Transcriptional Regulation of the Murine Caspase-3 Gene during T cell Activation

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Abstract

Caspases play an important role in shaping the developing organism. They are required to eliminate unwanted or damaged cells and therefore are able to prevent disease. Several reports have shown increased caspase-3 expression in different cell types undergoing apoptosis. We undertook to examine the role of T cell activation through the T cell receptor (TCR) in regulating caspase-3 gene expression. The KOX-14 murine T cell hybridoma was initially used as a model for activation-induced cell death. Caspase-3 mRNA levels increased by 3-fold following T cell activation and was independent of STAT1 activation and therefore of IFN-γ signaling in KOX-14 cells suggesting that the increase occurs early during T cell activation. Naïve T cells were then isolated from the lymph nodes of mice to determine the extent of the increase in caspase-3 mRNA levels in cells undergoing proliferation rather than apoptosis. Caspase-3 mRNA levels were selectively induced (13-fold) following TCR triggering. Furthermore, caspase-3 mRNA levels were the highest in effector T cells which are destined to undergo AICD, when compared to long-lived memory T cells. Interestingly, the levels of procaspase-3 were also induced (6-fold) in activated peripheral T cells. In addition, T cells deficient for caspase-3 were more resistant to different apoptosis inducing molecules when compared to T cells containing normal levels of caspase-3. Altogether, these results demonstrate that the levels of caspase-3 must be maintained in a cell to ensure efficient apoptosis. The caspase-3 promoter region was subsequently cloned to identify transcription factors responsible for the observed increased in caspase-3 mRNA levels during T cell activation. Several regions within the promoter had either positive or negative regulatory effects on reporter activity when deleted. TCR stimulation of KOX-14 cells containing the different caspase-3 promoter constructs did not show changes in reporter activity. These findings suggest that the elements required for the induction in caspase-3 mRNA levels during T cell activation are not present in the region investigated or other regulatory mechanisms are involved. The present study represents the first characterization of the murine caspase-3 promoter during T cell activation. Overall, our data demonstrates the importance of maintaining caspase-3 levels in T cells and suggest a potential role for caspase-3 in the differentiation of memory T cells.

Sommaire

Les caspases ont un rôle important dans le développement de l'organisme. Elles éliminent les cellules endommagées et alors sont capables de prévenir les maladies. Plusieurs études ont démontré une augmentation de l'expression de caspase-3 dans différents types cellulaires mourant par apoptose. Nous avons envisagé d'examiner le rôle de l'activation des cellules T, par le récepteur des cellules T (TCR), dans la régulation de l'expression du gène de caspase-3. L'hybridome de cellule T murin KOX-14 a été utilisé en premier comme modèle de la mort cellulaire induite par l'activation (AICD). Les niveaux d'ARNm de caspase-3 ont été induits de 3-fois en conséquence de l'activation des cellules T et ceci indépendamment de l'activation de STAT1 et donc de la signalisation induite par l'IFN-γ dans les cellules KOX-14. Ces résultats suggèrent que l'augmentation survient tôt durant l'activation des cellules T. Ensuite, des cellules T naïves ont été isolées des ganglions lymphatiques de souris pour déterminer l'ampleur de l'augmentation des niveaux d'ARNm de caspase-3 dans des cellules qui prolifèrent plutôt que de cellules qui meurent par apoptose. Les niveaux d'ARNm de caspase-3 ont été uniquement induites (13-fois) suite à la réticulation du TCR. Par ailleurs, les niveaux d'ARNm de caspase-3 étaient le plus élevés dans les cellules T effectrices qui sont destinées à mourir par AICD, contrairement aux cellules T mémoires. Les niveaux de procaspase-3 ont aussi été induits (6-fois) dans des cellules T périphérique activées. De plus, les cellules T qui n'expriment pas la caspase-3 étaient plus résistantes à différents stimuli apoptotiques en comparaison aux cellules T exprimant des niveaux normaux de caspase-3. En résumé, ces résultats démontrent que les niveaux de caspase-3 doivent être maintenus dans une cellule pour assurer une apoptose efficace. Ensuite, le promoteur de caspase-3 a été cloné pour identifier les facteurs de transcription responsable de l'augmentation des niveaux d'ARNm de caspase-3 durant l'activation des cellules T. Plusieurs régions du promoteur avaient des effets régulateurs positifs ou négatifs sur l'activité rapporteuse dans certaines délétions. L'activation des cellules KOX-14 contenant les différentes délétions du promoteur de caspase-3 par le TCR n'a démontré aucun changement dans l'activité rapporteuse. Ces résultats suggèrent que les éléments requis pour l'induction des niveaux d'ARNm de caspase-3 durant l'activation ne sont pas presents dans la région étudiée ou que d'autres mécanismes de régulation sont impliqués.

Cette étude est la première à caractériser le promoteur murin de caspase-3 durant l'activation des cellules T. Dans l'ensemble, ces données démontrent l'importance de maintenir les niveaux de caspase-3 dans les cellules T et suggèrent un rôle potentiel pour caspase-3 dans la différentiation des cellules T mémoires.

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Contributions of authors

Chapter 2: Selective upregulation of caspase-3 gene expression precedes the onset of apoptosis following TCR triggering. Laurent Sabbagh, Luchino Y. Cohen, Martin Bourbonnière and Rafick-Pierre Sékaly.

This chapter is written in manuscript format and will be submitted for publication to the European Journal of Immunology. Part of the results presented in figure 1 and all the experiments presented in figures 2, 3, 5 and 6 were done by Laurent Sabbagh. Laurent Sabbagh also developed the real-time RT-PCR assay. The second author, Luchino Y. Cohen, performed part of the experiments in figure 1 and generated the data for figure 4. Martin Bourbonnière performed the Western blots and was involved in planning the experiments. All authors contributed to the writing of the manuscript.

Chapter 3: The selective increase in caspase-3 expression in effector but not memory T cells allows susceptibility to apoptosis. Laurent Sabbagh, Susan M. Kaech, Martin Bourbonnière, Minna Woo, Luchino Y. Cohen, Nathalie Labrecque, Rafi Ahmed, and Rafick-Pierre Sékaly.

This chapter is written in manuscript format and will be submitted for publication to the Journal of Experimental Medicine. All the experiments presented in this manuscript were done by Laurent Sabbagh except for the generation of effector and memory T cells and the Affymetrix data that were generated by Susan M. Kaech in Rafi Ahmed's laboratory. The third author, Martin Bourbonnière participated in the analysis of the data and the planning of the experiments along with Luchino Y. Cohen. Minna Woo and Nathalie Labrecque are collaborators who provided the caspase-3 knockout mice and the 2C TCR transgenic mice, respectively. Laurent Sabbagh, Susan M. Kaech, Martin Bourbonnière, Luchino Y. Cohen, and Rafick-Pierre Sékaly contributed to the writing of the manuscript.

Chapter 4: Cloning and functional characterization of the murine caspase-3 gene promoter during T cell activation. Laurent Sabbagh, Martin Bourbonnière, François Denis and Rafick-Pierre Sékaly.

This chapter is written in manuscript format and will be submitted for publication to the journal DNA and Cell Biology. The generation of the caspase-3 promoter deletion constructs, sequencing of deletion mutants, transient transfections, cloning of the promoter region into the bottom strand retroviral vector and infection of KOX-14 cells along with flow cytometry analysis were done by Laurent Sabbagh. Also a 5 Kb region of intron 1 and the caspase-3 promoter were sequenced by Laurent Sabbagh. Martin Bourbonnière identified the transcriptional start sites, cloned the caspase-3 gene and promoter region and performed bioinformatics analysis. François Denis sequenced the murine caspase-3 gene. Laurent Sabbagh, Martin Bourbonnière and Rafick-Pierre Sékaly contributed to the writing of the manuscript.

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Abbreviations

AICD:	activation-induced cell death
AIF:	apoptosis-inducing factor
ALS:	amyotrophic lateral sclerosis
AnV:	Annexin V
AP-1:	activator protein-1
APC:	antigen presenting cell
BH:	Bcl-2 homology
BIR:	baculoviral IAP repeat
CAD:	caspase-activated DNase
CARD:	caspase recruitment domain
Cdk:	cell-cycle-dependent kinase
cFLIP:	cellular FADD-like IL-1β-converting enzyme inhibitory protein
CFSE:	5-(and-6)-carboxyfluorescein diacetate succinimidyl ester
ChIP:	chromatin immunoprecipitation
CTL:	cytotoxic T lymphocyte
DAG:	diacylglycerol
DD:	death domain
DED:	death-effector domain
DIABLO:	direct IAP-binding protein with low pI
DISC:	death-inducing signaling complex
EndoG:	endonuclease G
ER:	endoplasmic reticulum
FADD:	Fas-associated death domain
FADD-DN:	dominant negative mutant of FADD
FasL:	Fas ligand
FLIP:	FLICE/caspase-8 inhibitory protein
γc:	common γ-chain
GrB:	granzyme B
GSK3:	glycogen synthase kinase-3

HD:	Huntington disease
HID:	head involution defective
HN:	humanin
IAP:	inhibitor of apoptosis protein
ICAD:	inhibitor of caspase-activated DNase
ICE:	interleukin-1β converting enzyme
IFN:	interferon
IL:	interleukin
Ins(1,4,5)P3	inositol 1,4,5-triphosphate
IRF-1:	interferon regulatory factor-1
ITAM:	immunoreceptor tyrosine-based activation motif
JNK:	Jun kinase
LCMV:	lymphocytic choriomeningitis virus
LPS:	lipopolysaccharide
MAP:	mitogen-activated protein
MAKKK:	MAP kinase kinase kinases
MHC:	major histocompatibility complex
MOMP:	mitochondrial outer membrane permeabilization
NFAT:	nuclear factor of activated T cells
NF-κB:	nuclear factor κB
PBMC:	peripheral blood mononuclear cells
PHA	phytohaemaglutinin A
PI:	propidium iodide
PKA:	protein kinase A
PKB:	protein kinase B
PKC:	protein kinase C
PLCγ1:	phospholipase Cγ1
pRB:	retinoblastoma protein
PT:	permeability transition
RPA:	RNase protection assay
RTE:	recent thymic emigrants

SEB: staphylococcal enterotoxin B

Smac: second mitochondria-derived activator of caspases

SP-1 secretory protein-1

STAT1: signal transducer and activator of transcription 1

TCR: T cell receptor

TCM: central memory T cells

TEM: effector memory T cells

T_H T helper

TNF-R: tumor necrosis factor receptor

TRAIL: TNF-related apoptosis-inducing ligand

RPR: reaper

zDEVD-fmk: benzyloxycarbonyl (Cbz)-Asp(OMe)-Glu(OMe)-Val-Asp(OMe) fluoromethylketone

zVAD-fmk: benzyloxycarbonyl (Cbz)-Val-Ala-Asp(OMe)-fluoromethylketone

Chapter 1: Literature Review

1.1 T cell activation

The immune system is a highly specialized defense mechanism, its main function being the elimination of foreign microorganisms from an infected host. Moreover, it plays a crucial role in the rejection of tumors. The early response to an infection depends on innate immunity in which a variety of innate mechanisms recognize and respond to the presence of a pathogen. Innate immunity is present in all individuals at all times, and does not increase with repeated exposure to a given pathogen. However, adaptive immune responses, which depend on lymphocytes (B and T cells), lead to the production of antibodies against a particular pathogen and confer protection during the lifetime of an individual against reinfection with the same pathogen. Both the innate and adaptive immune systems provide an effective defense mechanism against the presence of pathogens present in our environment which ensures that we rarely become ill.

The works of Edward Jenner, Robert Koch and Louis Pasteur demonstrated the concept of long lived protection and introduced vaccination as a means of creating what is now known as immunological memory. The majority of infections are able to be successfully eliminated by the innate system without causing disease. However, infections that are not resolved by innate immunity lead to the initiation of adaptive immune responses which are capable of successfully clearing the infection and generating long lasting immunological memory.

Within an organism a variety of cell populations are involved in immunity. The precursor cells originate from the bone marrow and subsequently migrate to peripheral or secondary lymphoid organs. Lymphoid organs consist of tissues within a host that contain a large number of lymphocytes in an environment of nonlymphoid cells. Interactions of lymphocytes with nonlymphoid cells are important during lymphocyte development, the initiation of adaptive immune responses and the maintenance of lymphocytes. Lymphocytes are generated in primary lymphoid organs (bone marrow and

thymus) and adaptive immune responses are initiated and maintained in secondary lymphoid organs (spleen and lymph nodes).

Although B and T lymphocytes originate in the bone marrow only B cells mature there. T cell precursors migrate to the thymus and undergo positive and negative selection, which are mechanisms that eliminate T lymphocytes that are autoreactive to self-antigen and generate diversity in the T cell repertoire capable of recognizing foreign antigens. Once both B and T cell maturation are completed, both types of lymphocytes migrate to peripheral lymphoid organs through the bloodstream.

1.1.1 Immune response

T cells that survive thymic selection circulate through secondary lymphoid organs and monitor for the presence of foreign antigens. Mature recirculating T cells that have not yet encountered their antigens are known as naïve T cells. Naïve T cells are metabolically quiescent (Sprent and Tough, 1994) and require constant contact with self-peptide/major histocompatibility complex (MHC) complexes on dendritic cells (Tanchot et al., 1997) and the presence of the interleukin (IL)-7 cytokine, which has been shown to be critical for the survival of naïve T cells (Krajewski et al., 1999). The requirement for these molecules for the maintenance of naïve T cells is probably due to low-level signaling which allows the cells to maintain some intracellular activity and avoid passive cell death (Sprent and Surh, 2002). The function of MHC molecules is to bind peptide fragments derived from pathogens and display them on the cell surface of dendritic cells for recognition by the appropriate T cells which lead to their activation and clonal expansion.

Immature precursors of dendritic cells are found throughout the body, and ingest antigens at the site of infection. They are highly efficient at antigen processing, in contrast to the mature dendritic cells found in the T cell zones which are poor at capturing and processing native proteins into peptides (Jenkins et al., 2001). Immature dendritic cells migrate from the site of infection to secondary lymphoid tissues, process the antigen

into immunogenic peptides which are presented to naïve T cells in the context of MHC molecules and differentiate into mature dendritic cells. Antigen presenting cells (APC), such as dendritic cells, are also activated by inflammatory cytokines and proteins (heat shock proteins) released by cells undergoing necrosis (Galucci et al., 1999).

A large number of APC are activated, at the beginning of an immune response, due to the initial replication of pathogens (Sprent and Surh, 2002). These highly stimulatory APCs induce naïve T cells to proliferate and differentiate into effector T cells capable of eliminating the antigen. Effector cells are known as T helper (T_H) cells or cytotoxic T lymphocytes (CTLs) for $CD4^+$ and $CD8^+$ T cells, respectively. At the termination of an immune response, when the pathogen has been eliminated at the site of infection, the majority (>90%) of effector T cells die and a small proportion of surviving T cells become long-lived memory T cells (Fig. 1).

An immune response therefore involves three stages of T cell differentiation. The first stage consists of clonal expansion of antigen-specific T cells upon an encounter with an antigen. Following clearance of the pathogen a large number of effector T cells are eliminated, and this stage is referred to as the contraction phase. Finally, the remaining T cells differentiate into memory T cells and are maintained for long periods of time (Fig. 1) (Kaech et al., 2002b). Each of these phases will be discussed in greater detail in the sections below.

1.1.2 The immunological synapse

Initial interaction of the T cell receptor (TCR) with a peptide-MHC complex on the surface of an APC leads to the rapid clustering of TCR molecules bound to peptide-MHC complexes and the formation of an immunological synapse (Monks et al., 1998; Grakoui et al., 1999; Lanzavecchia and Sallusto, 2000). The immunological synapse is defined by a specific pattern of receptor segregation with a central cluster of TCRs surrounded by a ring of integrin family adhesion molecules (Monks et al., 1998). Three stages for TCR engagement during the formation of a stable immunological synapse have

been identified: junction formation, peptide-MHC transport, and stabilization (McHeyzer-Williams and Davis, 1995). During the formation of the junction (stage 1), LFA-1 anchors the central region of the nascent immunological synapse forcing an outermost ring of the T cell membrane which allows the TCRs to interact with peptide-MHC complexes. This stage is followed by peptide-MHC transport into the central cluster which might be mediated by actin-based transport mechanisms. Finally, the clustered peptide-MHC complexes are stabilized and sequestered from the pool of free peptide-MHC complexes (Grakoui et al., 1999).

Synapse formation and rapid clustering of TCRs binding to peptide-MHC complexes may be the last stage in which the TCR participates in T cell activation. It is also associated with the accumulation of intracellular signaling molecules such as Lck, LAT and protein kinase C (PKC) θ (Fig. 2) (Sprent and Surh, 2002). Coreceptor molecules (CD4 and CD8) participate in TCR triggering and it has been demonstrated that CD4 increases the efficiency of junction formation of the synapse (Grakoui et al., 1999). Whereas adhesion molecules play an important role in synapse stabilization, interaction of costimulatory molecules (CD28) on T cells with their ligands (CD80 and CD86) on the APC surface enhance T cell activation and provide essential secondary signals for IL-2 synthesis (Bromley et al., 2001; Lanzavecchia and Sallusto, 2001).

1.1.2.1 Function of the immunological synapse

The precise role of the immunological synapse however, has been the subject of much debate. Grakoui A. *et al.,* had initially shown that formation of the immunological synapse provided a mechanism for sustained TCR engagement and signaling (Grakoui et al., 1999). They also demonstrated that this molecular reorganization allows T cells to distinguish potential antigenic ligands. In contrast to these initial findings, a second group has observed that T cell engagement and activation occurs before the formation of the mature immunological synapse, as demonstrated by the presence of active Lck only in the immature synapse, and argues that formation of the immunological synapse does not initiate or enhance TCR signaling (Lee et al., 2002). This latter group favors the idea that

the immunological synapse is involved in TCR down-regulation and endocytosis and suggests that it could potentially be required for the activation of receptors other than the TCR. Finally, it has been proposed that formation of the immunological synapse may be required for polarized secretion of cytokines by helper CD4 T cells directed towards B cells and polarized secretion of the contents of lytic granules by cytotoxic CD8 T cells directed towards target cells (Davis and Van der Merwe, 2001; van der Merwe and Davis, 2002).

1.1.3 T cell expansion: proliferation

Activation of T cells in response to foreign antigen ensures antigen-specific T cell clonal expansion and differentiation. This activation is controlled by signaling pathways initiated by the antigen receptor along with the help of CD4 and CD8 co-receptors and co-stimulatory molecules such as CD28, which is very important for inducing IL-2 production. Interaction of the TCR with its nascent peptide-MHC complex leads to the association of signaling molecules with TCR-CD3 complexes which together with other intracellular molecules, initiate downstream signaling pathways that lead to cytokine production (IL-2), T cell proliferation, and the generation of effector cells (Fig. 2).

As mentioned earlier, naïve T cells circulate through the body in a metabolically inactive state. However, upon activation they enter the cell cycle and expand to differentiate into effector T cells. Their proliferation and differentiation is dependent on IL-2 production, which is produced by the activated T cell itself. At the same time, the IL-2 receptor α chain is synthesized which associates with the β and γ chains giving rise to a high affinity receptor which responds to very low concentrations of IL-2 (Cerdan et al., 1995). This in turn allows T cells to progress through the cell cycle, clonally expand and generate thousands of daughter cells bearing the same TCR.

1.1.3.1 Expression of the IL-2 cytokine

Production of IL-2 is dependent on CD28 ligation by B7 on the surface of the APC. CD28 signaling has been attributed to the stabilization of IL-2 mRNA which increases IL-2 synthesis by 20- to 30-fold (Jain et al., 1995). CD28 signaling has also been shown to promote phosphorylation, and thus inactivation, of the nuclear factor of activated T cells (NFAT) nuclear export kinase glycogen synthase kinase-3 (GSK3), which is coincident with enhanced dephosphorylation of NFATc proteins. This suggested that enhancement of transcriptional activation by NFAT, through inhibition of its nuclear export, plays a key role in mediating CD28 co-stimulatory signaling (Diehn et al., 2002).

The transcription factor NFAT binds to the promoter region of the IL-2 gene and is required for its transcription (Jain et al., 1995). In addition, nuclear factor κB (NF-κB) and the heterodimeric transcription factor activator protein-1 (AP-1; Jun and Fos) are also needed for IL-2 transcriptional activation (Clements et al., 1999). The initiation of signaling pathways following ligation of the TCR couples proximal biochemical events with more distal signaling pathways which lead to the activation of the transcription factors required for IL-2 production. Engagement of the TCR by antigen presented on MHC molecules leads to the activation of the Src family kinase Lck which phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) on the ε, δ, γ and ζ subunits of the TCR. This promotes the recruitment and subsequent activation of ZAP-70, another tyrosine kinase (Fig. 2) (Weil et al., 1995).

