%) of DP thymocytes (red, blue, middle) die by neglect (violet) if they do not interact with TECs while a smaller percentage (5%) die by negative selection as a consequence of an overly strong interaction with TECs (violet). Thymocytes expressing Themis survive by positive selection as a consequence of an appropriately weak interaction with TECs. 3) These thymocytes differentiate into various $CD4^+$ SP subsets (NK-T, $CD4^+$, or Treg) (red) or $CD8^+$ SP (blue) cells. The growth factor TSLP produced by TECs is responsible for allergic diseases, whereas the PLZF is responsible for NK-T cell differentiation.

Figure 2: Thymus-related medical conditions. The hypertrophic or hypotrophic thymus can be the consequence of different pathologies. Thymomas are often associated with either autoimmunity or immunodeficiency (myasthenia gravis, PRCA, hypogammaglobulinemia, Good's syndrome, or APS-1). A hypotrophic thymus is the consequence of diseases such as cancers, acute infections, and thymic GVHD or physiologic conditions such as aging.

Figure 3: Importance of caspase-8 in the sensitization of cancer cells to chemotherapy. On the left side, A cancer cell resistant to a chemotherapeutic DNA damaging agent in which the caspase-8 pathway is not activated (light gray dotted arrows, left). The same cancer cell treated sequentially with a chemotherapeutic DNA damaging agent (time 1) followed by an epidermal growth factor receptor (EGFR) inhibiting agent (time 2). This cell becomes highly sensitive to apoptosis and low tumorigenic (light gray dotted lines) due to activation of the caspase-8 pathway.

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Table1: Effect of different compounds on caspase-8 pathway

Compound	Caspase-8 pathway activation	Caspase-8 pathway inhibition	Ref
Glucocorticoids	+		**Pozzesi et al, Cannarile et al., Ayroldi et al, 2009, Ayroldi et al. 2012, Delfino et al.**
1, 4 benzothyazine	+		**Marchetti et al, Fringuelli et al.**
Curcumin		+	**Bhattacharyya et al.**
Yo-2	+		**Lee et al.**
EtxB	+		**Salmond et al.**
Indoleamine 2,3-dioxygenase (IDO)	+		**Fallarino et al.**
Beta-estradiol.17-valerate (E2)	+		**Do et al.**
Nickel(II)	+		**Kim et al.**
Gamma-tocopheryl quinine (gamma-TQ)	+		**Calviello et al.**
Etoposide	+		**Boesen-de Cock et al.**
Terfenadine	+/-		**Enomoto et al.**
Cannabinoids	+		**Lombarde t al.**
Methamphetamine			**Fujikawa et al.**
Geldanamycin	+		**Ohta et al.**
Cyclophosphamide (CY)	-		**Strauss et al.**
Lipopolysaccharide (LPS)	-		**Xiang et al.**
Organotin compounds	+		**Tomiyama et al.**
5-azacytidine (5AzC)	+		**Tochitani et al.**
3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1)	+		**Hashimoto et al.**