1.1.3.2 Signaling molecules mediating T cell activation and proliferation

Two adapter molecules, LAT and SLP-76, are phosphorylated on their tyrosine residues by ZAP-70 which recruits many proteins involved in the activation of the Ras pathway, calcium mobilization and cytoskeletal reorganization. LAT recruits and activates phospholipase Cγ1 (PLCγ1) which is responsible for the production of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (Ins(1,4,5)P3). DAG in turn activates PKC and Ras (Acuto and Cantrell, 2000). Ras leads to the activation of several

serine/threonine kinases that are responsible for the activation of the mitogen-activated protein (MAP) kinases Erk1/2, Jun kinase (JNK) and p38 (Martelli et al., 2000). These MAP kinases directly phosphorylate transcription factors involved in the formation of the AP-1 transcription factor (Cantrell, 1996). Upon binding of Ins(1,4,5)P3 to its receptors on the surface of the endoplasmic reticulum (ER), Ca^{2+} stores are released into the cytoplasm leading to the activation of calcineurin and the dephosphorylation of NFAT, allowing it to enter the nucleus and activate transcription (Acuto and Cantrell, 2000).

Another transcription factor that is also dependent on TCR stimulation and CD28 co-stimulation is NF-κB (Jones et al., 2000). The serine/threonine kinase Akt, also known as protein kinase B (PKB), and the MAP kinase kinase kinases (MAPKKKs) are involved in the activation of IκB kinase complex which phosphorylates IκB leading to its ubiquitination (Baldwin, Jr., 1996). This in turn dissociates IκB from NF-κB which migrates to the nucleus and activates transcription of IL-2, among other genes. Recruitment and activation of PLCγ1 by LAT, and subsequent production of DAG and activation of PKC are also able to activate the MAPKKK pathway, leading to NF-κB activation (Ghosh et al., 1998; Isakov and Altman, 2002).

When the TCR on the surface of a naïve T cell recognizes an antigen in the absence of co-stimulation, the cell is induced into a state of anergy or functional inactivation without cell death. Therefore, differentiation and proliferation of the T lymphocytes are inhibited. The mechanisms underlying anergy or immunologic tolerance ensure the tolerance of T cells to self-antigens. These tolerant T cells are unable to produce IL-2 which prevents them from proliferating and differentiating into effector T cells (Chai et al., 1999; Bachmann et al., 1997). Although some T cells are eliminated by programmed cell death when activated in the absence of co-stimulation, anergic T cells do persist *in vivo*. One possible role for these cells has been suggested, where they prevent responses by nonanergic T cells to foreign antigens that mimic self peptides presented in the context of self MHC complexes. This would prevent inadvertent activation of autoreactive T cells by pathogens (Bachmann et al., 1997).

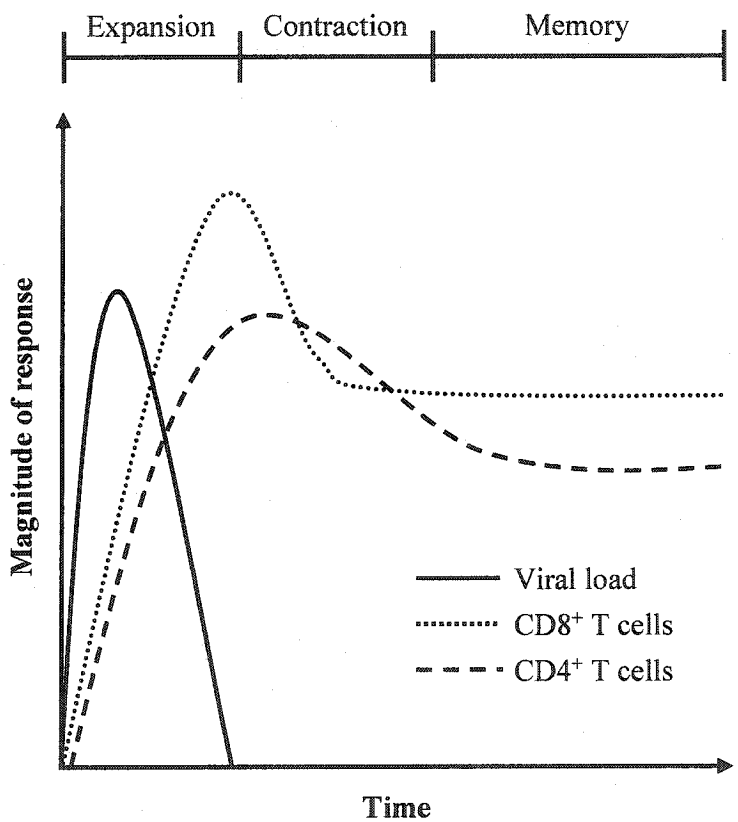

Figure 1-1. Three stages of a T cell response
Clonal expansion of activated antigen-specific naïve T cells is followed by a contraction phase and subsequently the generation of memory T cells (Kaech et al., 2002b).

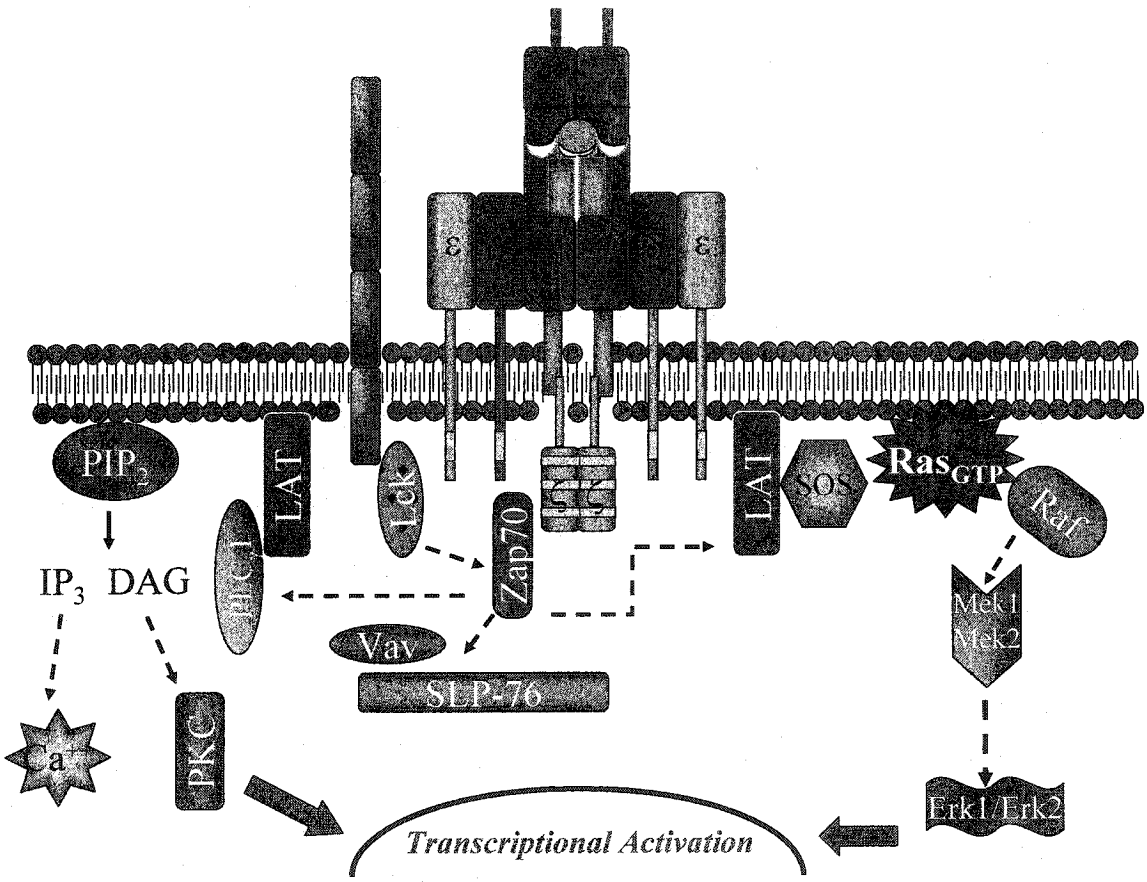

Figure 1-2. Signaling molecules recruited to the TCR during T cell activation
Following TCR ligation, ZAP-70 is activated and phosphorylates several downstream substrates. The initiation and activation of the different signaling pathways leads to the transcriptional activation of a number of genes many of which are involved in T cell proliferation.

1.1.4 T cell homeostasis

Homeostasis refers to a self-regulating process which maintains the stability of a biological system. T cell homeostasis insures that T cell numbers are preserved either by survival or to a balance between proliferation and death (Jameson, 2002). The persistence and homeostasis of naïve and memory T cells appears to be tightly regulated by internal stimuli, cytokines and self-peptide-MHC ligands. As mentioned earlier, a response to an antigen leads to the expansion of antigen-specific T cells and increases the size of this population by approximately 1000-fold during the primary response. However, this is followed by a contraction phase where less than 10% of the cells survive and become long-lived memory T cells. Furthermore, even the memory T cell pool changes in composition since pre-existing memory T cells are depleted to allow new cells to occupy the periphery, a process that is known as attrition (Selin et al., 1999). Therefore, there are size constraints that also play a significant role in shaping the different T cell subsets.

1.1.4.1 Homeostatic proliferation

Several models have been used to show that naïve T cells persist longer than originally thought and are maintained for months to years (Tough and Sprent, 1994; Michie et al., 1992; Hellerstein et al., 1999). Transfer experiments of naïve T cells into lymphopaenic mice found that cells not only survived but proliferated, however to a lesser extent than in response to an antigen (Viret et al., 1999; Bender et al., 1999; Oehen and Brduscha-Riem, 1999; Goldrath and Bevan, 1999). Such a proliferative response has been termed "homeostatic proliferation" or "homeostatic expansion" (Jameson, 2002). It has been suggested that interaction of TCR with self-peptide-MHC is responsible for naïve and memory T-cell homeostatic proliferation and not foreign antigen. Indeed, it was shown that in order for CD4 T cells to persist in the periphery, they required contact with self-MHC class II ligands (Brocker, 1997). Tanchot *et al.* also demonstrated that disruption of the interactions between TCR-transgenic CD8 T cells and specific self-MHC class I molecules leads to a decrease in the survival of CD8 T cells (Tanchot et al., 1997).

Cytokines have also been shown to play an important role in T cell homeostasis, especially the ones that signal through the common γ-chain (γc), such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Although IL-4, IL-7 and IL-15 are able to maintain homeostatic proliferation of naïve T cells *in vitro*, only IL-7 seems to be required *in vivo* (Schluns et al., 2000). However, IL-15 can increase homeostatic proliferation of naïve T cells following exposure to IL-7 since essential components of the IL-15 receptor are only expressed following IL-7-induced homeostatic proliferation (Jameson, 2002).

1.1.4.2 Shaping the T cell compartment

Several reports have demonstrated that co-transfer of increasing numbers of T-cells in a lymphopaenic host reduces proliferation of T cells (Cho et al., 2000), thus suggesting that the size of the T cell compartment plays an important role in shaping the different T cell subsets. One explanation for these findings could be that the factors required for homeostatic proliferation are limiting and there is competition for these limited resources (Jameson, 2002). An inverse correlation was found between the number of T cells and the levels of IL-7 in the serum of HIV-1 patients. This study indicated that the loss of T cells, mediated by HIV-1, was able to stimulate IL-7 production as part of a homeostatic response (Harris et al., 2002). Therefore competition for the amount of available IL-7 and access of the TCR to self-peptide MHC molecules could determine the size of the naïve T cell compartment. In several cases naïve T cells are able to acquire phenotypic and functional properties of memory T cells through homeostatic proliferation without going through an effector stage. In the case of CD8 T cells that underwent homeostatic expansion, they are capable of cytotoxic functions when responding to foreign antigen and the magnitude of the responses is similar to those of memory T cells (Kieper and Jameson, 1999; Cho et al., 2000; Goldrath et al., 2000).

The factors and the requirements for the survival and proliferation of memory T cells differ from that of naïve T cells and each subset appears to be regulated differently. Many reports have indicated that CD4 and CD8 memory T cells do not require foreign antigens or MHC molecules for homeostatic proliferation and persistence (Murali-

Krishna et al., 1999; Lau et al., 1994; Swain et al., 1999; Kassiotis et al., 2002). The need of cytokines for homeostatic expansion of memory T cells greatly differs from that of naïve T cells. Although CD8 memory T cells require IL-15 to support their proliferation (Prlic et al., 2002; Zhang et al., 1998c), CD4 memory T cells do not seem influenced by the presence or absence of cytokines (Tan et al., 2002). However, the number of CD4 memory T cells declines over time in contrast to CD8 memory T cells that persist longer (Jameson, 2002).

1.1.5 Generation of memory T cells

Memory T cells have recently been the subject of much study since they have very important implications in designing and optimizing new vaccine strategies. Vaccines have been designed to develop long-lived immunological protection where a recurrence of a pathogen would be prevented or the extent of a disease would be less severe. Understanding the pathway(s) of memory T cell generation will be critical in developing new vaccines which could enhance the expansion of effector T cells and increase the number of memory T cells.

As described in section 1.1.1, the majority of effector T cells die at the end of an immune response while the remaining T cells differentiate into memory T cells which are maintained for long periods of time. Memory T cells are able to respond more effectively and rapidly to a previously encountered antigen, in contrast to effector T cells (Champagne et al., 2001a). Several *in vivo* studies have shown that memory T cells divided after a shorter lag time and showed a more rapid acquisition of effector functions, in comparison to naïve T cells. Initial priming of naïve T cells resulted in cells expressing mutually exclusive effector functions, whereas memory T cells were multifunctional after reactivation, with each individual cell acquiring different effector functions simultaneously (Veiga-Fernandes et al., 2000). Another report demonstrated that cell cycle progression, kinetics of TCR down-modulation, and CD25 and CD69 up-regulation were identical in naïve and memory T cells after antigen recognition. However, memory CD8 T cells could be more rapidly induced to become cytolytic and secrete high levels of

IL-2 and interferon (IFN)-γ relative to naïve T cells (Zimmermann et al., 1999). It has been suggested that memory T cells respond more rapidly to rechallenge with an antigen than naïve T cells since the latter cell type has a requirement to interact with antigen before they can efficiently migrate to inflammatory sites, while this is not required for memory T cells (Kedl and Mescher, 1998).

1.1.5.1 Memory T cell subsets

Different subsets of memory T cells have been recently described in the literature. Expression of CCR7, a chemokine receptor that controls homing to secondary lymphoid organs and CD62L, which interacts with peripheral-node addressins on high endothelial venules, have been used to define two functionally distinct subsets of human memory T cells (Sallusto et al., 1999). $CD62L^{lo}CCR7^{-}$ memory T cells, known as effector memory T cells (TEM), express receptors for migration to inflamed tissues and display immediate effector function. In contrast, $CD62L^{hi}CCR7^{+}$ memory T cells, named central memory T cells (TCM), express lymph-node homing receptors and lack immediate effector function, but efficiently stimulate dendritic cells and differentiate into $CCR7^{-}$ effector cells upon secondary stimulation (Sallusto et al., 1999).

Another study identified four different subsets of HIV- and CMV-specific CD8+ T lymphocytes based on *ex vivo* analysis of the expression of CD45RA and CCR7 antigens (Champagne et al., 2001b). The prememory subset consists of antigen-specific T cells that possess a naïve-like phenotype or $CD45RA^{+}CCR7^{+}$. These cells then differentiate and reach the maturation stages to become TCM and TEM ($CD45RA^{-}CCR7^{+}$ and $CD45RA^{-}CCR7^{-}$, respectively). Finally, they may reach a terminally differentiated effector stage with a $CD45RA^{+}CCR7^{-}$ phenotype (Champagne et al., 2001b). The following lineage differentiation pattern appears to occur in a two-step process characterized initially by a phase of proliferation largely restricted to the $CCR7^{+}$ $CD8^{+}$ cell subsets, followed by a phase of functional maturation encompassing the $CCR7^{-}$ $CD8^{+}$ cell subsets.

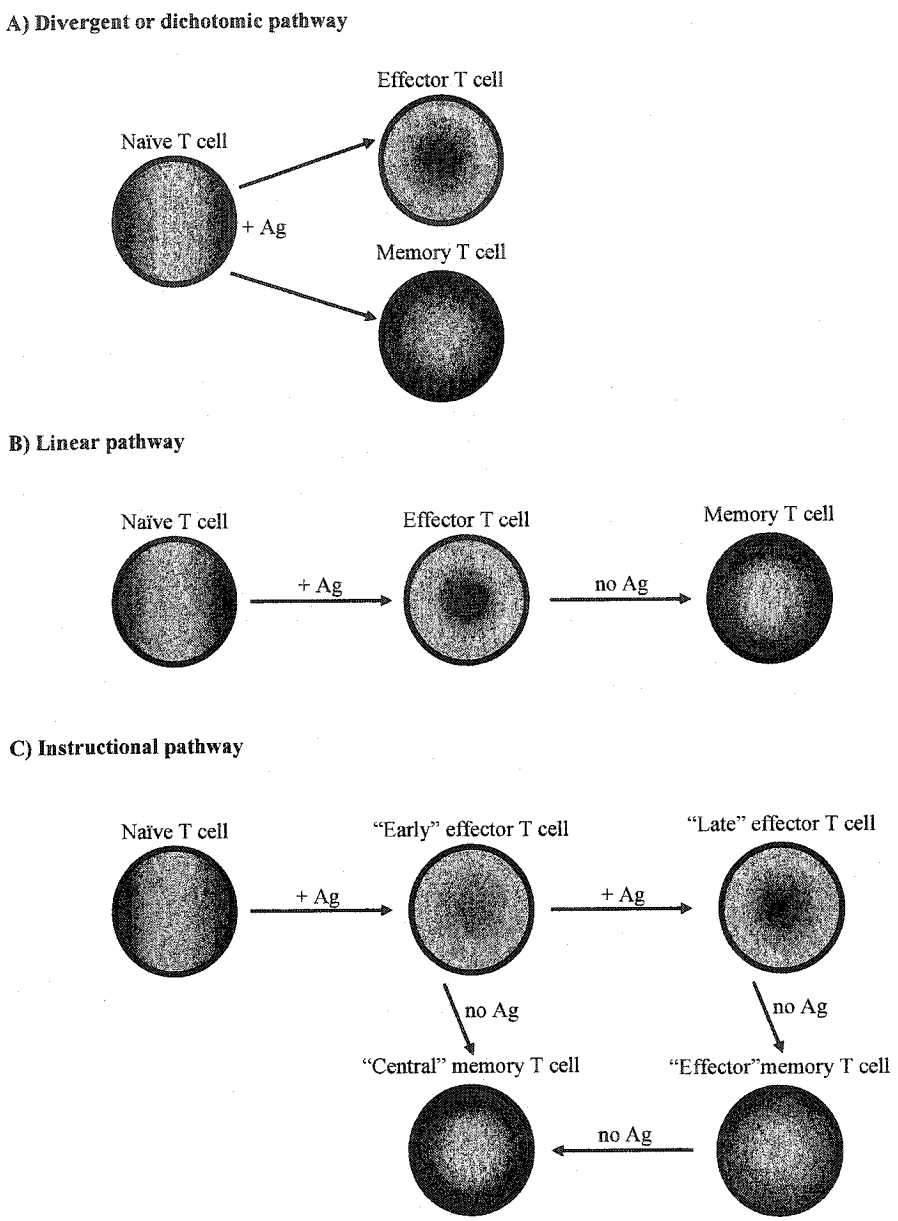

Figure 1-3. Different pathways for the generation of memory T cells
A) The divergent pathway suggests that memory T cells arise directly from naïve T cells without passing through an effector stage. B) The linear pathway favors the notion where a small number of effector T cells give rise to memory T cells. C) An instructional model proposes that differences in the initial signals that a naïve T cell receives determines the generation of different memory T cell subsets (Kaech et al., 2002b).

1.1.5.2 Models of memory T cell lineage commitment

The lineage of memory T cell development is still unclear. Several models explaining the generation of memory T cells have been proposed (Fig. 3). One model referred to as the dichotomic differentiation pathway or divergent pathway suggests that memory T cells arise directly from naïve T cells without acquiring effector function, which are also known as TCM (Fig. 3A) (Sallusto et al., 1999; Manjunath et al., 2001; Iezzi et al., 2001). It has been shown that T cells cultured in high-doses of IL-2 develop into cells with effector phenotype and function. In contrast, cells cultured in the presence of IL-15 or low-dose of IL-2 do not develop immediate effector and cytotoxic functions but acquire a memory T cell phenotype and are able to mount a potent secondary response after adoptive transfer and antigen rechallenge (Manjunath et al., 2001). These results suggest that the generation of memory T cells occurs independently of effector T cell differentiation.

The Cre/loxP system in a transgenic mouse model was used to follow the generation of memory T cells by labeling activated cells (Jacob and Baltimore, 1999). It was demonstrated that 5% of activated T cells were labeled with the reporter gene and became long-lived memory T cells showing that they originated directly from effector cells. Furthermore, a recent study suggested that TCM and TEM develop from effector T cells and are part of a linear pathway of differentiation and do not represent different subsets of memory T cells (Fig. 3B) (Wherry et al., 2003). When purified 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled TCM and TEM cells were adoptively transferred to naïve irradiated recipients, more cell division was observed by the transferred TCM than TEM cells. The memory T cell pool was shown to differentiate from a TEM population that is $CD62L^{lo}CCR7^{-}$ to a TCM population that is $CD62L^{hi}CCR7^{+}$ that responds quickly to antigen stimulation and produces IL-2 (Wherry et al., 2003). Both these studies favor a linear model of memory T cell differentiation where following antigen encounter, naïve T cells proliferate and differentiate into effector T cells of which a small proportion survive as long-lived memory T cells (Fig. 3B).

An instructional model of memory T cell generation has also been suggested. This pathway of differentiation proposes that differences in the initial signals that different naïve T cells receive will generate different subsets of memory T cells (TCM or TEM) (Fig. 3C) (Manjunath et al., 2001). Kaech and Ahmed have demonstrated that naïve $CD8^+$ T cells could commit to effector and memory T cells following a brief period (24 hours) of stimulation (Kaech and Ahmed, 2001). These effector T cells seemed to be instructed to develop into long-lived memory T cells without further antigenic stimulation (Kaech and Ahmed, 2001; Manjunath et al., 2001). The presence of different cytokines, the different affinity/avidity of the interaction between peptide-MHC and TCR, and differences in signals provided by costimulation could therefore all contribute to whether a newly activated naïve T cells develops as central or effector memory (Champagne et al., 2001a).

1.2 Apoptosis

Programmed cell death or apoptosis is a highly ordered physiological process for eliminating or killing cells. It is an essential component of animal embryonic development and maintenance of tissue architecture and plays a crucial role in immune responses. To preserve tissue homeostasis within a developing organism a balance between cell proliferation and death must occur. The programmed cell death of a cell is controlled by different intracellular pathways that could be initiated either by the binding of ligands to their respective receptors on the cell surface or by extracellular factors that disrupt the proper functioning of a cell. These highly regulated mechanisms of apoptosis eliminate unwanted cells without eliciting an inflammatory response.

1.2.1 Apoptosis in development and homeostasis

During the development of an organism, numerous tissues are formed to be removed later by apoptosis. This allows the formation of structures that are required at specific stages of development and can later be eliminated to achieve proper organ function. For instance, the Müllerian duct which gives rise to the uterus and oviduct in

females is not needed in males and is therefore removed by apoptosis (Meier et al., 2000). Different model organisms have been studied to understand the role of cell death in development and homeostasis, such as the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster* and the mouse *Mus musculus*.

1.2.1.1 Apoptosis in *C. elegans*

Most of what we know about apoptosis was initially gained from studies in *C. elegans* in which 131 of the 1090 somatic cells die by apoptosis, giving rise to an adult hermaphrodite worm with 959 cells (Sulston et al., 1983). Genetic screening for somatic mutations which lead to defects in cell death identified *ced-3* and *ced-4* genes. The *ced-9* gene was also identified and found to inhibit the function of the former genes (Vaux et al., 1992). These genes are required for the regulation and execution of apoptosis. Loss-of-function mutations in *ced-3* or *ced-4* results in the survival of the 131 cells destined to die, demonstrating the essential role of these molecules in cell death (Sulston et al., 1983). In contrast, a functional defect in *ced-9* leads to ectopic cell death, whereas a gain-of-function mutation blocks all death of 131 cells, implicating this gene in inhibiting apoptosis. *ced-3* was shown to encode a cysteine protease (CED-3), now known as caspases, which cleaves specific cellular substrates that eventually destroys the cell (Thornberry et al., 1992).

1.2.1.2 Apoptosis in *D. melanogaster*

The apoptotic machinery appears to be conserved but with some complexity among different organisms. In both *Drosophila* and mammals, multiple caspases have been identified along with various homologues of CED-4 such as Apaf-1 in humans and dApaf-1/DARK/HAC-1 in flies (Kanuka et al., 1999). *Drosophila* apoptosis is regulated somewhat differently where three pro-apoptotic proteins have been identified: reaper (RPR), GRIM and head involution defective (HID) (White et al., 1994). No homologues of these proteins have been identified yet in mammals however, they do interact with components of the apoptotic machinery that are conserved such as the inhibitor-of-

apoptosis protein (IAP) family. Deletion of the *rpr*, *grim* and *hid* loci eliminates embryonic apoptosis but leads to death towards the end of embryogenesis due to an excess of cells (White et al., 1994). Furthermore, loss-of-function mutation in the *Drosophila* IAP DIAP-1 also results in embryonic death but this time as a result of extensive apoptosis (Goyal et al., 2000). These findings clearly demonstrate a requirement for a balance between pro- and anti-apoptotic molecules during development.

1.2.1.3 Apoptosis in vertebrates

Unlike *C. elegans* and *Drosophila* where development is restricted to early life, the critical role of apoptosis in development is more evident in vertebrates since it is sustained throughout their life spans. Therefore disruption in the apoptotic processes not only leads to developmental abnormalities but also to a variety of pathologies such as cancer, degenerative and autoimmune disorders (Meier et al., 2000). Although the apoptotic machinery is homologous to *C. elegans* and *Drosophila*, it is more complex involving many different caspases and IAP proteins as well as signaling pathways which have not yet been identified in less complex organisms, but also lead to apoptosis. When members of the TNF receptor family are ligated, they trigger the direct activation of initiator caspases which in turn activate downstream executioner caspases. A second pathway which leads to a cascade of caspase cleavage involves the release of pro-apoptotic molecules from the mitochondria, following apoptotic insults, such as Apaf-1, the homologue of the nematode CED-4 (Fig. 4) (Vaux, 1997).

Due to the complexity of the nervous and immune systems of mammals it is not surprising that functional disruptions of molecules within the apoptotic pathways lead to phenotypic aberrations within these tissues. This is most evident in models of caspase-3, caspase-9 or Apaf-1 knockout mice which all exhibit neuronal hyperproliferation evident by visible masses in their heads that represent ectopic masses of supernumerary cells, in place of pyknotic clusters that represent normal apoptosis that occur during brain development (Kuida et al., 1996; Woo et al., 1998; Kuida et al., 1998; Hakem et al.,

1998; Yoshida et al., 1998). Similarly, disruptions in immune function have been demonstrated from mutations of genes involved in apoptosis (Krammer, 2000). Mice lacking Fas-associated death domain (FADD), an adaptor molecule that recruits caspases to death receptors, were shown to have defects in T cell proliferation and Fas-mediated apoptosis (Zhang et al., 1998a).

1.2.2 Cell death: apoptosis versus necrosis

Whether a cell dies by necrosis or apoptosis, the consequences on the organism and immune system differ. Cell death by apoptosis occurs through an orderly process, involving the recruitment of different molecules and the activation of biochemical pathways, whereas necrosis is a disorderly process where a dying cell takes up water and swells causing the plasma membrane to burst (Rathmell and Thompson, 1999). However, during apoptosis the plasma membrane integrity is maintained and cytoplasmic contents are sequestered into apoptotic bodies. This in turn limits the release of intracellular pathogens and inflammation and does not lead to secondary tissue damage (Arch and Thompson, 1999). Although apoptotic cells release the proinflammatory cytokines IL-1 and IL-18 into the surrounding environment, the levels of inflammation are controlled. In contrast, necrosis of infected cells can spread the intracellular pathogen into the organism and the release of cytoplasmic contents can cause further inflammation. This would result in tissue damage, non-specific immune response and activation of autoreactive lymphocytes. It has been suggested that a balance between apoptosis and necrosis may influence the extent and length of an immune response (Rathmell and Thompson, 1999).

1.2.2.1 Hallmarks of apoptosis

Apoptosis is characterized by distinct morphological and molecular changes. When the cell is dying it loses contact with neighboring cells and becomes detached from surrounding tissues. Furthermore, there occurs nuclear and cytoplasmic condensation which causes the cell to shrink in size. Other organelles are affected by the process of apoptosis, such as the mitochondrion which loses its membrane potential, $\Delta\Psi_m$, and

permeability transition (PT). This in turn releases several factors that initiate a cascade leading to apoptosis. The nuclear envelope and the nucleolus break down as chromatin condenses and is cleaved intranucleosomally into fragments of ~180 base pairs. The DNA laddering pattern is characteristic of apoptosis since necrosis leads to a more continuous sizing of DNA fragments and occurs through a different mechanism.

Cells dying by apoptosis flip their phosphotidylserine molecules from a cytoplasmic to an extracellular orientation after the mitochondrial PT. The plasma membrane also begins to bleb, which divides the cell into smaller apoptotic bodies that contain condensed or normal organelles. These behaviors serve to signal phagocytes which can recognize apoptotic bodies and degrade them in lysosomes. Phagocytes that take up apoptotic bodies present peptides that may allow the activation of antigen-specfic T cells to enhance specific immune responses or regulatory T cells to cause immune suppression (Rathmell and Thompson, 1999). Furthermore, during the apoptotic process the proinflammatory cytokines IL-1α and β, as well as IL-18, which are normally found unprocessed in the cytosol, can be cleaved by caspase-1 and released into the surrounding environment.

1.2.3 Pathways leading to apoptosis

Many different signals and inducers of apoptosis have been identified and some appear to be cell-type specific. Under conditions of growth factor withdrawal, ionizing radiation, Ca^{2+} influx, tumor necrosis factor, viral infection and glucocorticoid treatment, a cell will initiate cell death through apoptosis. Several catalytic pathways will be initiated, including protease activation, which leads to the cleavage of cellular proteins and nuclease activation, which is responsible for DNA fragmentation and RNA degradation (King and Cidlowski, 1998). Members of the TNF receptor family (TNF-R) also initiate apoptosis through the recruitment of proteases such as caspases, which leads to the activation of downstream molecules that are responsible for the molecular changes that are characteristic to apoptosis (Fig. 4).

Apoptotic cell death plays a crucial role in shaping the repertoire of mature lymphocytes. In the thymus immature T lymphocytes that fail to rearrange an appropriate TCR undergo apoptosis early in their development. Those that acquire a functional receptor with autoreactive specificity are eliminated by negative selection. During an immune response, excess lymphocytes are eliminated after antigen clearance by activation-induced cell death (AICD). Furthermore, if a lymphocyte becomes a victim of irreversible DNA damage it will be eliminated by apoptosis. The different apoptotic pathways, which are responsible for maintaining lymphoid homeostasis, will be discussed in further details in the next section. When cell death occurs at a low frequency, this ensues autoimmunity or cancer, whereas excessive cell death can result in immunodeficiency (Thompson, 1995).

1.2.3.1 Extrinsic pathway: death receptors

One pathway leading to apoptosis has been shown to be initiated by cell surface receptors. These death inducing receptors are members of the TNF-R family. However, not all receptors belonging to the TNF-R family initiate cell death. The TNF-R family has been divided into two subfamilies; receptors that promote cell survival and those that lead to cell death (Nagata, 1997). Receptors that promote cell survival include CD27 (Camerini et al., 1991), CD30 (Durkop et al., 1992), CD40 (Stamenkovic et al., 1989), TNF-RII (Smith et al., 1990) and 4-1BB (Kwon and Weissman, 1989). Receptors that induce apoptosis within the TNF-R family include Fas (CD95/APO-1) (Itoh et al., 1991; Oehm et al., 1992), TNF-R1 (Loetscher et al., 1990; Schall et al., 1990), DR3 (Chinnaiyan et al., 1996), DR4 and DR5 (Chaudhary et al., 1997).

1.2.3.1.1 Death receptor-induced apoptosis

T cell apoptosis is primarily initiated by antigen-induced death cytokines, Fas ligand (FasL) and TNF (Dhein et al., 1995; Zheng et al., 1995) which bind the Fas and TNF-RI, respectively (Fig. 4). Binding of FasL to Fas leads to receptor trimerization and aggregation of intracellular signaling proteins through the death domains (DD) of Fas and

cytoplasmic proteins that contain similar domains (Scaffidi et al., 1999a). The expression of Fas can be increased by IFN-γ and TNF but also by T cell activation (Klas et al., 1993). CTLs can eliminate virus-infected cells by releasing FasL from lytic vesicles, which can interact with the Fas receptor on target cells, or by the joint action of perforin and granzyme B (GrB), also found in the vesicles (Medema et al., 1997; Golstein, 1995). Both these forms of inducing apoptosis lead to the initiation of the caspase cascade and eventual cell death. Furthermore, both FasL and TNF can be cleaved from the membrane by metalloproteases and hence a T cell can kill itself (Kayagaki et al., 1995). However, expression of these death-inducing ligands on the surface of T cells allows them to kill target cells involved in an immune response and maintain their cytotoxic functions (Kagi et al., 1994).

1.2.3.1.1.1 Death-inducing signaling complex formation

Upon binding of FasL to its receptor a death-inducing signaling complex (DISC) is formed (Kischkel et al., 1995). The adaptor FADD binds to the intracellular DD of Fas via its own homologous domain and recruits procaspase-8 through the interaction of death-effector domains (DED) found in both molecules (Yeh et al., 1998; Zhang et al., 1998a; Newton et al., 1998; Walsh et al., 1998). Aggregation of all three molecules together (Fas, FADD and procaspase-8) lead to the formation of the DISC. Following DISC formation, procaspase-8 is cleaved to its active form (caspase-8) which is then released from the DISC as a heterotetramer consisting of two small and two large subunits (Muzio et al., 1996; Muzio et al., 1998). The active form of caspase-8 then initiates the proteolytic cleavage of caspase-3, which in turn cleaves caspase-6 and -7 and a multitude of cellular substrates leading to apoptosis (Fig. 4) (Nicholson, 1999).

1.2.3.1.1.2 Consequences of death receptor crosslinking

One of the substrates of caspase-3 is the inhibitor of caspase-activated DNase (ICAD), which once cleaved releases the caspase-activated DNase (CAD) and leads to the activation of its catalytic subunit, which generates cuts in the genomic DNA between

nucleosomes, generating the DNA laddering typical of apoptosis (Sakahira et al., 1998). Furthermore, it was demonstrated that ICAD can be directly inactivated in a caspase-independent manner by GrB, which is released by CTLs (Thomas et al., 2000; Sharif-Askari et al., 2001). Cleavage of other substrates by caspases is also responsible for some of the morphological characteristics of apoptosis such as nuclear lamins which lead to nuclear shrinking and budding (Rao et al., 1996). In addition caspases process upon their activation many cytoplasmic proteins involved with the actin cytoskeleton, DNA metabolism and repair, regulation of cell cycle and proliferation, proteins involved in signal transduction pathways such as kinases, and several proteins implicated in human genetic diseases (Earnshaw et al., 1999).

1.2.3.1.2 Activation-induced cell death

The phenomenon of AICD in T lymphocytes refers to activation through the TCR resulting in apoptosis. AICD of activated lymphocytes ensures the rapid elimination of effector cells after their antigen-dependent clonal expansion. This form of cell death plays an important role in both central and peripheral deletion events required for tolerance and homeostasis. Originally it was observed that T cell hybridomas undergo apoptosis following activation through the TCR (Ashwell et al., 1987; Shi et al., 1989; Ucker et al., 1989; Shi et al., 1992). Mature T cells expand initially following TCR stimulation in the presence of IL-2 *in vitro*, but upon restimulation a few days later, undergo apoptosis through AICD (Lenardo et al., 1999a; Lenardo, 1991). This demonstrates that cell death mediated by AICD represents a major mechanism of clonal deletion in the immune system.

1.2.3.1.2.1 Death ligands involved in AICD

The activation-induced expression of FasL and subsequent binding to its death receptor Fas was then shown by several groups to induce apoptosis by AICD in activated T cell hybridomas (Dhein et al., 1995; Brunner et al., 1995; Ju et al., 1995). Furthermore, activation of T cells was also shown to sensitize cells to Fas-mediated apoptosis (Fig. 5)

(Brunner et al., 1995). Several studies performed *in vitro* and *in vivo* demonstrated a requirement for an interaction between FasL and Fas for AICD in mature T cells (Russell and Wang, 1993; Alderson et al., 1995a; Renno et al., 1996). However, other molecules also seem to be involved in AICD since mice deficient in Fas or FasL have a reduction but not an absence in peripheral deletion (Russell and Wang, 1993; Russell et al., 1993) which is blocked by inhibiting TNF (Chi et al., 1999; Tucek-Szabo et al., 1996). In addition, TNF has been implicated in mediating AICD in the majority of CD8 T cells (Zheng et al., 1995). The TNF-related apoptosis-inducing ligand (TRAIL) can also be produced by activated T cells which can eventually die when TRAIL binds to its receptor (Mariani and Krammer, 1998; Jeremias et al., 1998).

1.2.3.1.2.2 Transcriptional regulation of FasL

One of the mechanisms controlling AICD is the transcriptional regulation of FasL. The FasL promoter has been fairly studied and several transcription factors have been identified to play a role in AICD in mature peripheral T cells. The transcription factor c-Myc was initially shown to be required for AICD (Shi et al., 1992) and a site within the FasL promoter was shown to bind Myc-Max heterodimers and to be required for response to c-Myc (Kasibhatla et al., 2000). Dominant negative forms of Myc and Max were shown to be able to inhibit activation-induced FasL expression in T cells (Brunner et al., 2000). NFAT was also found to be required for FasL expression in activated T cells following engagement of the TCR (Latinis et al., 1997) and mediates its effect in cooperation with AP-1 (Fos/Jun) (Macian et al., 2000) and probably secretory protein-1 (SP-1) under certain conditions of stimulation (Xiao et al., 1999). NF-κB, which is ubiquitously expressed and inducibly activated, is also required for the induction in FasL expression following T cell stimulation (Kasibhatla et al., 1999). The most distal NF-κB site within the 1.2-kb proximal promoter was shown to be critical to drive the expression of FasL following TCR ligation (Green et al., 2003). These findings demonstrate how AICD can be tightly controlled and require the coordination of many different factors to mediate its effects.

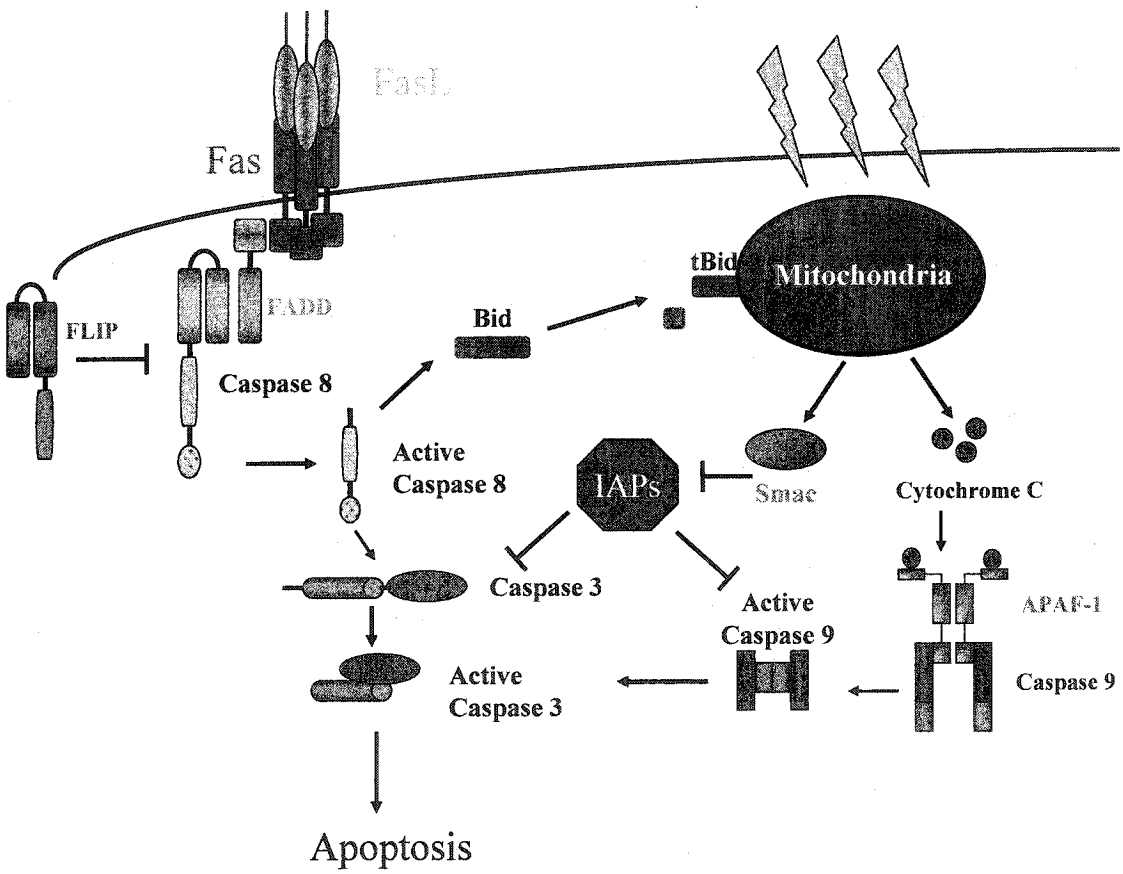

Figure 1-4. Pathways leading to apoptosis
The extrinsic pathway is initiated by the crosslinking of surface death receptors (e.g. Fas) by their respective ligands (e.g. FasL). The intrinsic pathway is dependent on the release of different apoptosis-inducing molecules (e.g. cytochrome *c*) from the mitochondria.