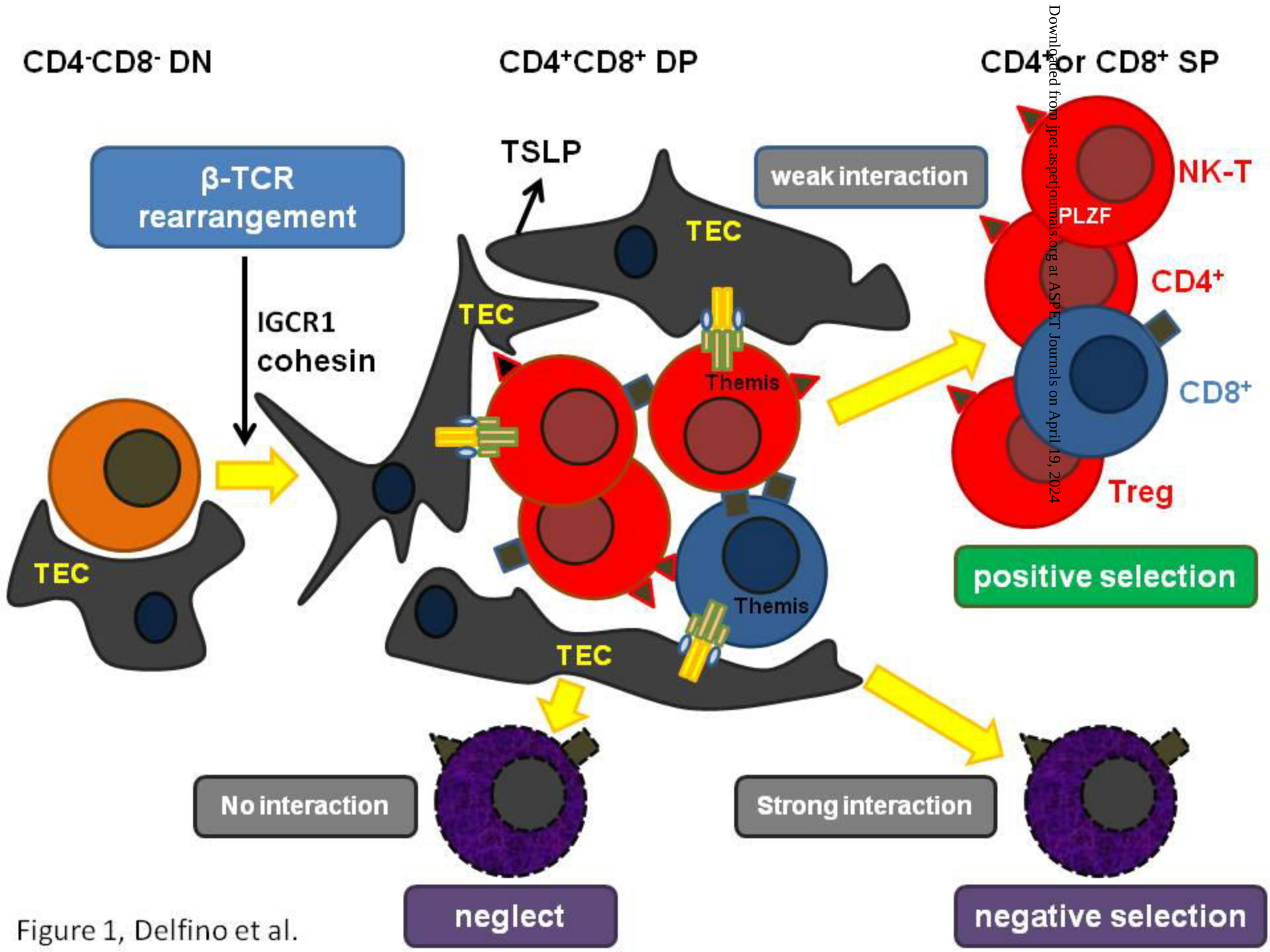

Figure 1, Delfino et al.