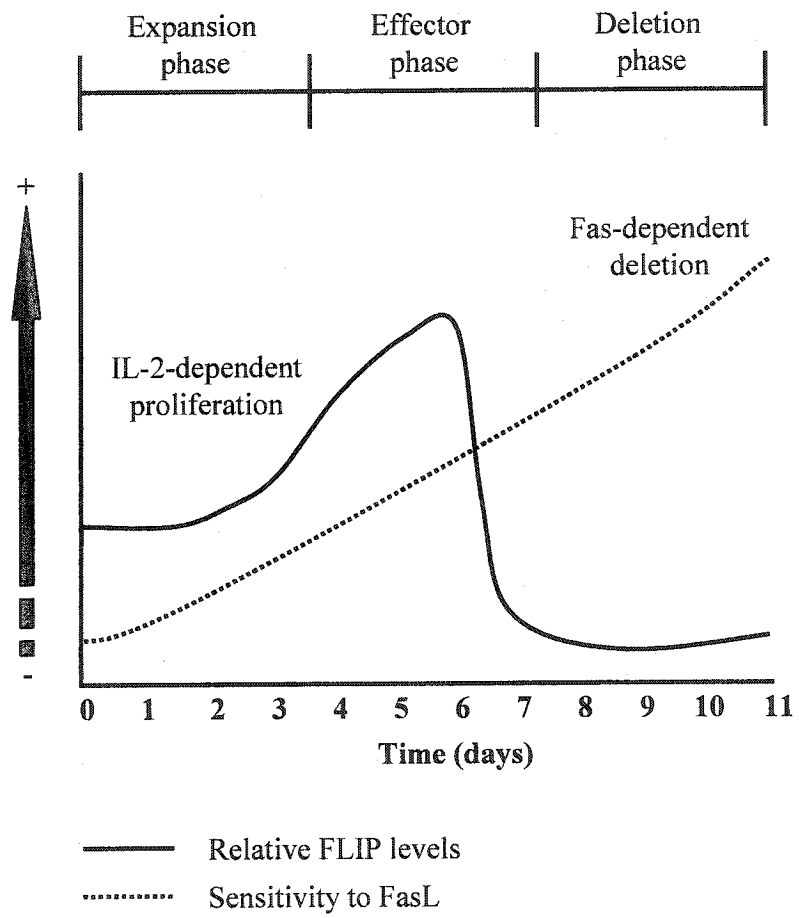

Figure 1-5. Elimination of clonally expanded T cells by AICD
Following antigen challenge and IL-2 dependent expansion, T cells acquire different degrees of sensitivity to FasL and express different levels of FLIP as a T cell progresses through the different phases of an immune response (Thome and Tschopp, 2001).

1.2.3.1.2.3 Inhibition of Fas-mediated apoptosis

Another molecule that was found to be involved in regulating the sensitivity of T cells to AICD was cellular FADD-like IL-1β-converting enzyme inhibitory protein (cFLIP). Overexpression of cFLIP inhibits Fas-mediated apoptosis (Scaffidi et al., 1999b) and cFLIP has been suggested to play a role in protecting activated peripheral T cells from Fas-mediated apoptosis and to play a role in the maintenance of memory T cells due to its high expression levels in memory T cell subset (Irmler et al., 1997; Thome and Tschopp, 2001). Primary T cells have also been shown to be resistant to AICD following the first stimulation because they express high levels of cFLIP (Fig. 5) (Algeciras-Schimnich et al., 1999). cFLIP which contains two death effector domains gets recruited to the DISC, like FADD and caspase-8, following stimulation of death receptors and inhibits Fas-mediated apoptosis by blocking caspase-8 activation at the DISC (Scaffidi et al., 1999b).

1.2.3.1.2.4 Mechanisms mediating AICD

Defects in AICD have been shown to result in lymphoproliferative diseases associated with autoimmune disorders, similar to *gld* and *lpr* mice (genetic defects in FasL and Fas, respectively) (Russell and Wang, 1993; Russell et al., 1993). However, different mechanism appear to be involved in AICD and negative selection in the thymus since there are no apparent defects in negative selection in *lpr* mice (Singer et al., 1994). New results have emerged supporting the role of TRAIL in thymic negative selection (Lamhamedi-Cherradi et al., 2003). TRAIL-deficient mice develop normally but have enlarged thymuses compared to wildtype littermates. Furthermore, it was demonstrated that TRAIL was required for anti-CD3 induced thymocyte apoptosis *in vivo* and *in vitro* and mediates negative selection against both exogenous and endogenous antigens. Finally, $TRAIL^{-/-}$ mice have a high susceptibility to autoimmune disorders (Lamhamedi-Cherradi et al., 2003). However, TRAIL does not appear to be the only molecule involved since transgenic mice expressing FADD-DN, which blocks death receptor signaling during apoptosis, is also required for TRAIL-induced apoptosis and these mice

do not shown any problems in negative selection (Newton et al., 1998). Bim has been proposed as another molecule to play some role in negative selection, since Bim-deficient mice have profound defects in negative selection (Bouillet et al., 2002). How TRAIL and Bim function together to mediate AICD and negative selection in the thymus remains to be explored but JNK activation via TRAIL has been proposed as a potential mechanism since transgenic mice expressing a dominant negative form of JNK (Rincon et al., 1998) and JNK2-deficient mice (Sabapathy et al., 1999) show defects in AICD in the thymus (Green, 2003b).

1.2.3.2 Intrinsic pathway: mitochondria

The mitochondrial pathway represents another mechanism which initiates apoptosis that could be induced following exposure of a cell to chemicals or radiation. In the case of the mitochondria procaspase-9 is the initiator caspase responsible for the triggering of apoptosis in a caspase-dependent manner (Fig. 4). Following mitochondrial outer membrane permeabilization (MOMP) and loss of the $\Delta\Psi_m$, cytochrome *c* is released from the intermembrane space and binds Apaf-1 (Green, 2003a). Apaf-1 has a poor affinity to dATP or ATP; however the binding of cytochrome *c* increases its affinity for the nucleotide by approximately 10 times (Jiang and Wang, 2000). The interaction formed by the nucleotide to Apaf-1 and the binding of cytochrome *c* leads to the oligomerization of the complex forming the apoptosome (Zou et al., 1999). Formation of the apoptosome leads to the recruitment of procaspase-9 through its caspase recruitment domain (CARD) with the CARD of the adapter molecule Apaf-1 (Cecconi, 1999). These series of events trigger the processing and activation of caspase-9 which is then able to efficiently cleave and activate caspase-3 (Fig. 4). Similar to apoptosis initiated through the death receptors, the active form of caspase-3 then cleaves cellular substrates involved in cellular integrity and metabolism.

Although the two pathways leading to apoptosis appear to be separate, there is a molecule that links both of them together. The pathway that involves the formation of the DISC induced by death receptor crosslinking and followed by rapid cleavage of caspase-

3 has been associated with so-called type I cells (Scaffidi et al., 1998). In type II cells, there is scarcely any DISC formation therefore the caspase cascade has to be amplified through the mitochondria (Scaffidi et al., 1998). This is achieved by the cleavage of Bid, a member of the Bcl-2 family, by caspase-8. The truncated form of Bid then translocates from the cytosol to mitochondria and induces cytochrome *c* release which leads to the activation of downstream events ending in cell death (Fig. 4) (Li et al., 1998b).

1.2.3.2.1 Apoptosis-inducing molecules

In addition to cytochrome *c*, other mitochondrial proteins have been identified whose release from the mitochondria lead to apoptosis. Both the apoptosis-inducing factor (AIF) (Susin et al., 1999) and endonuclease G (EndoG) (Li et al., 2001) are capable of inducing apoptosis in a caspase-independent manner. AIF is a 57-kDa flavoprotein that is found in the mitochondrial intermembrane space and translocates to the nucleus upon the induction of apoptosis, causing chromatin condensation and large-scale DNA fragmentation (Susin et al., 1999). It is also an oxidoreductase that could potentially play a role in normal mitochondrial physiology. Mice deficient in AIF have profound defects in their development and AIF appears to be essential for mouse morphogenesis (Joza et al., 2001). Whether the phenotype in AIF knockout mice is due to the loss of the apoptotic activity or its oxidoreductase function remains to be determined. EndoG is a 30-kDa nuclease which is able to induce nucleosomal DNA fragmentation once released from the mitochondria (Li et al., 2001). Unlike the cleavage of ICAD, which is dependent on caspase-3 cleavage, EndoG activity is independent of caspase activation (Liu et al., 1997; Enari et al., 1998; Li et al., 2001). ICAD-deficient MEF cells still showed DNA fragmentation after induction of apoptosis demonstrating that EndoG may be responsible for the observed nucleosomal cleavage (Zhang et al., 1998b; Li et al., 2001). AIF and EndoG could potentially initiate pathways leading to apoptosis that are similar to caspase activation.

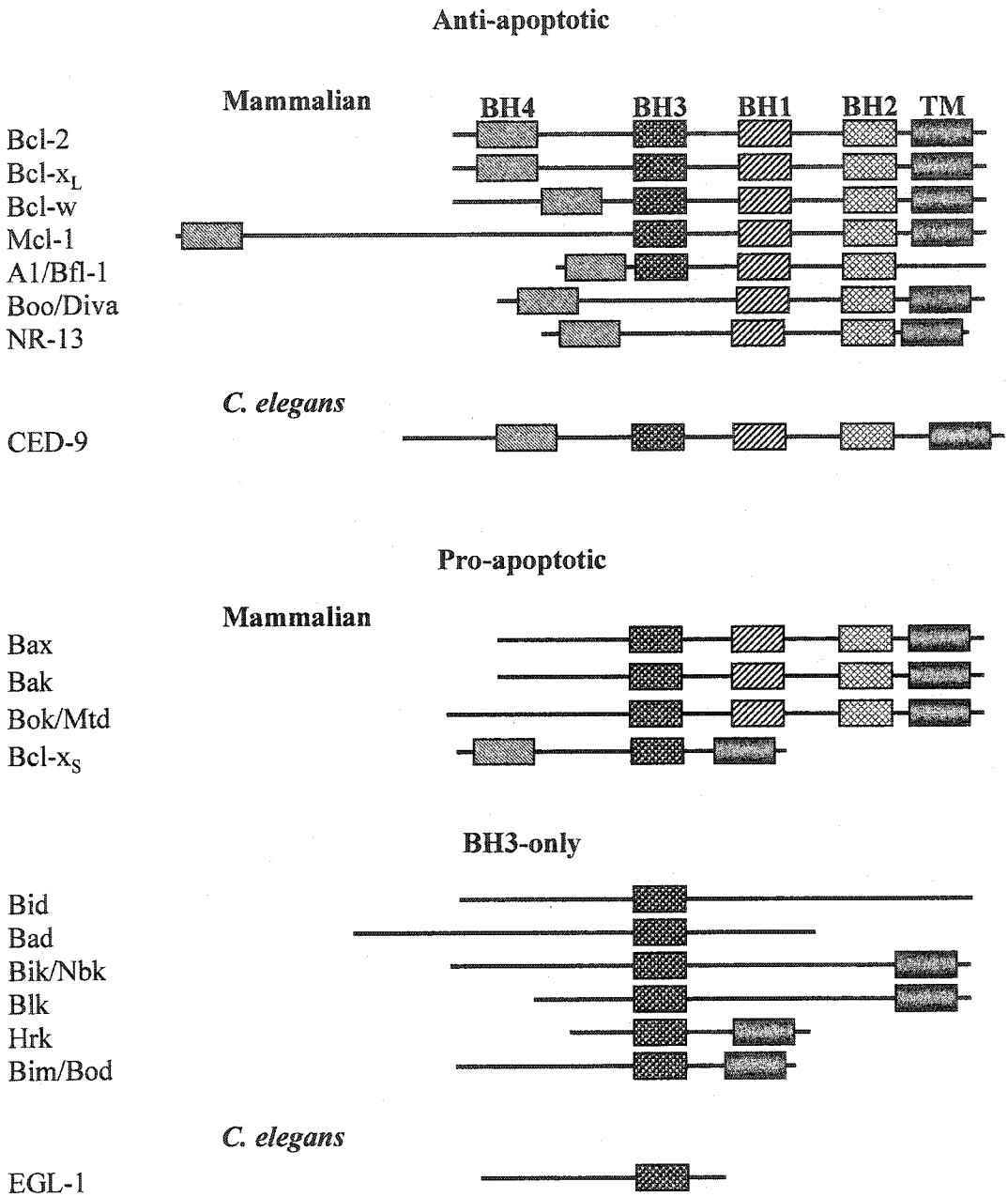

Figure 1-6. Anti- and pro-apoptotic Bcl-2 family members
The different proteins are classified based on their function and structural similarities of the Bcl-2 homology (BH) domains (Fesus, 1999).

1.2.3.2.2 Regulation of apoptosis by the Bcl-2 family

Apoptotic signals are regulated by pro- and anti-apoptotic Bcl-2 family members at the level of mitochondria. These molecules are found upstream of pathways leading to cell death but are localized in different compartments prior to the initiation of death signals. Anti-apoptotic members are initially found as integral membrane proteins in mitochondria, ER or nuclear membrane (Hockenbery et al., 1990; Krajewski et al., 1993; de Jong et al., 1994; Zhu et al., 1996) and include, in addition to Bcl-2, A1/Bfl-1, Bcl-w, Bcl-x_L, Boo/Diva, NR-13 and Mcl-1 (Marsden and Strasser, 2003). In contrast, the majority of pro-apoptotic members are confined to cytosol or cytoskeleton prior to cellular damage leading to apoptosis (Hsu et al., 1997; Gross et al., 1998; Puthalakath et al., 1999). Bcl-2 family members possess up to four conserved Bcl-2 homology (BH) domains referred to as BH1, BH2, BH3 and BH4 (Gross et al., 1999). The pro-apoptotic subfamily has been divided into two subgroups, those that posses two or three BH regions and those that share with the family only the short BH3 region (Fig. 6).

1.2.3.2.2.1 Pro-apoptotic Bcl-2 family members

The BH3-only members include Bad, Bid, Bik/Nbk, Bim/Bod, Blk, Bmf, Hrk, Noxa and PUMA/Bbc3 (Fig. 6) (Marsden and Strasser, 2003). These proteins are regulated in different ways. Bid, for example, is cleaved by caspase-8 to mediate its effects, as described above (Li et al., 1998b). Bad, on the other hand, is phosphorylated by Akt and mitochondrial-anchored protein kinase A (PKA), which dissociates its interaction with anti-apoptotic family members at the outer mitochondrial membrane, and is then sequestered to the cytosol by 14-3-3 protein (Datta et al., 1997; Datta et al., 2000; Harada et al., 1999). Bim, which is usually found associated with cellular microtubule complexes by binding to dynein light chain LC8 (Puthalakath et al., 1999; Wang, 2001), dissociates from the microtubule complex and translocates to the mitochondria to release cytochrome *c* following an apoptotic stimulus, such as cytokine deprivation, calcium ion flux and microtubule perturbation (Bouillet et al., 1999). Furthermore, Bim-deficient mice have an accumulation of lymphoid and myeloid cells and develop autoimmune

kidney disease, demonstrating that Bim is required for hematopoietic homeostasis (Bouillet et al., 1999). Several BH3-only proteins are regulated at the level of transcription. The levels of mRNA and protein of both Hrk (Sanz et al., 2000; Sanz et al., 2001) and Bim (Dijkers et al., 2000; Putcha et al., 2001; Shinjyo et al., 2001) are induced following cytokine withdrawal in hematopoieic progenitor cells. Two other BH3-only proteins which are involved in DNA-damage-induced apoptosis, Noxa and PUMA, have been shown to be upregulated in a p53-depedent manner in primary mouse cells exposed to X-ray irradiation (Oda et al., 2000; Nakano and Vousden, 2001; Yu et al., 2001).

Once the BH3-only proteins have translocated to the mitochondria they interact with Bax and Bak, which undergo conformational changes and oligomerize (Korsmeyer et al., 2000; Nechushtan et al., 2001). This response may lead to the formation of a pore that allows apoptotic inducing molecules to be released from mitochondria or destabilize the mitochondrial outer membrane (Wang, 2001). Bax has also been shown to interact with VDAC and ANT, two proteins of the outer and inner membranes of mitochondria, and has been suggested to form protein pores that allow molecules such as cytochrome *c* to pass through (Shimizu et al., 2000; Marzo et al., 1998). Although it remains unclear how Bax is activated, humanin (HN), an anti-apoptotic peptide of 24 amino acids encoded in mammalian genomes, has been recently shown to interact with Bax and prevent its translocation from the cytosol to mitochondria (Guo et al., 2003). Other proteins of this subgroup of pro-apoptotic Bcl-2 family members include Bok/Mtd, Bcl-X_S, and Bcl-G_L (Fig. 6) (Marsden and Strasser, 2003).

1.2.3.2.2.2 Anti-apoptotic Bcl-2 family members

Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-x_L, can neutralize proteins such as Bax and Bak by blocking their oligomerization and preventing apoptosis (Nechushtan et al., 2001; Sundararajan et al., 2001; Sundararajan and White, 2001; Wei et al., 2001). Furthermore they are able to sequester BH3-only proteins and therefore prevent Bax- and Bak-mediated mitochondrial apoptosis (Cheng et al., 2001). These findings further support the conclusions that the release of cytochrome *c* from

mitochondria is blocked by Bcl-2 (Kluck et al., 1997; Yang et al., 1997). Endogenous levels of expression of Bcl-2 can block apoptosis induced by cytokine withdrawal, glucocorticoids, phorbol esters, ionophores, γ-irradiation and anti-CD3 crosslinking on T lymphocytes (Sentman et al., 1991; Strasser et al., 1991). Several anti-apoptotic genes, such as Bcl-x_L (Boise and Thompson, 1997), Mcl-1 (Kozopas et al., 1993) and A1 (Lin et al., 1993) have been shown to be transcriptionally responsive under different conditions of stimulation. Bcl-2 expression has been shown to be dependent on IL-7 in earliest T lineage-committed cells (Freeden-Jeffry et al., 1997; Akashi et al., 1997). Using antigen-specific CD8 T cells, it was demonstrated that memory CD8 T cells express higher levels of Bcl-2 than naïve T cells which suggested that Bcl-2 may play a role in the generation and maintenance of memory CD8 T cells (Grayson et al., 2000; Grayson et al., 2001). Gene expression of pro- and anti-apoptotic proteins might be able to control the susceptibility of different cells types to apoptosis and thus play a major role in the homoeostasis of the immune system.

1.3 Caspases

Caspases are a family of closely related cysteine proteases that cleave proteins at aspartic acid residues and play important roles in apoptosis. The first caspase to be identified was caspase-1, previously known as interleukin-1β converting enzyme (ICE). Caspase-1 was found to be responsible for the proteolytic maturation of proIL-1β to its proinflammatory active form (Thornberry et al., 1992). To date, 14 mammalian caspases have been identified, 11 of them in humans (Fig. 7) (Nicholson, 1999). Several of these proteases have also been cloned from *C. elegans* and *D. melanogaster*. Caspases appear to be constitutively expressed and all cells express one or more of such caspases (Kumar et al., 1992; Kuida et al., 1996; Juan et al., 1996; Duan et al., 1996; Lippke et al., 1996; Krajewska et al., 1997; Velier et al., 1999; Alam et al., 1999; Ding et al., 1998). Although a great deal of information is available on the activation and function of caspases, little is known concerning the role of caspase-4, -5, -10, -12, -13 and -14 and their involvement in pathways leading to apoptosis.

1.3.1 Structure of caspases

The structural organization and requirement for an aspartic acid residue at the cleavage site is what distinguished these caspases from other cysteine proteases (Earnshaw et al., 1999). The general organization of caspases is an N-terminal prodomain which is followed by a large and then a small subunit. Caspase prodomains vary in length between different caspases with caspase-3, -6 and -7 (effector caspases) having small peptides with unknown function. Those that have large domains are involved in the recruitment and subsequent activation of caspases such as caspase-2, -8, -9 and -10 (initiator caspases) (Slee et al., 1999). Furthermore, the prodomains of caspase-8 and -10 contain DEDs which are required for recruitment and interactions with DEDs of FADD following the crosslinking of death receptors on the cell surface (Boldin et al., 1996). On the other hand, caspase-1, -2, -4 and -9 all have CARD motifs which allows them to interact with other caspases and adapter molecules such as Apaf-1 (Fig. 7) (Hofmann et al., 1997).

1.3.1.1 Caspase substrate specificity

Caspases are synthesized as catalytically-inactive proenzymes that require cleavage between the different subunits to generate the active form of the protease. They recognize a core tetrapeptide motif corresponding to four residues (P_4-P_3-P_2-P_1) with an absolute requirement of an aspartic residue at P_1 (Sleath et al., 1990; Howard et al., 1991), with P_2 and P_3 having a limited effect on substrate cleavage since their side chains point away from the caspase active site (Sleath et al., 1990; Thornberry et al., 1992). The presence of aspartic acid residues at the cleavage sites of procaspases allows them to be activated by other caspases or even be activated by themselves. The large and small subunits of procaspases form the active enzyme and come together by cleavage at Asp(P_1)-X(P_1') bonds (Nicholson, 1999). The active caspase consists of homodimers of heterodimers which is achieved from the association of two processed procaspases and thus contains two active sites (Walker et al., 1994; Mittl et al., 1997). The active site, which is formed by cysteine and histidine residues, is found within the large subunit,

whereas residues which form the S_1 subsite that recognizes the Asp(P_1) residue are derived from the large and small subunits (Nicholson, 1999). The substrate binding site (S_4-S_1) is formed from residues from both the large and small subunits; however the S_4 subsite to which the P_4 residue binds to appears to dictate substrate specificities of caspase-1 and -3 (Earnshaw et al., 1999).

Caspases are important mediators of programmed cell death and play a central role in the different apoptotic pathways (death receptor and mitochondrial pathways; Fig. 4). AICD is a critical mechanism in the shaping and homeostasis of the immune system and is responsible for the removal of expanded T cells following clearance of a pathogen and establishment of T cell memory. This response is dependent on the activation of caspases. Knockout mice for different caspases have been generated and described in the literature, but defects in the immune system were sometimes subtle, as will be further discussed, demonstrating that there is a redundancy in substrate specificity and function between caspases. However, their expression appears to be regulated and some caspases are essential in different tissues during development demonstrating that a particular caspase may be required at a specific point in time and may not be substituted by another.