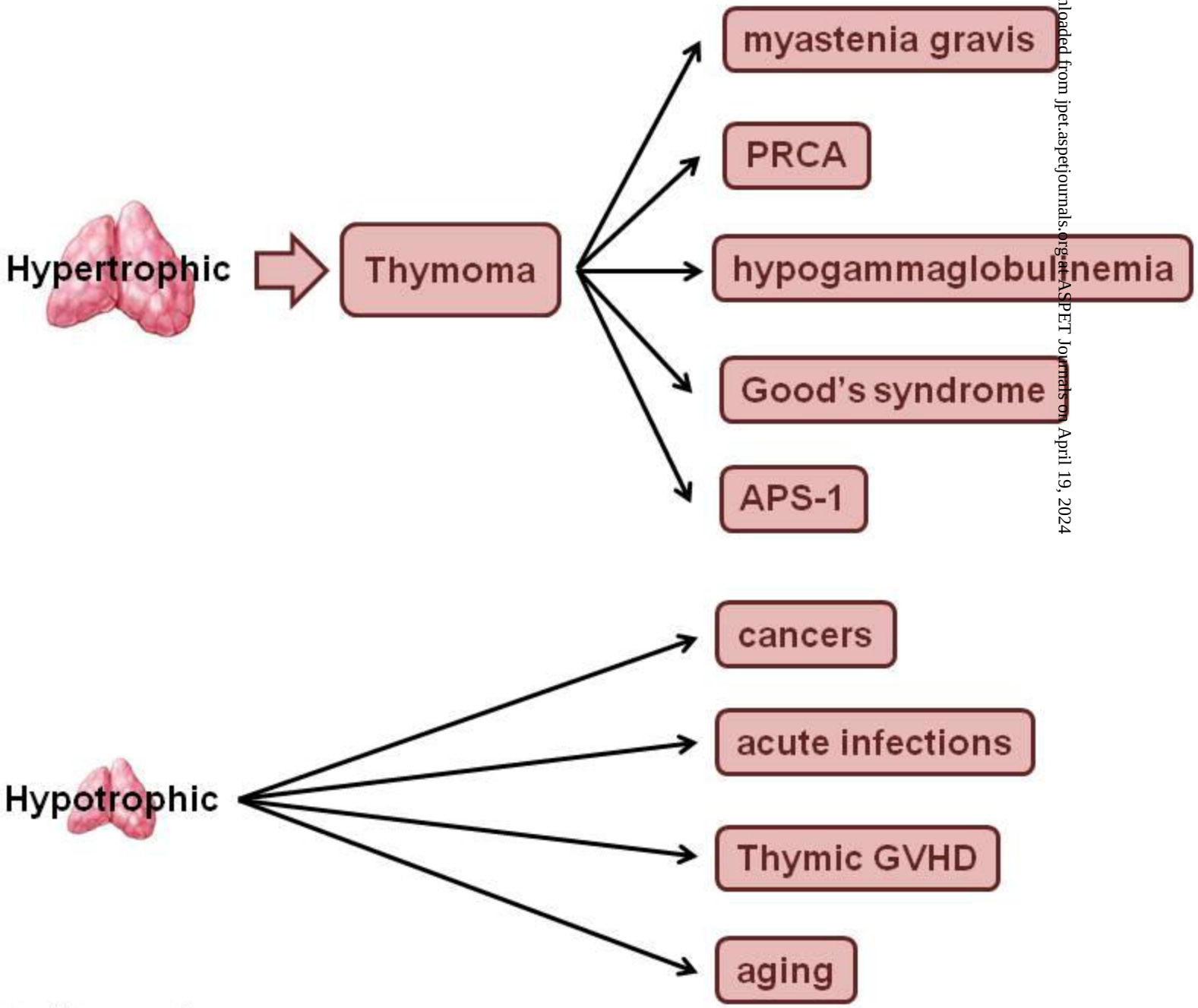

Figure 2, Delfino et al.

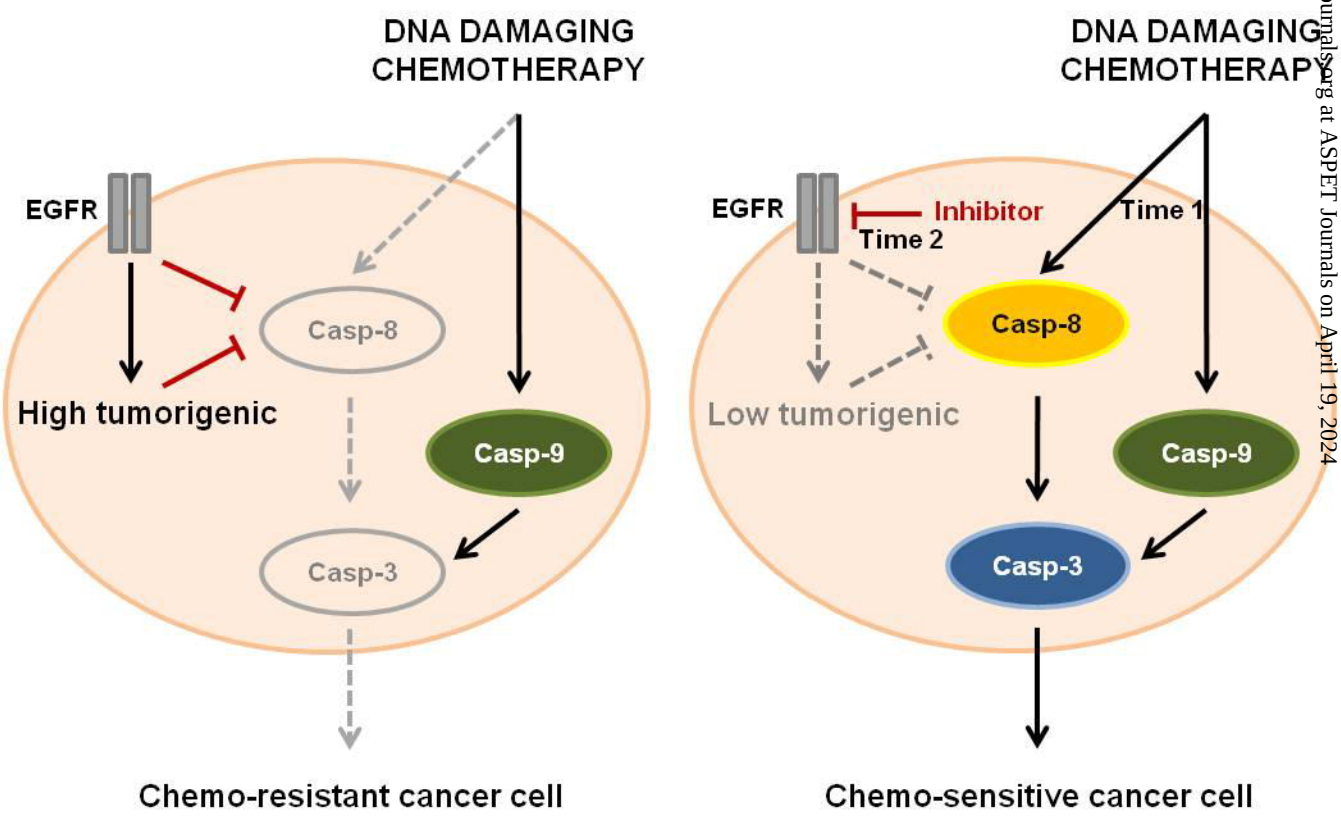

Figure 3, Delfino et al.