1.3.2 Family of caspases

Phylogenetic analysis originally demonstrated that the caspase gene family can be subdivided in two major sub-families, those related to caspase-1 or caspase-3. Another way of subdividing the caspases was based on the lengths of the prodomains with initiator caspases having long prodomains and effector caspases short ones (see above; Fig. 7). Positional scanning combinatorial substrate library technique further subdivided the caspases into three subgroups based on their substrate specificities at the S_4 subsite (Thornberry et al., 1997; Rano et al., 1997). Caspase-1, -4, -5 and -13, which form group I, are not stringent for a residue in P_4 but prefer bulky hydrophobic amino acids such as tyrosine or tryptophan. The group II caspases (2, 3 and 7) have an absolute requirement for an aspartic acid residue in P_4, which is identical to the specificity of CED-3 of *C.*

elegans. Finally group III, which consists of caspase-6, -8, -9 and -10, have a preference for branched chain aliphatic amino acids in P_4 (Thornberry et al., 1997).

Grouping the different caspases into three sub-families reconciles initial forms of classifying them. Caspase members belonging to group III correspond to initiator caspases and those belonging to group II correspond to effector caspases. Amino acid residues found at the cleavage sites of group II and III caspases match the residue specificity of group III caspases (Nicholson, 1999). Support for this form of classification is best demonstrated in the initiation of the caspase cascade through death receptors and mitochondria by the initial activation of caspase-8 (group III), which in turn processes and activates caspase-3 and -7 (group II), and the activation of caspase-9 (group III), which mediates the cleavage of caspase-3, respectively. However, there are some caspases that seem to be the exception to the rule when grouping these cysteine proteases. Caspase-6 has been shown to mediate effector functions (Lazebnik et al., 1995) but belongs to group III, and caspase-2, which appears to be self-activating such as initiator caspases has been classified in group II (Thornberry et al., 1997; Duan and Dixit, 1997; Ahmad et al., 1997).

Based on their functional properties caspases can be subdivided into two subfamilies (Fig. 7). The caspase-1 subfamily, which includes caspase-1, -4, -5, -11, -12, -13 and -14, appears to be involved in cytokine processing and the control of inflammation. Furthermore, these caspases all belong to group I and they don't appear to be required for cell death, since substrates that are cleaved during apoptosis do not contain hydrophobic amino acids in P_4, which dictates their specificity (Nicholson, 1999). In contrast, the caspase-3 subfamily, which consist of caspase-2, -3, -6, -7, -8, -9 and -10, all process molecules leading to apoptosis and belong to group II and III demonstrating that they are the major players of programmed cell death (Earnshaw et al., 1999).

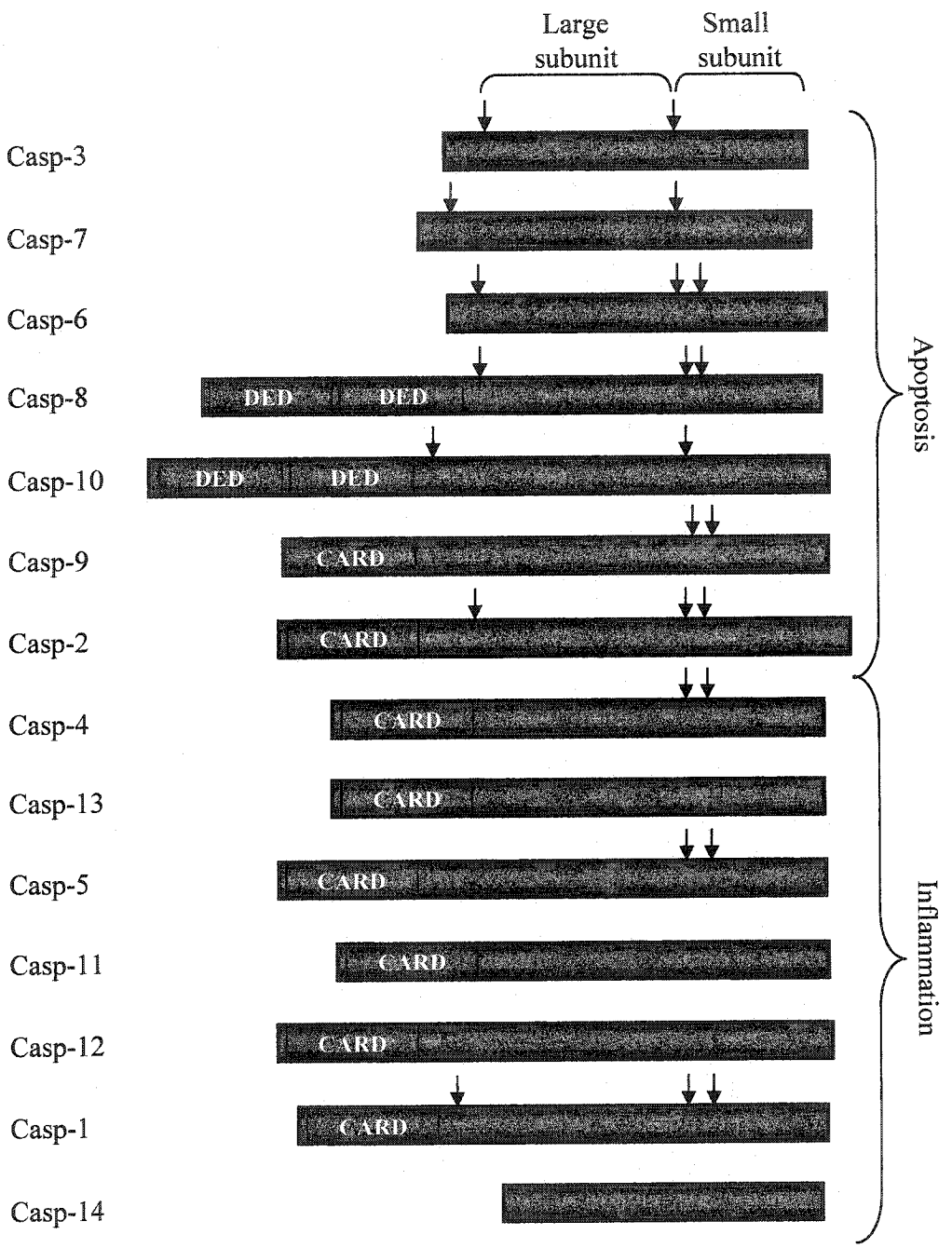

Figure 1-7. Mammalian caspases
All caspases are of human origin except caspase-11 and -12 which have been identified in mice only and caspase-13 is of bovine origin. The different cleavage sites are represented by arrows (Shi, 2002).

1.3.3 Caspase activation

It is well established that caspases are activated by proteolytic cleavage mediated through transactivation by other caspases or by activation following recruitment to an oligomeric complex such as the DISC or apoptosome. Caspases interact with different adaptor molecules through conserved homologous domains (e.g. DEDs and CARDs) found in both molecules. It has been previously shown that forced oligomerization of effector caspases results in their activation (MacCorkle et al., 1998; Colussi et al., 1998) which suggested that caspases have some intrinsic protease activity (Kumar, 1999).

1.3.3.1 Activation of caspase-1 and -2

The prodomains of caspase-1 and -2 contain a conserved CARD which allows these proteases to form oligomers. Furthermore, overexpression of both caspases has been shown to induce apoptosis (Miura et al., 1993; Kumar et al., 1994). The CARDs of these molecules are able to interact with CARD in the adaptor molecules RAIDD (Ahmad et al., 1997; Duan and Dixit, 1997) and in CARDIAK/RIP2/RICK, a RIP-like serine/threonine kinase (Thome et al., 1998; McCarthy et al., 1998; Inohara et al., 1998). CARDIAK appears to play a role in the recruitment of procaspase-1 to the Fas and TNF-R1 signaling complex (McCarthy et al., 1998). These findings provided the first evidence of an interaction between a procaspase and a kinase though the protease interacts with the CARD rather than the kinase domain (Thome et al., 1998). Although several studies have suggested that apoptosis can be regulated by phosphorylation/dephosphorulation mechanisms, there are no direct examples that have demonstrated that caspases can be phosphorylated (Martins et al., 1998).

1.3.3.2 Initiator caspase activation

Caspase-8 and -10 both contain two DEDs in their prodomain region (Fig. 7). Currently, there are no known studies that have focused on the function and activation of caspase-10. However, given the structural similarities between the two procaspases, it

won't be surprising if both proteases appear to be activated by a similar mechanism. An induced proximity model has been proposed for the activation of caspase-8 (Muzio et al., 1998; Salvesen and Dixit, 1999). The model was introduced to explain how initiator caspases (8 and 9) can become activated in the absence of a protease upstream in the apoptotic pathway. Using a chimeric caspase-8 molecule that can be oligomerized *in vivo* or a nonprocessable form of the zymogen, it was demonstrated that procaspase-8 possesses low level proteolytic activity which is able to activate downstream caspases at a certain concentration when brought into close proximity to the DISC (Muzio et al., 1998). A similar mechanism for procaspase-9 activation applies but is mediated by the apoptosome (Salvesen and Dixit, 1999). However, the mechanism by which the recruitment of procaspases to an activation complex renders them catalytically competent remained unclear.

Recently, two different groups have attributed the activation of procaspase-8 and -9 to the formation of dimers following the recruitment of the monomeric forms to the DISC and apoptosome, respectively (Donepudi et al., 2003; Boatright et al., 2003). An uncleavable form of procaspase-8 was produced and was mainly found as monomers, however when a small amount of the mutant dimerizes they become as active as the wildtype proenzyme (Donepudi et al., 2003). A single amino acid substitution at the procaspase dimer interface was shown to abrogate the activity of caspase-8 and -9 (Boatright et al., 2003). Furthermore, it was established that the appearance of dimers leads to the formation of the active site and that subsequent cleavage within the interdomain linker is responsible for the stabilization of initiator caspase dimers (Boatright et al., 2003). These results demonstrated that dimerization is responsible for the activation of caspase-8 and -9.

Previous work has shown that procaspase-9 can be activated without proteolytic processing, but required the recruitment to the apoptosome (Stennicke et al., 1999; Zou et al., 1999). Recombinant forms of procaspase-9 with mutations in one or both of its interdomain cleavage sites were able to initiate apoptosis as efficiently as the wildtype form (Stennicke et al., 1999). It became evident that procaspase-9 does not require any

processing to be activated but is dependent on cytosolic factors. These findings are in agreement with the model presented above where dimerization of inactive monomers of procaspase-8 and -9 leads to their activation (Donepudi et al., 2003; Boatright et al., 2003). In contrast, executioner caspases (3 and 7) pre-exist as inactive dimers and require proteolytic cleavage within the interdomain linker for their activation (Stennicke et al., 1998; Zhou and Salvesen, 1997).

1.3.3.3 Executioner caspase activation

The reason why initiator caspase activation is independent of proteolysis in contrast to executioner caspase has been partially attributed to the length of the interdomain linker within the procaspases. Caspase-8 and -9 have long interchain linkers which may not block the translocation of the activation loop whereas the short linker in caspase-3 and -7 appears to hinder this movement (Boatright et al., 2003). Atomic resolution studies were able to show that the interdomain linker partially obstructs translocation of an activation loop (Chai et al., 2001; Riedl et al., 2001) and its cleavage allows conformational stabilization for substrate binding (Boatright et al., 2003). Moreover, formation of stable caspase-3 or -7 dimers can be partly explained by the presence of extremely hydrophobic residues at the dimer interface in contrast to the weak hydrophobic nature of the dimer interface of caspase-8 and -9 forcing them to remain as monomers until they are recruited to an oligomeric complex allowing them to dimerize (Bose and Clark, 2001).

When looking at a simplified scheme of caspase activation either through death receptors or mitochondria, activation of caspase-8 leads to the cleavage of caspase-3 and then caspase-6 or activation of caspase-9 to caspase-3 and -7 and then caspase-6, respectively (Earnshaw et al., 1999). However, several studies have demonstrated that some pathways can occur in reverse *in vitro* where caspase-6 has been shown to activate caspase-3 (Srinivasula et al., 1996b; Liu et al., 1996) and caspase-3 can activate caspase-9 (Srinivasula et al., 1996b). Furthermore, caspase-8 is capable of cleaving procaspase-3, -4, -7 and -9 (Srinivasula et al., 1996a; Muzio et al., 1997; Stennicke et al., 1998) and

caspase-10 has been proven to cleave procaspase-3, -7 and -8 (Fernandes-Alnemri et al., 1996) *in vitro*.

1.3.3.4 Caspase activation during T cell activation and proliferation

Caspases have always been associated with pathways leading to cell death. The large majority of the studies concerning caspase-3-like proteases have associated their activation with the onset of cell death. Indeed, they do play an essential role in regulating and controlling cell numbers at the termination of an immune response. Interestingly, several reports have shown caspase cleavage and activation during T lymphocyte stimulation and proliferation in the absence of cell death (Miossec et al., 1997; Wilhelm et al., 1998; Alam et al., 1999; Kennedy et al., 1999; Boissonnas et al., 2002).

1.3.3.4.1 Caspase activation in the absence of apoptosis

Miossec *et al.* were the first group to show evidence of caspase-3 activation, in the absence of cell death, in phytohaemaglutinin A-(PHA)-stimulated T lymphocytes (Miossec et al., 1997). They demonstrated the cleavage of the caspase-3 proenzyme into its active form following stimulation of T cells, which was also associated with caspase-3-like activity in cell lysates. No cell death was detected in stimulated T lymphocytes however the cells were actively proliferating (Miossec et al., 1997). These observations were further confirmed in mixed lymphocyte reactions. A second group also demonstrated caspase-3-like activity in the absence of apoptosis in human and murine T lymphocytes stimulated with mitogens and IL-2 (Wilhelm et al., 1998). Mice were challenged with the staphylococcal enterotoxin B (SEB) superantigen, which stimulates $V\beta8^+$ T cells (White et al., 1989), as a model for *in vivo* T cell activation. Caspase-3-like activity correlated well with the activation and expansion of SEB-reactive $V\beta8^+$ T cells (Miethke et al., 1996), after analysis of the spleen and lymph nodes from these mice (Wilhelm et al., 1998). The authors of this latter study suggested the presence of a checkpoint downstream of caspase activation in the apoptotic pathway.

Despite these observations, another report hypothesized that the activation of caspase-3 in stimulated T cells in the absence of cell death is due to the release of granzyme B or other serine proteases with specificity to aspartic acid, following lysis of the cells (Zapata et al., 1998). The source of granzyme B would come from activated $CD8^+$ cells and release from cytotoxic granules during lysis of peripheral blood mononuclear cells (PBMCs). However, Alam *et al.* performed experiments under conditions that ruled out the possibility of caspase-3 processing by proteases released from surrounding cells (Alam et al., 1999). This latter group observed complete processing of caspase-3 into its active forms following TCR triggering of PBMCs in the absence of cell death (Alam et al., 1999). To further confirm their findings, Western blot analysis on Annexin V negative (AnV^-) sorted activated PBMCs showed the processing of caspase-3, -6 and -7, as well as two caspase substrates, PARP and Wee1. The 45-kDa subunit of the DNA fragmentation factor was not processed, which excluded the possibility of contamination by dead cells in the protein lysates (Alam et al., 1999).

1.3.3.4.2 Caspases and T cell activation

Caspase processing and activation has also been shown to be required for T cell activation and proliferation. The presence of the broad caspase inhibitor benzyloxycarbonyl (Cbz)-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk) in anti-CD3 stimulated cultures of PBMCs blocked proliferation, MHC class II expression, and blastic transformation but not surface expression of the early activation marker, CD69 (Alam et al., 1999). This demonstrated that inhibition of caspase activity results in defective T cell activation and proliferation following stimulation of naïve T cells through the TCR. This suggests that caspases are involved in the early steps leading to lymphocyte proliferation. It was also shown that caspase-8 and FasL, two molecules involved in the induction of apoptosis, play crucial roles in T cell proliferation (Kennedy et al., 1999). This latter report concluded that T cell proliferation was caspase dependent since they also demonstrated a significant block in the stimulation of resting T lymphocytes by anti-CD3 antibody in the presence of the caspase inhibitors IETD-fmk and zVAD-fmk. Furthermore, FasL was shown to have a co-stimulatory function in the

cleavage of caspase-8. The presence of a Fas-Fc fusion protein blocked T cell proliferation, whereas soluble FasL enhanced anti-CD3-induced proliferation by increasing IL-2 production and cell cycling (Kennedy et al., 1999). These findings demonstrated another role of death receptors and downstream molecules in promoting T cell proliferation in a caspase-dependent manner.

A recent paper has shown that TCR signaling regulates caspase activation during T cell proliferation (Boissonnas et al., 2002). Using altered peptide ligands they demonstrated that caspase activation was tightly regulated by the strength of antigenic stimulation during naïve CD4 T cell proliferation. All the data presented above is in agreement with several findings where a disruption in the apoptotic pathways leading to caspase activation resulted in impaired T cell proliferation. Activation-induced proliferation was found to be impaired in T cells lacking FADD, despite production of IL-2. The similarities between FADD-/- mice and mice lacking the beta-subunit of the IL-2 receptor suggest a link between cell proliferation and apoptosis (Zhang et al., 1998a). In accordance with these findings, transgenic mice expressing a dominant negative mutant of FADD (FADD-DN) inhibited T cell activation by increasing apoptosis (Newton et al., 1998). Furthermore, expression of a *bcl-2* transgene reduced proliferation of thymocytes and delayed cell cycle entry of mitogen-stimulated B and T lymphocytes (O'Reilly et al., 1996). These observations provide evidence that inhibition or disruption of molecules normally involved in the apoptotic pathway, influence T cell activation and cell cycle entry.

1.3.4 Inhibitors of caspases

As discussed above, caspases are synthesized as inactive precursors that require recruitment or cleavage for their activation, which represents one form of regulating caspase activity. Several caspase inhibitors are also synthesized by cells and interact with proteases to inhibit their activities. Others appear to compete with caspases for binding to adaptor molecules at the DISC and inhibit caspase activation. Given the potentially

devastating effect of inadvertent caspase activation, these inhibitors of apoptosis represent another way of regulating caspase activity.

1.3.4.1 Inhibition of caspase activity

IAPs were originally identified in the baculoviral genome and were able to block apoptosis by interacting with and inhibiting the protease activity of the cleaved forms of caspases (Deveraux and Reed, 1999). The mammalian IAP family consists of eight members that target the enzymatic activity of caspase-3, -7 and -9 but not other caspases, such as caspase-6 and -8 (Deveraux and Reed, 1999). XIAP, c-IAP1 and c-IAP2 contain three conserved baculoviral IAP repeat (BIR) domains, and each domain mediates a highly specific inhibition of a caspase (Takahashi et al., 1998). The BIR3 domain potently inhibits the activity of the cleaved form of caspase-9 (Shiozaki et al., 2003) but not caspase-3 and -7, and the linker region between BIR1 and BIR2 is able to selectively inhibit caspase-3 and -7 (Shi, 2001). ML-IAP/Livin, which is an IAP family member highly expressed in melanoma and lymphoma, contains only a single BIR domain capable of inhibiting both caspase-3 and -9 (Ashhab et al., 2001; Kasof and Gomes, 2001; Vucic et al., 2000). Survivin, another single BIR-containing IAP, has been shown to inhibit caspase activity (caspase-3, -7 and -8) and apoptosis induced by Fas (Tamm et al., 1998). However, its function does not appear to regulate caspase activity like other IAP family members, since its expression changes as cells progress through the cell cycle and is highest in the G2/M phase and therefore appears to mediate events in mitosis (Altieri, 2001; Li et al., 1998a).

1.3.4.1.1 Degradation of caspases by IAPs

In addition to inhibition of caspase activity by IAPs, the active forms of certain caspases can be eliminated by the ubiquitination-mediated proteasome degradation pathway. XIAP (Suzuki et al., 2001) and cIAP2 (Huang et al., 2000), have been found to contain Ring finger domains, highly homologous to ubiquitin-protein ligases (E3s), which are directly involved in substrate recognition. Both these anti-apoptotic molecules

have been demonstrated to promote the degradation of both themselves and their substrates (active forms of caspase-3 and -7) through their ubiquitin-protein ligase activity (Suzuki et al., 2001; Huang et al., 2000). The inhibition of caspases by the BIR domain of IAPs has been suggested to target the active forms of caspases for Ring finger-mediated ubiquitination and degradation (Huang et al., 2000). The ubiquitin-protein ligase function of IAP family members along with their inhibitory roles might together ensure that caspase activity is kept to a minimum in a cell in order to prevent premature apoptosis and allow T cells to perform their functions before AICD.

1.3.4.1.2 Smac/DIABLO: inhibitors of IAPs

The BIR domains within the IAPs are essential not only to inhibit caspase activity but provide a site for Smac/DIABLO binding. Second mitochondria-derived activator of caspases (Smac) (Du et al., 2000) also known as direct IAP-binding protein with low pI (DIABLO) (Verhagen et al., 2000) is a pro-apoptotic protein released from the mitochondria together with cytochrome *c* during apoptosis. Upon its release from mitochondria Smac/DIABLO interacts with the BIR2 and BIR3 domains of XIAP and relieves IAP-mediated inhibition of caspases (Chai et al., 2000). Its function is dependent on a seven residue peptide in the N-terminal sequence which is exposed upon removal of the N-terminal mitochondria-targeting sequence (Du et al., 2000). These residues have been found to be responsible for the removal of IAP-mediated inhibition of caspases (Chai et al., 2000). A tetrapeptide motif was also identified in the N terminus of cleaved caspase-9 and is responsible for the interaction between caspase-9 and XIAP and subsequent inhibition of the protease (Srinivasula et al., 2001). These findings demonstrate that conserved IAP-binding motifs in both caspase-9 and Smac/DIABLO regulate caspase activity.

1.3.4.2 Viral caspase inhibitors

Besides IAPs, six viral inhibitors that are present in several γ-herpesviruses and molluscipoxviruses and two forms of a cellular homologue have been identified and

designated FLIP (FLICE/caspase-8 inhibitory protein) (Thome et al., 1997; Irmler et al., 1997). Two different splice variants of cellular FLIP ($cFLIP_S$ and $cFLIP_L$) have been identified. Both contain two DEDs which bind the FADD adaptor molecule and caspase-8 and interfere with the activation of caspase-8, thereby inhibiting death receptor-mediated apoptosis (Thome et al., 1997; Thome and Tschopp, 2001). Whereas the short splice variant is structurally related to viral $FLIP_S$, $cFLIP_L$ is structurally similar to caspase-8 and -10 and contains a caspase-like domain, except the cysteine and histidine residues found in the active site of the caspase are substituted by other amino acids rendering the inhibitor proteolytically inactive (Irmler et al., 1997; Boldin et al., 1996; Vincenz and Dixit, 1997). FLIP has also been associated in the regulation of proliferation of Fas-stimulated cells mediated by the activation of the NF-κB and Erk signaling pathways (Kataoka et al., 2000; Hu et al., 2000; Chaudhary et al., 1999; Chaudhary et al., 2000).

Other natural caspase inhibitors have been identified such as the p35 baculoviral protein and the serpin CrmA derived from the cowpox virus. p35 is a pan-caspase inhibitor capable of blocking the apoptotic response of insect cells to viral infection (Clem et al., 1991). It can inhibit *C. elegans* CED-3 and mammalian caspases-1, -3, -6, -7, -8 and -10 both *in vivo* and *in vitro* (Miller, 1999). The cleaved form of p35, generated by caspases, is responsible for the inhibition of caspase activity. Processing at the P_1 residue (Asp87) of the inhibitor leads to the translocation of the N terminus of p35 into the active site of caspases thus forming an inhibitory complex (Bump et al., 1995; Zhou et al., 1998). CrmA mediates its inhibition in a similar covalent modification mechanism and acts as a pseudosubstrate that binds to the active protease. However, in contrast to p35, CrmA is able to efficiently inhibit the activities of caspase-1 and -8 only and not caspase-3, -6 and -7 *in vivo* (Zhou et al., 1997; Garcia-Calvo et al., 1998). Furthermore, CrmA can prevent maturation of IL-1β and IL-18 by caspase-1 and was shown to inhibit apoptosis triggered by death receptors which is dependent on caspase-8 activity (Tewari and Dixit, 1995).

1.3.5 Lessons learned from knockout mice

Mice in which individual caspases were knocked out were generated to better understand the function of each caspase and to determine whether they are responsible for specific events in a cell. Originally, disruption of the *ced-3* gene abolished all developmental cell death in *C. elegans* (Yuan and Horvitz, 1990). Thus far, studies have been performed on caspase-1, -2, -3, -8, -9 and -11 knockout mice and none of them eliminated all developmental apoptosis. However, regardless of the caspase targeted, defects in apoptosis appeared to be cell type- and stimulus-dependent demonstrating their nonredundant role in apoptosis. Some of the knockouts, as will be discussed further, demonstrated prenatal lethality eliminating the possibility of studying caspases in the adult organism. Therefore, the generation of conditional knockout mice will allow us to better understand the *in vivo* function of each caspase during development and the homeostasis of the immune system.

1.3.5.1 Caspase-1 and -11 knockout mice

Mice with a disruption in caspase-1 and -11 are developmentally normal but show several defects in cytokine processing, such as the maturation of IL-1β and the synthesis of IL-1α, in response to lipopolysaccharide (LPS) (Kuida et al., 1995; Li et al., 1995; Wang et al., 1998). As such, these mice are highly resistant to endotoxic shock induced by injection of LPS (Li et al., 1995; Wang et al., 1998). Caspase-1-deficient thymocytes were as sensitive as their wildtype counterparts to dexamethasone and γ-irradiation, however were more resistant to Fas-induced apoptosis (Kuida et al., 1995). Despite these observations, caspase-1 appears to be dispensable for the majority of apoptotic pathways (Kuida et al., 1995; Li et al., 1995). Furthermore, caspase-11-deficient cells were resistant to caspase-1 overexpression, suggesting that caspase-1 activation requires caspase-11 (Wang et al., 1998). The similarities between the defects observed in caspase-1- and -11-deficient mice suggested that these two proteases play an important role in inflammation and appear to interact together to mediate their effects.

1.3.5.2 Caspase-2 knockout mice

Previously, two alternatively spliced variants of caspase-2 had been identified, a short form (caspase-2_S) which protects cells from apoptosis and a long form (caspase-2_L) which promotes death (Wang et al., 1994). The long form was mostly expressed in female germ cells, whereas both caspase-2_S and -2_L were expressed in the brain (Bergeron et al., 1998). Caspase-2 knockout mice had both forms eliminated and developed normally with their cells undergoing apoptosis to various stimuli (Bergeron et al., 1998). However, several developmental defects were observed. An excess number of germ cells were found in the ovaries of caspase-2-deficient mice, and oocytes were resistant to doxorubicin-induced apoptosis. Furthermore, B-lymphoblasts from mutant mice were also more resistant to apoptosis mediated by GrB and perforin but died in response to anti-Fas, etoposide, γ-irradiation, staurosporine and the combination of TNF-α and cycloheximide (Bergeron et al., 1998). In contrast, sympathetic neurons from caspase-2 knockout mice were more sensitive to apoptosis than the wildtype cells in the absence of nerve growth factor (Bergeron et al., 1998). These findings demonstrated a dual role for caspase-2, both as a positive and negative regulator of apoptosis during development.

1.3.5.3 Caspase-3 knockout mice

Caspase-3-deficient mice survive birth but at a frequency lower than wildtype mice. In addition, they exhibit major defects in brain development due to decreased levels of apoptosis in the brain. The brain from caspase-3 knockout mice shows multiple indentations and ectopic cell masses (Kuida et al., 1996; Woo et al., 1998). No apparent abnormalities were observed in the heart, lung, liver or kidney (Kuida et al., 1996). Although caspase-3 was shown to play a role in thymocyte apoptosis (Alam et al., 1997), the absence of caspase-3 had no effect on T and B cell development (Kuida et al., 1996). Similar percentages for the different populations of immature T cells were observed in the thymus of wildtype and knockout mice. Thymocytes from caspase-3 knockout mice were as sensitive as wildtype cells to apoptosis induced by anti-Fas, dexamethasone,

ceramide, staurosporine and γ-irradiation (Kuida et al., 1996; Woo et al., 1998). However, peripheral T cells from caspase-3-deficient mice were less susceptible to AICD, anti-CD3- and Fas-induced apoptosis (Woo et al., 1998). Furthermore, activated peripheral T cells lacking caspase-3 showed higher levels of proliferation compared to wildtype T cells, due to decreased cell death (Woo et al., 1998). These results clearly indicate the essential role of caspase-3 in peripheral T cell apoptosis, suggesting that this protease could contribute significantly to T cell homeostasis.

1.3.5.4 Caspase-9 knockout mice

Elimination of caspase-9 in mice resulted in embryonic lethality and severe defects in brain development, similar to caspase-3 knockout mice, however the phenotype was more severe in caspase-9-deficient mice (Hakem et al., 1998; Kuida et al., 1998). Different abnormalities were observed in apoptosis in a cell type- and stimulus-specific manner. Mouse embryonic fibroblasts lacking caspase-9 were resistant to apoptosis induced by γ-irradiation and chemotherapeutic drugs. Caspase-9-deficient lymphocytes were sensitive to anti-CD3, Fas-induced apoptosis, UV irradiation, sorbitol-induced osmotic shock, and several chemotherapeutic drugs but surprisingly were resistant to γ-irradiation (Hakem et al., 1998). As was observed in caspase-3 knockout mice (Kuida et al., 1996), T cell development was normal in cells lacking caspase-9 (Hakem et al., 1998; Kuida et al., 1998). However, there was a requirement for caspase-9 during apoptosis of thymocytes in response to γ-irradiation and dexamethasone (Hakem et al., 1998).

1.3.5.5 Caspase-8 knockout mice

Finally, caspase-8 knockout mice had an accumulation of blood throughout the animal and suffered from defective heart development and early embryonic lethality (Varfolomeev et al., 1998). Furthermore, caspase-8-deficient embryos have decreased numbers of myeloid progenitor cells. Since the mice were lethal *in utero* continuous fibroblast strains from wildtype and knockout embryos were established (Varfolomeev et

al., 1998). Cultured fibroblasts derived from caspase-8 mutant mice were more resistant than wildtype cells to apoptosis induced by TNF receptors, Fas and DR3. In contrast, caspase-8-deficient fibroblasts were as sensitive as wiltype derived cells to inducers of apoptosis such as UV irradiation, staurosporine, etoposide and ceramide (Varfolomeev et al., 1998). These findings demonstrate a requirement for caspase-8 in death receptor-induced apoptosis and an important role in embryonic development.

1.3.6 Regulation of caspases

As described above, several caspases have been identified and their role in the different pathways leading to apoptosis (extrinsic and intrinsic) is well established. Programmed cell death has been shown to play a critical role in the developing organism and different caspases appear to be required at different stages of development and some mediate their effects in a tissue- and stimulus-dependent manner (Kuida et al., 1995; Li et al., 1995; Wang et al., 1998; Bergeron et al., 1998; Kuida et al., 1996; Woo et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Varfolomeev et al., 1998). AICD, which is responsible for the elimination of expanded T cell populations at the termination of an immune response, appears to be dependent on caspase-3 activity. Caspase-3-deficient peripheral T cells were resistant to AICD (Woo et al., 1998) demonstrating the central role that caspase-3 plays in T cell homeostasis. The importance of the executioner caspase-3 in apoptosis is further highlighted by the fact that it is central to both death receptor- and mitochondria-mediated apoptosis (Fig. 4).

The activation of caspases is also required for T cell proliferation and IL-2 production since the presence of caspase inhibitors blocked blastic transformation (Alam et al., 1999; Kennedy et al., 1999). In addition, caspase-3 and -8 have been shown to be activated in response to T cell activation in the absence of apoptosis (Miossec et al., 1997; Wilhelm et al., 1998; Alam et al., 1999; Kennedy et al., 1999) and to be tightly regulated by the strength of antigenic stimulation (Boissonnas et al., 2002). Similar to the elimination of expanded T cell population through AICD in response to antigenic stimulation, it was recently shown that homeostatic control of antigen-independent IL-7-

induced proliferation of recent thymic emigrants (RTEs) was driven by caspase activation (O'Neill et al., 2003). Again the presence of caspase inhibitors reduced the levels of RTE proliferation in response to IL-7. Furthermore, inhibition of Fas signaling had little effect on cell proliferation or caspase-8 activation of IL-7-treated RTEs. However, RTEs were more susceptible to anti-Fas-induced apoptosis in the presence of IL-7 (O'Neill et al., 2003). These findings demonstrate a Fas-independent pathway of caspase processing in response to IL-7-mediated homeostatic proliferation of RTEs and emphasize the importance of caspase proteolytic activity in controlling different forms of T cell expansion.

1.3.6.1 Transcriptional regulation of caspases

As discussed above, several cellular proteins regulate caspase activity by inhibiting the catalytic function of caspases (Deveraux and Reed, 1999; Ekert et al., 1999) or mediate the degradation of the active forms of caspases (Huang et al., 2000; Suzuki et al., 2001), which could explain the initial absence of apoptosis in activated T cells. It has been suggested that low levels of activated caspases are tolerable and do not induce apoptosis because of the presence of IAPs (Salvesen and Dixit, 1999). Consequently, a certain concentration of the active forms of caspases must be attained in order to overcome this inhibition. Therefore, there appears to be a balance between pro- and anti-apoptotic molecules within a cell and a certain threshold concentration of caspase expression must be reached in order to induce apoptosis which can be enhanced by a decrease in the levels of apoptosis inhibitors (Salvesen and Dixit, 1999; Fesus, 1999).

1.3.6.1.1 Regulation of caspases in disease models

Caspases can additionally be regulated through transcription. Increase in caspase mRNA levels has been demonstrated under conditions leading to apoptosis. Two studies have reported an induction of caspase-3 mRNA levels after transient cerebral ischemia and have suggested that transcriptional activation of the caspase-3 gene may be involved

in apoptosis of CA1 pyramidal neurons (Ni et al., 1998; Chen et al., 1998). Interestingly, an increase in caspase transcription has been associated in two mouse models of neurodegenerative disorders (Li et al., 2000b; Smith et al., 1990). In an amyotrophic lateral sclerosis (ALS) transgenic mouse model it was shown that inhibition of caspase-1 activity delays disease onset and mortality (Li et al., 2000b). In addition, the upregulation of caspase-1 and -3 mRNA levels were significantly reduced in the presence of zVAD-fmk. It was also demonstrated that caspase-1 and -3 are transcriptionally regulated in the R6/2 transgenic mouse model (Chen et al., 2000). R6/2 mice develop a progressive neurological phenotype with features of Huntington disease (HD) and other neurological pathologies which lead to early mortality (Davies et al., 1997). Administration of minocycline, a tetracycline derivative, to R6/2 mice inhibited caspase-1 and -3 mRNA upregulation, decreased inducible nitric oxide synthetase activity and delayed mortality of R6/2 mice (Chen et al., 2000).

1.3.6.1.2 Caspase expression levels determines sensitivity to apoptosis

Decreased levels of caspase mRNA has also been associated with resistance to apoptosis in human tumor cells (Droin et al., 1998; Devarajan et al., 2002). One group showed a heterogeneous pattern of expression of caspase genes in human leukemic cell lines (Droin et al., 1998). Etoposide is a topoisomerase II-reactive agent which produces double-stranded breaks in DNA leading to cell death and is commonly used for the treatment of different human tumors. The HL60 cells (human leukemic cell line) which are sensitive to etoposide-induced apoptosis increased the mRNA levels of caspase-2 and -3 upon treatment with etoposide (Droin et al., 1998). In contrast, gene expression of the different caspases remained unchanged in the K562 cell line which is more resistant to etoposide treatment. Furthermore, following the induction of caspase-2 and -3 mRNA levels, synthesis of the corresponding procaspases was observed before the onset of apoptosis (Droin et al., 1998). It was also determined that the majority of breast and cervical tumors had undetectable levels of caspase-3 mRNA and decreased levels in the case of ovarian tumors (Devarajan et al., 2002). Reconstitution of caspase-3 expression in the caspase-3-deficient MCF-7 breast cancer cell line restored sensitivity to different

apoptotic stimuli (Devarajan et al., 2002). Altogether, these findings demonstrate a correlation between the levels of caspase gene expression and cell sensitivity to apoptosis.

1.3.6.1.3 Transcription factors regulating caspase gene expression

Several studies using cell lines deficient for certain transcription factors or signaling components required for the activation of transcription factors have shed some light on potential candidates that could regulate the expression of caspases. The interferon regulatory factor (IRF)-1 was shown to be required for certain apoptotic pathways in T lymphocytes (Tamura et al., 1995). The levels of caspase-1 mRNA were significantly reduced in IRF-1-deficient splenocytes however overexpression of IRF-1 enhanced the sensitivity of cells to radiation-induced apoptosis. In addition, it was suggested that susceptibility of IFN-γ-pretreated cells to apoptosis occurred through the induction of caspase-1 (Tamura et al., 1996) and caspase-8 (Fulda and Debatin, 2002) following IRF-1 expression.

The signal transducer and activator of transcription 1 (STAT1) was also found to be required for constitutive expression of caspase-1, -2 and -3 in human fibroblasts (Chin et al., 1997; Kumar et al., 1997). Low levels of expression of caspases due to the absence of STAT1 correlated with resistance of cells to TNF-α-induced apoptosis (Kumar et al., 1997). The JAK-STAT pathway was shown to be essential for caspase-1 mRNA expression and induction of apoptosis in response to IFN-γ (Chin et al., 1997). STAT activity and caspase-1 mRNA were undetectable in a JAK1 kinase-deficient cell line which was also resistant to IFN-γ-induced apoptosis (Chin et al., 1997). An increase in caspase-1, -3, -4, -7, -8 and -10 was observed in the human colon adenocarcinoma cell line, HT-29, following treatment with IFN-γ and different cytotoxic drugs (Ossina et al., 1997). IFN-γ induced the upregulation and activation of caspase-1, -3 and -8 in human erythroid progenitor cells which resulted in apoptosis (Dai and Krantz, 1999).

A recent report has demonstrated a requirement for IFN-γ during AICD and T cell homeostasis (Refaeli et al., 2002). Anti-CD3 and anti-CD28 activated T cells from mice deficient for IFN-γ or STAT1 were resistant to apoptosis when exposing the cells to anti-CD3 in order to mimic repeated antigenic stimulation (Refaeli et al., 2002). Although the levels of Fas and FasL were normal in IFN-γ- and STAT1-deficient T cells, they remained resistant to anti-Fas-mediated apoptosis. The defect in AICD was attributed to reduced mRNA levels of caspase-3, -6 and -8 and was restored upon addition of IFN-γ in IFN-γ-deficient cells but not STAT1-deficient lymphocytes. The levels of caspase-8 protein were also increased under the same conditions. The cytokine appears to be responsible for basal levels of caspase expression, since supplementing wildtype T cells with IFN-γ, which already produce the cytokine, does not increase the levels of caspase-8 (Refaeli et al., 2002). It was also shown that caspase-3 mRNA levels are upregulated *in vitro* in T cells tolerized following exposure to ionomycin and *in vivo* in a model of oral tolerance (Macian et al., 2002). The increase in caspase-3 gene expression as well as other genes identified was shown to be dependent on NFAT. T cells deficient in NFAT1 were resistant to the induction of anergy and did not induce the expression of caspase-3 or anergy-associated genes (Macian et al., 2002).

1.3.6.1.4 Regulation of caspases during cell cycle entry

Profiling of the cell cycle on a genome-wide scale in human fibroblasts identified genes that display transcriptional fluctuations with a periodicity consistent with that of the cell cycle (Cho et al., 2001). Many genes involved in diverse biological functions were identified and fell into different categories such as those required for cell cycle control, apoptosis, DNA replication and chromosome segregation. Among the genes that belonged to the apoptosis cluster, the Bcl-2 homologue Bak1, caspase-3 and several caspase substrates such as α-fodrin, CdkN1 and plectin, were detected (Cho et al., 2001).

Activation of members of the E2F family is sufficient to induce a cell to undergo DNA replication demonstrating their crucial role in the control of cellular proliferation (Trimarchi and Lees, 2002). E2F is inhibited by its association with a

hypophosphorylated form of the retinoblastoma protein (pRB). Mitogenic growth factors induce the activation of cell-cycle-dependent kinase (Cdk) complexes such as Cdk4/Cdk6-cyclin D and Cdk2-cyclin E which phosphorylates pRB and allow the release of E2F. The transcription factor E2F then activates E2F responsive genes during late G1 phase of the cell cycle thus committing a cell to divide (Trimarchi and Lees, 2002). While certain members of the E2F family may positively regulate cell cycle progression, E2F-1 has been demonstrated to regulate apoptosis and suppress cell proliferation. Mice deficient for the E2F-1 gene developed normally but exhibited several defects in T cell development and E2F-1 was shown to be required for normal thymic negative selection (Field et al., 1996; Garcia et al., 2000). E2F-1 was able to activate the p53 homologue p73 and induce apoptosis in the absence of p53 (Irwin et al., 2000). Furthermore, using TAT-fusion proteins of a dominant-negative form of the E2F-1 protein it was clearly demonstrated that E2F-1 is a mediator of TCR AICD which occurs from a late G1 cell-cycle checkpoint and is also dependent on p73 activity (Lissy et al., 2000). Both E2F-1- and p73-deficient cells failed to undergo apoptosis (Lissy et al., 2000; Irwin et al., 2000). Altogether, these findings indicate that a common pathway mediates AICD where p73 is a downstream target of E2F-1.

One study using oligonucleotide arrays to monitor changes in gene expression following activation of E2F-1, E2F-2 or E2F-3 has identified genes involved in differentiation, development, proliferation and apoptosis (Muller et al., 2001). E2F-1 induced the expression of several mediators of apoptosis, such as Apaf-1 (3.3-fold), caspase-3 (14.1-fold) and caspase-7 (2.4-fold). All three genes were induced in the absence of *de novo* protein synthesis, which demonstrated that E2F transcription factors are directly responsible for the observed increase in gene expression of these genes involved in apoptosis (Muller et al., 2001). Nahle *et al* then showed that loss of pRb or enforced expression of E2F-1 resulted in the accumulation of procaspases through a direct transcriptional mechanism which was independent of p53 signaling (Nahle et al., 2002). MEFs deficient for pRB expressed higher protein levels of caspase-3, -7, -8 and -9 when compared to wildtype cells. Additionally, it was demonstrated that cells expressing

higher levels of the caspases responsive to E2F-1 were sensitized to apoptosis induced by the release of cytochrome *c* (Nahle et al., 2002).

1.3.6.1.5 Functional characterization of caspase promoters

Analysis of the promoter regions of caspase-3, -7, -8 and -9 identified several putative E2F-binding sites (Nahle et al., 2002). Using chromatin immunoprecipitation (ChIP), which allows the *in vivo* detection of protein bound to specific DNA regions, it was demonstrated that E2F-1 was bound to the caspase-7 promoter in cells destined to undergo apoptosis. In addition, the caspase-7 promoter was responsive to E2F transactivation (Nahle et al., 2002). The promoter regions of the human caspase-8 gene and the rat caspase-3 gene have also been studied to date (Liu et al., 2002; Liedtke et al., 2003). It was demonstrated that both SP-1 and Ets-1-like binding motifs are necessary for sustained basal transcriptional activity of both genes (Liu et al., 2002; Liedtke et al., 2003).

Although the studies overexpressing E2F-1 demonstrated that the increase in procaspase expression occurred through a direct transcriptional mechanism independently of p53 signaling (Nahle et al., 2002) Liedtke *et al* identified a p53 responsive element downstream of the transcriptional start site of the human caspase-8 promoter. Furthermore, they demonstrated that the p53 binding site induced the caspase-8 promoter independently from the transcription factors required for basal activity (Liedtke et al., 2003). The tumor suppressor p53 also appears to be involved in the transcriptional regulation of caspase-1 (Gupta et al., 2001). A site within the caspase-1 promoter was shown to be required for transcriptional activation by p53. MCF-7 cells expressing a mutant form of p53 could not induce caspase-1 promoter activity nor increase caspase-1 endogenous mRNA levels following doxorubicin treatment (Gupta et al., 2001).

1.4 Project rationale and research objectives

When T cells respond to foreign pathogens they expand and perform their effector functions. However, at the termination of an immune response more than 90% of effector T cells die by AICD except for a small proportion of surviving T cells that become long-lived memory T cells. Caspases play central roles in apoptosis and have been shown to be required for AICD of T lymphocytes. Given the potentially devastating effect of inadvertent caspase activation, it is not surprising that caspases are under several levels of regulation. Besides regulating their activity with cellular inhibitors caspases appear to be regulated at the transcriptional level. Increase in caspase mRNA levels has been demonstrated under conditions leading to apoptosis. More importantly, decreased levels of caspase mRNA has been associated with resistance of human tumor cells to apoptosis. Therefore certain levels in caspase expression are required to ensure the elimination of unwanted or harmful cells.

This thesis is concerned with characterizing the regulation of caspases during the activation of T lymphocytes. Understanding the effect of TCR on the levels of gene expression of caspases will be essential in determining the signaling pathways involved in T cell homeostasis, since caspases are important mediators of immune homeostasis through AICD. A better insight into the regulation of caspases will be critical in understanding the contribution of caspase gene expression in the generation of different T cell subsets (effector versus memory T cells) and T cell homeostasis, which could lead to designing and optimizing more efficient vaccine strategies.

The first Chapter of the thesis gives a literature review of the different mechanisms, pathways and molecules involved from the initiation to the termination of immune responses and the generation of different T cell subsets. The different processes involved in the cell death machinery along with the mediators of apoptosis will also be discussed. Finally, a brief review of the regulation of caspases in different models will be described.

The objectives of Chapter 2 were: (i) to characterize the KOX-14 murine T cell hybridoma as a model of AICD and demonstrate the roles of the death receptor Fas and caspase-3 in apoptosis in this cell line; (ii) to determine the effect of T cell activation of KOX-14 cells on caspase mRNA levels using RNase protection assays, semi-quantitative and real-time-RT-PCR analysis; and (iii) to demonstrate whether the regulation of caspases are dependent on their activity.

The objectives of Chapter 3 were: (i) to determine the effect of T cell stimulation during the activation of primary T cells isolated from different models of mice; (ii) to demonstrate the selective increase in caspase-3 mRNA levels in contrast to other members of the caspase family of proteases in live activated peripheral T cells; (iii) to evaluate the levels of caspase-3 mRNA in an *in vivo* model of infection and in different T cell subsets; (iv) to determine the kinetics of the increase in caspase-3 mRNA levels and when, during T cell activation, does the increase occur; (v) to show the effects of T cell activation on the levels of proteins in the absence of cell death; and (vi) to correlate the levels of caspase-3 expression with the sensitivity of T lymphocytes to different inducers of apoptosis.

The objectives of Chapter 4 were: (i) to identify the promoter region of the caspase-3 gene; (ii) to clone the caspase-3 promoter upstream of a luciferase reporter gene and generate deletion constructs to isolate a region within the promoter responsible for the inductions in caspase-3 levels and show reporter activity in different cell lines; (iii) to develop a retroviral based reporter system which will allow us to integrate the reporter constructs in KOX-14 cells; and (iv) study the changes in reporter activity in KOX-14 cells following T cell activation, by flow cytometry.

Chapter 2: Selective upregulation of caspase-3 gene expression precedes the onset of apoptosis following TCR triggering

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Preface

Several groups have shown that caspase-3 expression levels are increased in cells undergoing apoptosis under different conditions of culture. Caspase-3 plays an essential role in T cells and during AICD. Therefore we investigated the effects of TCR crosslinking on caspase expression in KOX-14 cells, a model of AICD.

Abstract

A key step during activation-induced cell death in T lymphocytes is the expression of Fas-ligand, which triggers the apoptotic process after binding to its receptor Fas. Apoptosis is then dependent on the activation of cysteine proteases of the caspase family and caspase-3 has been shown to play a critical role during this response. The regulation of caspase expression was studied in T cell hybridomas following the induction of activation-induced cell death to determine whether T cell receptor (TCR) signaling has an effect on caspase gene expression. A 3-fold increase in caspase-3 mRNA levels during AICD was observed by semi-quantitative and real-time RT-PCR analysis. Caspase mRNA levels were assessed by RNase protection assay which revealed that caspase-3 expression was selectively increased among five different caspases following TCR stimulation. This was further confirmed by real-time RT-PCR, where a 3-fold upregulation in caspase-3 mRNA levels was observed and caspase-8 mRNA levels remained unchanged, following TCR triggering. Furthermore, the increase in caspase-3 mRNA levels following T cell activation occurred before cleavage and activation of caspase-3 and in the absence of apoptosis. Therefore, TCR stimulation induces the simultaneous increase of Fas-ligand and caspase-3 mRNA levels independently of caspase-3 activity and before the onset of apoptosis. Finally, TCR-mediated induction in caspase-3 expression is not dependent on STAT1 activation, since following stimulation of KOX-14 cells this transcription factor is not phosphorylated. Altogether these results suggest that early events dependent on TCR signaling are responsible for the selective increase in caspase-3 mRNA levels independently of apoptosis.

2.1 Introduction

Recognition of a peptide/MHC complex by the TCR results in the activation and proliferation of mature T lymphocytes. However, chronic stimulation through the TCR leads to apoptosis through activation-induced cell death (AICD), a response initially described in a T cell hybridoma activated *in vitro* with a specific antigen [1]. AICD is responsible for the deletion of self-reactive T lymphocytes in the periphery [2] and of effector T cells during the resolution of an immune response [3]. In mature $CD4^+$ T cells, AICD is dependent on the upregulation of Fas-ligand (FasL) expression [3-6]. Defects in the Fas pathway, such as in *lpr* mice (mutated in the Fas receptor) [7], *gld* mice (defective for FasL) [8] or in the human autoimmune lymphoproliferative syndrome (ALPS) [9], result in lymphoproliferation and autoimmune disease. These observations support the role of AICD and Fas in the maintenance of peripheral tolerance [10-12].

Interaction of FasL with its receptor Fas leads to the activation of cysteine proteases of the caspase family which now includes 14 members [13]. Oligomerization of Fas by its ligand triggers the activation of caspase-8 and caspase-3, the latter being a major executioner caspase during AICD in peripheral T cells [14]. Although a great deal of information is available on the function of caspases, their transcriptional regulation remains poorly understood. STAT1 was shown to be required for the basal transcriptional regulation of both caspase-1 and caspase-3 since cell lines defective in STAT1 express very low levels of both caspases [15]. IFN-γ, which can activate STAT1, has been shown to upregulate caspase-1, -3, -4, -7, -8 and -10 suggesting a role for this cytokine in the regulation of caspase gene expression [15-20]. Interestingly, it has also been demonstrated that caspase-1 and -3 gene expression are modulated in several models where apoptosis is triggered, including after transient cerebral ischemia in rats [21], in murine models for Huntington disease [22] and amyotrophic lateral sclerosis (ALS) [23] and in cell lines undergoing apoptosis after treatment with etoposide, which leads to DNA damage [24]. The transcription factors involved in these responses have not yet been identified.

Caspases play a central role in apoptosis and the levels of procaspase-3 must be maintained to insure the elimination of expanded T cell populations at the termination of an immune response through AICD. Since the expression levels of several caspases increase in different models of apoptosis and that decreased levels rendered T cells resistant to apoptosis [14], we examined whether the levels of caspases were modulated by TCR signaling. The KOX-14 murine T cell hybridoma was characterized and shown to undergo a caspase-3-dependent AICD upon T cell activation through the TCR. Furthermore, caspase-3 mRNA levels were selectively induced following TCR stimulation and occurred independently of STAT1 activation. Finally, the increase in caspase-3 gene expression was shown to be independent of caspase activity and the pathways leading to apoptosis suggesting that an early TCR-mediated response is responsible for the selective increase in caspase-3 mRNA levels.

2.2 Materials and Methods

2.2.1 Reagents and apoptosis assay

The murine anti-CD3 antibody was purified from 145-2C11 hybridoma supernatants (hybridoma provided by Dr. M. Julius, University of Toronto, Ontario, Canada). KOX-14 cells were resuspended at 10^6 cells/ml in culture medium and cultured in 6-well plates in the presence or absence of 1 μg/ml of immobilized anti-CD3 antibody in 0.05M Tris buffer (pH 9.3). FITC-conjugated Annexin V (AnV; Biosource International) and Propidium iodide (PI; Sigma) were used to determine the percentage of AnV^- (viable) and AnV^+ (dead) cells. The Fas-Fc and TNFR-Fc chimeric proteins were kindly provided by Dr. D.H. Lynch (Immunex Corp., Seattle, WA, USA) and the human polyclonal IgG was purchased from Sigma. KOX-14 cells were pre-incubated with different caspase inhibitors with 100 μM of DEVD-CHO and YVAD-CHO (Peptide International Inc.), 50 μM of zDEVD-fmk and zFA-fmk or 100μM of zVAD-fmk (Enzyme Systems Products), when indicated.

2.2.2 Western blotting

Cells were washed and lysed in RIPA buffer (2% NP-40, 0.5% deoxycholic acid, 50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM NaF, 10 mM NaH_2PO_4 , 2 mM EGTA, 10 mM EDTA, 0.5 mM Na_3VO_4) and 30 μg of proteins were subjected to SDS-PAGE before transfer to PVDF membrane (Boehringer Mannheim). The membranes were blocked overnight at 4°C in Tris Buffered Saline with 0.05% Tween-20 (TBST), containing 5% of non-fat dried milk. Membranes were then probed with antibodies specific for caspase-3 (New England Biolabs), cleaved caspase-3 (New England Biolabs), β-actin (Sigma), or PARP (BIOMOL) and the PhosphoPlus STAT1 antibody kit, (Cell Signaling Technology), washed three times in TBST and incubated with the HRP-conjugated anti–rabbit Ig or anti-mouse Ig antibody, respectively. Signals were revealed with the enhanced chemiluminescence ECL kit (Amersham) and visualized by autoradiography.

2.2.3 Semi-quantitative RT-PCR

Cells treated as indicated were lysed with Trizol reagent (Invitrogen) for total RNA isolation. Semi-quantitative RT-PCR were performed with the Mu-MLV RT (Gibco BRL, Life Technologies) and pd(N)6 random hexamers (Amersham Pharmacia Biotech) at 65 °C for 10 min and then at 37 °C for 1 hour. PCR reaction mixture contained 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 1 U of Taq polymerase, and 0.5 μM of the following primers: β-actin-Fwd, 5'-GGGAATGGGTCAGAAGGA-3'; β-actin-Rev, 5'-AAGAAGGAAGGCTGGAAA-3'; FasL-Fwd, 5'-TACCACCGCCATCACAAC-3'; FasL-Rev, 5'-GAGATCAGAGCGGTTCCA-3'; Casp-3-Fwd: 5'-TGGGAGCAAGTCAGTGGA-3'; Casp-3-Rev, 5'-CACCATGGCTTAGAATCA-3'. After an initial template denaturation at 94 °C for 2 min, PCR mixtures were submitted to 25 cycles of denaturation at 95 °C (45 sec), annealing at 52 °C (45 sec), and extension at 72 °C (1 min). PCR products were quantified by ethidium bromide staining on 1.2% agarose gels. Samples were standardized using the β-actin signal and results are expressed as fold increase relative to the non-stimulated sample at 0 hour.

2.2.4 RNase protection assay (RPA)

Total cellular RNA was isolated using the RNAwiz reagent (Ambion) from cells treated with the anti-CD3 antibody. The expression of various cytokines, apoptosis-related molecules and caspases was measured by multiprobe RPA template sets (Pharmingen). [^{32}P]-UTP-labeled probes were prepared using the MAXIscript T7 kit from Ambion. Briefly, 50 ng of DNA from multiprobe set was used to generate ^{32}P-labeled riboprobes with T7 RNA polymerase in the presence of 50 μCi of [^{32}P]-UTP (Perkin Elmer). Template DNA was eliminated by digestion with DNase 1 (Pharmingen). Unincorporated [^{32}P]-UTP was removed with MicroSpin G25 columns (Pharmacia). Total RNA (10 μg) was then added to ^{32}P-labeled riboprobe mixture in hybridization buffer and incubated at 90 °C for 2 min followed by 56 °C for 16 h. The hybridized RNA duplexes were then treated with an RNase A and T1 mixture followed by proteinase K digestion (Pharmingen). RNase-resistant duplex RNA was extracted once with phenol:chloroform

and precipitated by the addition of an equal volume of 4M ammonium acetate and 2 volumes of 95% ethanol. The RNA pellet was then solubilized in RNase-free water, heated at 90 °C for 3 min and cooled on ice. Protected transcripts were separated on a 6% sequencing gel, which was dried and exposed to a phosphor screen (Molecular Dynamics) to perform densitometry analysis. Each time point was first normalized to L32 intensity to account for variability in loading. The initial time point (T0) was then given an arbitrary value of one and samples were normalized to the T0.

2.2.5 Real-time RT-PCR

Real-time RT-PCR was performed on 400 ng of total RNA, using ThermoScript One-Step RT-PCR with Platinum *Taq* (Invitrogen). The RT-PCR amplifications were performed as a multiplex with 100 μM of the external primers (caspase-3 or -8 and β-actin) being present in the reaction mix (Table 1). The control plasmid (Fig. 6 A) was amplified in the same run as the experimental samples, and used to generate a standard curve for real-time PCR using LightCycler™ technology (Roche Diagnostics). Following the first round of linear amplification (12 cycles), PCR products were diluted 10-fold prior to on-line, nested real-time PCR using internal primers and fluorescent probes for detection of amplification products (Table 1). For the LightCycler™ amplifications, caspase-3 or -8 and β-actin PCR products were performed in separate capillaries, but quantified on the same first-round serially diluted standard curve. All samples were normalized to the relative levels of β-actin, and results are expressed as the fold increase of relative levels of caspase-3 or -8 in stimulated samples relative to non-stimulated cells, as determined by real-time amplification.

2.3 Results

2.3.1 AICD is mediated by Fas-Ligand in the murine KOX-14 T cell hybridoma

The response of KOX-14 cells to TCR crosslinking was first characterized to determine whether this T cell hybridoma could undergo a Fas-dependent AICD as was previously shown with other T cell hybridomas [3-5]. KOX-14 cells were cultured on anti-CD3-coated plates for 12 hours and cell viability was assessed by AnV/PI staining. Viability started to decrease after 6 hours of stimulation and dropped to 47% live cells (AnV^-) after 12 hours of TCR crosslinking (Fig. 1 A). FasL expression was also monitored by RT-PCR following T cell activation of KOX-14 cells. Figure 1 A shows that FasL mRNA levels increased within 2 hours of anti-CD3 stimulation and peaked at 6 hours, preceding the induction of apoptosis. The requirement for the Fas pathway in this T cell hybridoma was then confirmed using a soluble Fas receptor fused with the Fc domain of the human IgG (Fas-Fc), which inhibits Fas-mediated apoptosis, as previously demonstrated [25]. The presence of 100 μg/ml of Fas-Fc fusion protein prior to stimulation reproducibly resulted in a dramatic inhibition of apoptosis (68% AnV^- cells compared to 17% AnV^- cells in the absence of Fas-Fc) induced by TCR crosslinking (n=3), whereas the control human IgG antibody had no inhibitory effect at a concentration of 100 μg/ml (Fig. 1 B). Under these conditions a TNFR-Fc fusion protein did not suppress cell death in KOX-14 cells (Fig. 1 B) showing that AICD induced by TCR crosslinking is strictly Fas/FasL-dependent in the KOX-14 T cell hybridoma.

2.3.2 Caspase-3 activity is required for AICD in KOX-14 cells

The essential role of caspase-3 in different models of cell death, including AICD of peripheral T cells has been clearly demonstrated in caspase-3 knockout mice [14]. In order to determine the contribution of caspase-3 to apoptosis induced by TCR crosslinking in the KOX-14 cell line, caspase-3 processing and activation was analyzed by Western blot analysis. Cleavage of caspase-3 to its 17-kDa form was observed 8 hours after T cell activation and its activity was confirmed by the cleavage of the caspase-3

specific substrate PARP, in apoptotic cells (Fig. 1 C). The requirement for caspase-3 activity was further demonstrated using the caspase-3-like protease inhibitor DEVD-CHO or the caspase-1-like protease inhibitor YVAD-CHO. The DEVD-CHO inhibitor considerably reduced AICD whereas YVAD-CHO only had a marginal inhibitory effect when compared to cells stimulated in the absence of caspase inhibitors (Fig. 1 D). Altogether, these findings demonstrate that caspase-3 is activated following TCR crosslinking and its proteolytic activity is required for AICD in a Fas-dependent manner in KOX-14 cells.

2.3.3 Effect of TCR signaling on gene expression of cytokines, and apoptosis-related molecules in KOX-14 cells

AICD requires *de novo* RNA and protein synthesis [4] and is characterized by the induction in Fas and FasL expression (RNA and protein) [3;5], the cleavage of caspase-3 and externalization of phosphatidylserine (PS). We first looked at whether the expression profile of cytokines in KOX-14 cells is characteristic of activated T lymphocytes following anti-CD3 stimulation [26]. RNase protection assays were performed on total RNA isolated from non-stimulated (T0) and anti-CD3 stimulated KOX-14 cells using a probe set specific for interleukin genes (Fig. 2 A). Stimulation through the TCR induced within 2 hours the expression of IL-4 and IL-13 which were both absent prior to T cell activation. The expression of IL-2, IL-6, IL-10, and IFN-γ also increased however gradually over time. In contrast, IL-5 and IL-9 mRNA levels were not affected by TCR crosslinking (Fig. 2 A). This observed expression profile of cytokines in activated KOX-14 cells is characteristic of activated T lymphocytes [26]. The expression profile of genes involved in apoptosis was also investigated using the same approach (Fig. 2 B). Induction of FasL expression, a hallmark of AICD, occurred within 2 hours of T cell activation and Fas mRNA levels were increased on average 2-fold over the course of the stimulation. In contrast, caspase-8, FADD, FAF, TNFRp55, TRADD and RIP did not show any variations in mRNA levels in TCR stimulated KOX-14 cells (Fig. 2 B). Together, these results demonstrate that T cell activation leads to an increase in the expression levels of

different cytokines and to an induction in FasL and Fas expression, in the KOX-14 murine T cell hybridoma.

2.3.4 Caspase-3 mRNA levels are selectively increased following T cell activation

We then investigated whether T cell activation leads to the upregulation of caspase-3 mRNA levels required to replenish the pool of procaspase-3, which is cleaved upon TCR triggering [27-30]. RPA analysis was performed on KOX-14 cells using a panel of probes complementary to nine different caspases (Fig. 3 A). Quantification of mRNA levels was performed for caspase-2, -3, -6, -7 and –8 since they are the only proteases that appeared to be expressed in KOX-14 cells by RPA. Caspase-3 was the only one to show increased levels of mRNA 4 hours after TCR ligation whereas caspase-2, -6, -7 and –8 levels did not increase over the same period of time (Fig. 3 B). RPA experiments demonstrated a relative increase of 5.6-fold in caspase-3 mRNA levels, 4 hours after TCR ligation, when normalized to the non-stimulated sample (time 0 hours) and was selective to caspase-3 (Fig. 3 B). These results clearly demonstrate the selective increase in caspase-3 mRNA levels in KOX-14 cells following activation through the TCR.

2.3.5 Caspase-3 expression is induced in the absence of STAT1 phosphorylation

Several studies have suggested a role for IFN-γ signaling through STAT1 in the regulation of caspase-3 expression levels [15-17;20]. RPA analysis on anti-CD3 stimulated KOX-14 cells demonstrated a slight increase in IFN-γ mRNA levels following 2 hours of activation (Fig. 2 A), prior to the increase observed in caspase-3 mRNA levels. We therefore examined the phosphorylation state of STAT1 to determine whether the induction in caspase-3 mRNA levels was dependent on the activation of this transcription factor following T cell activation. Western blot analysis revealed that resting KOX-14 cells (T0) contained significant levels of STAT1 but not the phosphorylated form (STAT1-P) (Fig. 4). Although caspase-3 expression levels peaked following activation of KOX-14 cells after 4 hours of culture on anti-CD3 coated plates (Fig. 3), we did not

observe any STAT1 phosphorylation. However, the cells did undergo apoptosis as demonstrated by the decrease in cell viability with only 20% live (AnV^-) cells remaining after 6 hours of stimulation (Fig. 4). In order to ensure that the lack of STAT1 phosphorylation was not due to the onset of apoptosis, KOX-14 cells were stimulated in the presence of the broad caspase inhibitor zVAD-fmk. Stimulation of KOX-14 cells in the presence of zVAD-fmk significantly inhibited apoptosis with 78% live (AnV^-) cells remaining after 6 hours of stimulation. Nevertheless, phosphorylation of STAT1 was not observed in the absence of apoptosis (Fig. 4). These results clearly show that the induction in caspase-3 mRNA levels following T cell activation of KOX-14 cells is not dependent on STAT1 phosphorylation.

2.3.6 Induction of Caspase-3 gene expression precedes apoptosis

Since caspase-3 activity is required during AICD in mature T cells [14], the regulation of its expression by the TCR was further characterized. The levels of caspase-3 mRNA were determined by semi-quantitative RT-PCR following the induction of AICD (Fig. 5). As previously demonstrated in other T cell hybridomas [31], TCR crosslinking induced a rapid expression of Nur77 mRNA levels peaking 2 hours after stimulation (data not shown). Nur77 expression was followed by an increase in FasL mRNA levels, which reached highest expression levels 6 hours after TCR triggering, when 36% of the cells were already undergoing apoptosis (Fig 5 A). Caspase-3 mRNA levels also increased after TCR triggering and showed an average increase of 3-times 6 hours after stimulation (n=3; Fig. 5 B). However, mRNA levels of caspase-3 began to decrease as the cells started undergoing apoptosis. Similar results were obtained on total mRNA isolated from the DO11.10 T cell hybridoma stimulated with anti-CD3 (Fig. 5 A and B).

Western blot analysis was performed on lysates of anti-CD3 activated KOX-14 cells using antibodies directed against the full length (32-kDa) and cleaved forms (20- and 17-kDa) of caspase-3. The disappearance of procaspase-3 and the appearance of the active forms of caspase-3, 6 hours following stimulation, correlated with the externalization of PS as observed by AnV staining (Fig. 5 C). Caspase-3 gene expression consistently

peaked between 4 to 6 hours, before the decrease in cell viability and activation of caspase-3, suggesting a regulation independent of the mechanisms leading to cell death. These results demonstrate that the upregulation in caspase-3 mRNA precedes the onset of apoptosis which occurs through AICD following T cell activation in the KOX-14 murine T cell hybridoma.

2.3.7 Upregulation of Caspase-3 mRNA occurs in the absence of apoptosis

Real-time PCR has proven to be a very efficient tool to quantitatively measure gene expression. In this study we developed an assay taking advantage of this technology to measure the upregulation in caspase-3 mRNA levels during T cell activation. Two sequence specific oligonucleotides labeled with fluorescent dyes are used for DNA detection of the amplification product which allows us to quantitatively determine changes in gene expression (Table 1). We have designed a construct which contains, within the same plasmid, a portion of the cDNA of the gene of interest (Caspase-3 or -8) and a housekeeping gene (β-actin) (Fig. 6 A). This allows us to maintain the same number of copies of the target gene and the internal control when generating a standard curve and quantitatively measure changes in gene expression for caspase-3 or -8, following TCR stimulation in KOX-14 cells. Furthermore, it was determined that only 12 cycles were required for the first round of PCR which ensured linear amplification of the PCR products for caspase-3 and -8 and β-actin (data not shown).

Cells were stimulated with the anti-CD3 antibody over a course of 10 hours, in the presence or absence of 50 μM of the caspase-3 inhibitor, zDEVD-fmk. Addition of zDEVD-fmk inhibited apoptosis for up to 8 hours, with 87% of the cells remaining AnV^- whereas in the absence of the inhibitor only 60% of the cells remained AnV^- after 6 hours of stimulation, as determined by AnV and PI staining (Fig. 6 B). The negative control inhibitor, zFA-fmk, did not inhibit apoptosis when KOX-14 cells were treated with anti-CD3 (data not shown).

Induction in caspase-3 mRNA levels were measured by real-time PCR and showed a 2.5-fold increase after 4 hours of TCR ligation which appeared before the onset of apoptosis (89% AnV^- cells; Fig. 6 B). There was a dramatic drop in caspase-3 gene expression when the percentage of AnV^- cells decreased to 60% after 6 hours of culture with anti-CD3. However, caspase-3 levels were slightly higher (4-fold) after 4 hours of receptor crosslinking in the presence of 50 μM of zDEVD-fmk, and were maintained for 8 hours in the presence of the caspase-3 inhibitor. In contrast, there was a decline in caspase-8 gene expression in the presence or absence of zDEVD-fmk (n=2; Fig. 6 B). These results confirmed data obtained by semi-quantitative RT-PCR (Fig. 5) and demonstrate that the upregulation in caspase-3 gene expression occurs early during the activation phase following T cell stimulation. Furthermore, the increase in caspase-3 mRNA levels occurred between 2 and 4 hours following stimulation, in the presence of a caspase-3 inhibitor and before the onset of apoptosis showing that the increase in caspase-3 gene expression is independent of its activity.

2.4 Discussion

Expression of members of the caspase family is known to vary in a tissue- and development-dependent manner. For instance, caspase-2, -3 and -7 are expressed in the developing brain, but their levels are very low in the adult brain [32-37], whereas caspase-8 and -12 are expressed in adult cortical neurons [38]. In contrast, almost all caspases are expressed in mature lymphocytes [29;39]. These observations indicate that caspases are differentially regulated during development. In addition, the expression of caspases has been shown to increase following various treatments leading to apoptosis [15;17;21;24;40]. The present study now demonstrates that caspase-3 mRNA levels are selectively increased during AICD triggered by TCR crosslinking in the KOX-14 T cell hybridoma.

The magnitude of TCR-mediated increase in caspase-3 expression was similar to the one reported in the U937 tumor cell line treated with etoposide [24] or the upregulation of caspase-3 expression by IFN-γ [16;17;20] which represented a three-fold increase when compared to baseline levels. Although post-transcriptional regulation cannot be excluded, several observations suggest that caspase expression is regulated at the transcriptional level during apoptosis. First, caspase-3 transcription is increased by etoposide which was demonstrated by nuclear run-on experiments [24]. Second, several stimuli can enhance caspase-3 expression, including IFN-γ [16;17;20] and now TCR crosslinking (present study). Unlike Fas-mediated cell death which is independent of transcription, one mechanism controlling AICD is the transcriptional regulation of FasL which is dependent on TCR signaling [3-5]. The data presented in this report demonstrate that T cell activation of KOX-14 cells also leads to the transcriptional upregulation of the caspase-3 gene. Furthermore, signaling pathways and transcription factors activating the FasL promoter could be mediating their effects on the caspase-3 promoter.

The IFN-γ receptor signaling pathway and particularly STAT1 phosphorylation have been suggested to be required for caspase-3 expression and AICD [20]. Although STAT1 was found to be required for basal levels of caspase-3 expression,

phosphorylation and dimer formation of STAT1 are not required to maintain gene expression of this protease [15]. Interestingly, STAT1 knockout mice [41;42] did not show any developmental abnormalities as the ones observed in caspase-3 knockout mice [14;33] suggesting that this transcription factor is not essential for caspase-3 expression during development. Our results in KOX-14 cells clearly show that the induction in caspase-3 expression is independent of the activation state of STAT1 following TCR crosslinking (Fig. 4). Furthermore, there is no evidence in the literature showing the binding of STAT1 to a region within the caspase-3 promoter to support a direct role for this transcription factor in the regulation of caspase-3 gene expression [43;44].

The increase in caspase-3 mRNA levels always preceded the activation of caspase-3 (Fig. 5) and was maintained in the presence of caspase-3-specific inhibitors (Fig. 6 B). Following a 3-fold induction in caspase-3 mRNA levels a sharp decrease was observed after 6 hours of stimulation which is attributed to the onset of apoptosis. Similar results were obtained with anti-CD3 stimulated thymocytes which also showed a 3-fold increase in caspase-3 mRNA levels before the induction of apoptosis (data not shown). Inhibition of caspase activity with zDEVD-fmk and subsequent apoptosis maintained the increased levels and even showed higher levels of caspase-3 expression (Fig. 6 B). Similarly, it was previously shown that inhibition of etoposide-induced apoptosis with zVAD-fmk and zDEVD-fmk, leads to increased levels of caspase-2 and -3 mRNA which were also maintained for longer periods of time [24]. These findings clearly demonstrate that signaling pathways independent of caspase-3 activity are responsible for the observed increase in caspase-3 gene expression.

We have previously shown that caspase-3 and other caspases are rapidly cleaved following T lymphocyte stimulation, although the cells remain alive and proliferate [29]. It has been suggested that the half-life of the cleaved forms of caspases is shorter than the full length proenzymes [45] and it was also demonstrated that the active forms of caspase-3 disappear rapidly through XIAP mediated proteosomal degradation [46]. Therefore, once T cells are stimulated, the pool of procaspase-3 may rapidly decrease due to the conversion into active subunits and subsequent degradation or even because of cell

division. In human fibroblasts it was demonstrated that external stimuli that induce cell division affected the expression of hundreds of genes, including caspase-3 [47]. This finding suggests that a cell will increase the expression of some genes when entering the cell cycle to ensure that a certain level of the products of such genes are maintained to allow a cell to perform its biological function and could dictate the fate of a cell in the future. For instance, the E2F-1 transcription factor which plays a critical role in cell cycle entry and T cell proliferation has been shown to be a mediator of AICD [48]. Interestingly, several studies have demonstrated a potential role of this transcription factor in the induction of caspase-3 mRNA levels [43;49]. Whether the E2F-1 transcription factor is directly responsible for the increase in caspase-3 mRNA levels during T cell activation remains to be determined.

Thus, TCR-induced caspase-3 upregulation observed in the KOX-14 T cell hybridoma may reflect a mechanism essential for the maintenance of minimal levels of procaspase-3, during the initial phase of activation of resting T cells, in order to ensure the elimination of expanded T cell populations, in response to an antigen following subsequent stimulations, through AICD.

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Table 2-1. Oligonucleotides for quantification of caspase gene expression by real-time RT-PCR

RNA quantification primers		
β-actin	5'-In:	AAGGACTCCTATGTGGGTGA
	5'-Out:	GGGAATGGGTCAGAAGGA
	3'-In:	CCATCACAATGCCTGTGGTA
	3'-Out:	AAGAAGGAAGGCTGGAAA
caspase-3	5'-In:	GACCATACATGGGAGCAAGT
	5'-Out:	GGAGAACAACAAAACCTCAGTGGA
	3'-In:	CCTTCATCACCATGGCTTAGA
	3'-Out:	CAGGCCTGAATGATGAAGAGT
caspase-8	5'-In:	GATGACTTGAGCCTGCTTGA
	5'-Out:	TGGAGGAAGGCAATCTGTC
	3'-In:	CGAGGTTTGTTCTTCATTTGGT
	3'-Out:	ATTTGCAGTGCAGTCGTCGT
LightCycler™ probes		
β-actin	P1:	GTGAAAAGATGACCCAGATCATGTTTGAGACC-f
	P2:	(Red-705)TCAACACCCCAGCCATGTACGTAGCCAT-p
caspase-3	P1:	GCATATGCCCATTTCAGGATAATCCATTTTGTA-f
	P2:	(Red-640)CTACTGTCCAGATAGATCCCAGAGTCCA-p
caspase-8	P1:	TGGATGAGATGAGCCTCAAAATGGCGGAACTG-f
	P2:	(Red-640)GTGACTCGCCAAGAGAACAAGACAGTGA-p

f, fluoresceine labeled probe, p, phosphate.

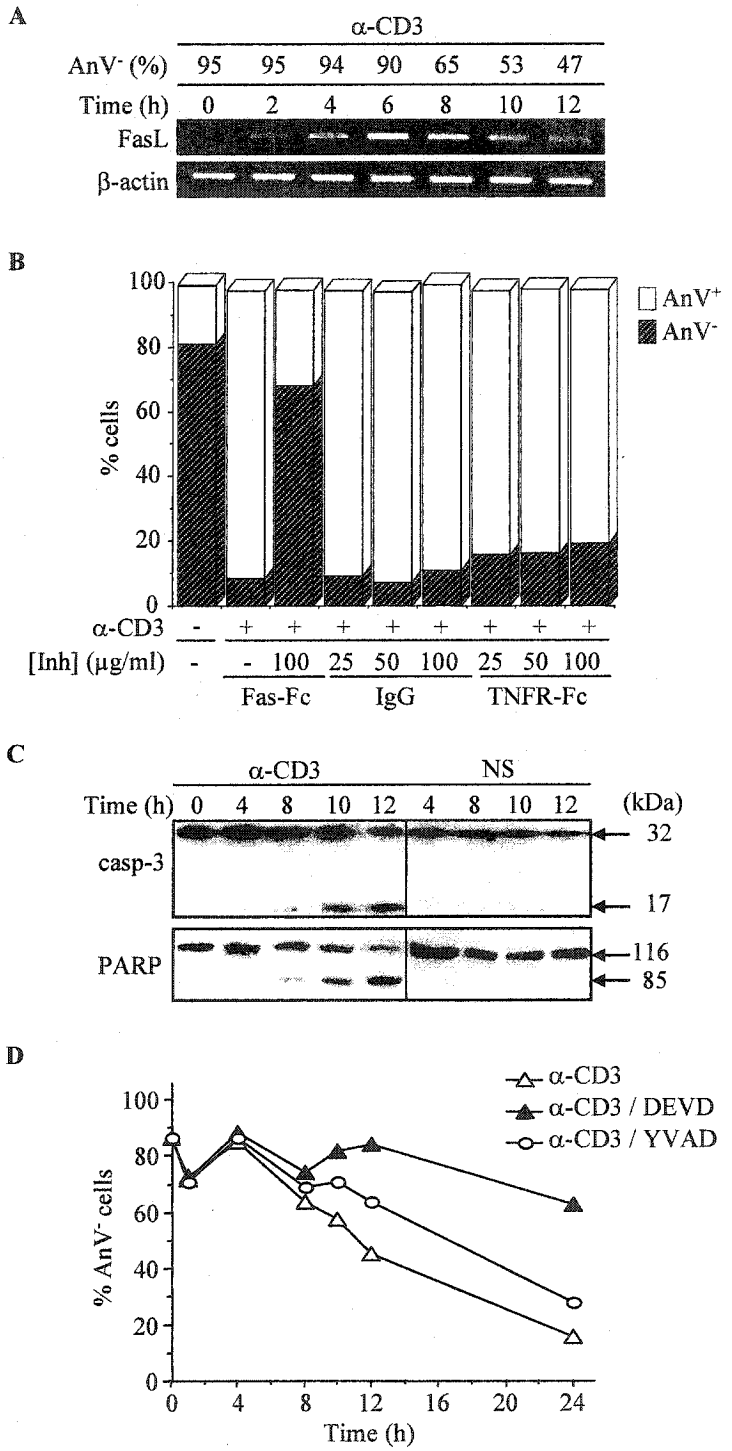

A
α-CD3
AnV⁻ (%) 95 95 94 90 65 53 47
Time (h) 0 2 4 6 8 10 12
FasL
β-actin
B
100
80
60
40
20
0
% cells
AnV⁺
AnV⁻
α-CD3 - + + + + + + + +
[Inh] (μg/ml) - - 100 25 50 100 25 50 100
Fas-Fc
IgG
TNFR-Fc
C
α-CD3
NS
Time (h) 0 4 8 10 12 4 8 10 12
(kDa)
casp-3
32
17
PARP
116
85
D
α-CD3
α-CD3 / DEVD
α-CD3 / YVAD
100
80
60
40
20
0
% AnV⁻ cells
0 4 8 12 16 20 24
Time (h)

Figure 2-1. Fas- and caspase-3-dependent AICD in KOX-14 cells
(A) KOX-14 cells were cultured on anti-CD3-coated (α-CD3) plates for 12 hours. Apoptosis was quantified by AnV/PI staining and the percentage of AnV negative (viable) cells is shown. Total RNA was extracted from cells in the same experiment and RT-PCR was performed using primers specific for the murine FasL or β-actin gene. (B) KOX-14 cells were resuspended in medium containing 100 μg/ml of soluble Fas-Fc fusion protein, 25 to 100 μg/ml of human IgG or 25 to 100 μg/ml of TNFR-Fc and cultured in the presence or absence of anti-CD3 for 12 hours. Percentage of viable (AnV^-) and dead (AnV^+) cells is shown. (C) Proteins were extracted from KOX-14 cells cultured in the presence or absence of the anti-CD3 antibody for 12 hours, and cleavage of caspase-3 and PARP was analyzed by Western blot. (D) Cells were resuspended in medium containing DEVD-CHO (DEVD; caspase-3-like protease inhibitor), YVAD-CHO (YVAD; caspase-1-like protease inhibitor) or medium alone, and cultured on anti-CD3-coated plates for 24 hours. Cell viability was determined by AnV/PI staining and the percentage of AnV^- cells is shown. Results shown are representative of three independent experiments.

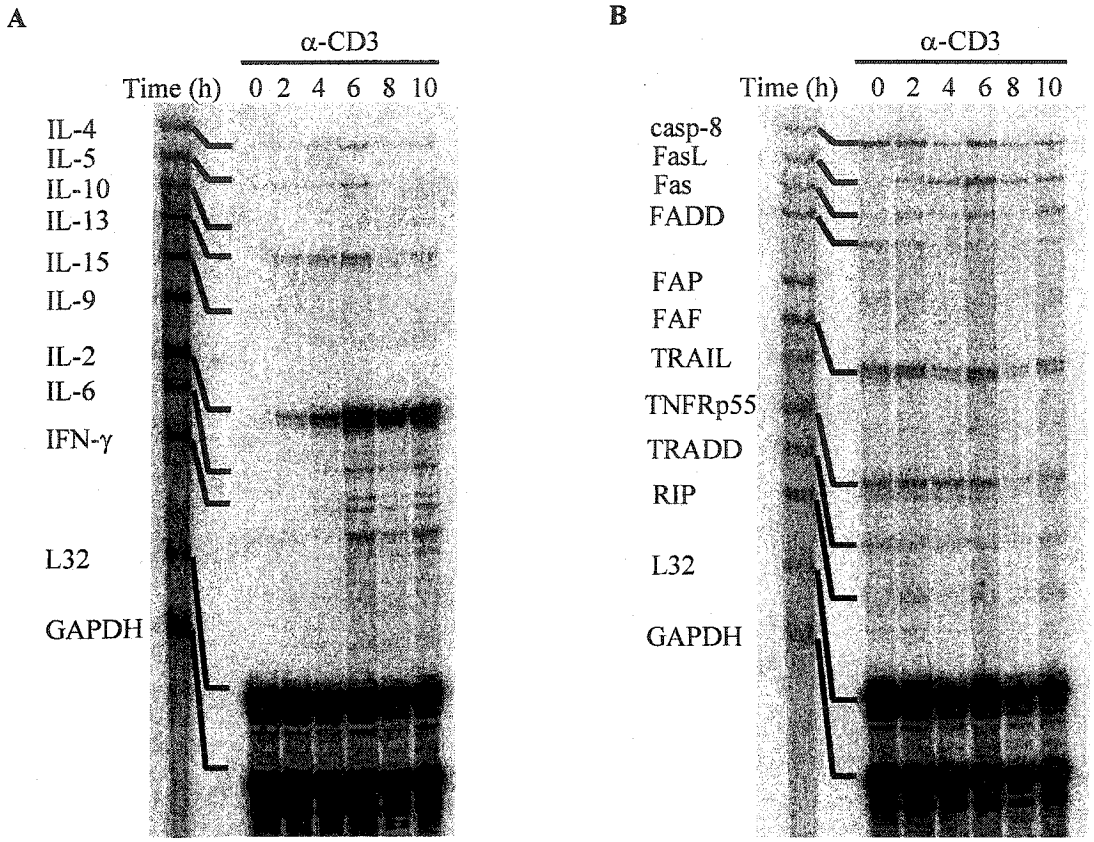


Figure 2-2. Activation of KOX-14 cells induces the expression of cytokines, Fas and FasL

KOX-14 cells were stimulated with immobilized anti-CD3 (α-CD3) for 10 hours and total RNA was isolated at two hour intervals. RNase protection assay (RPA) was then performed on 10 μg total RNA hybridized to radiolabeled RNA probes corresponding to (A) murine cytokines genes and (B) apoptosis-related genes. Results shown are representative of three independent experiments.

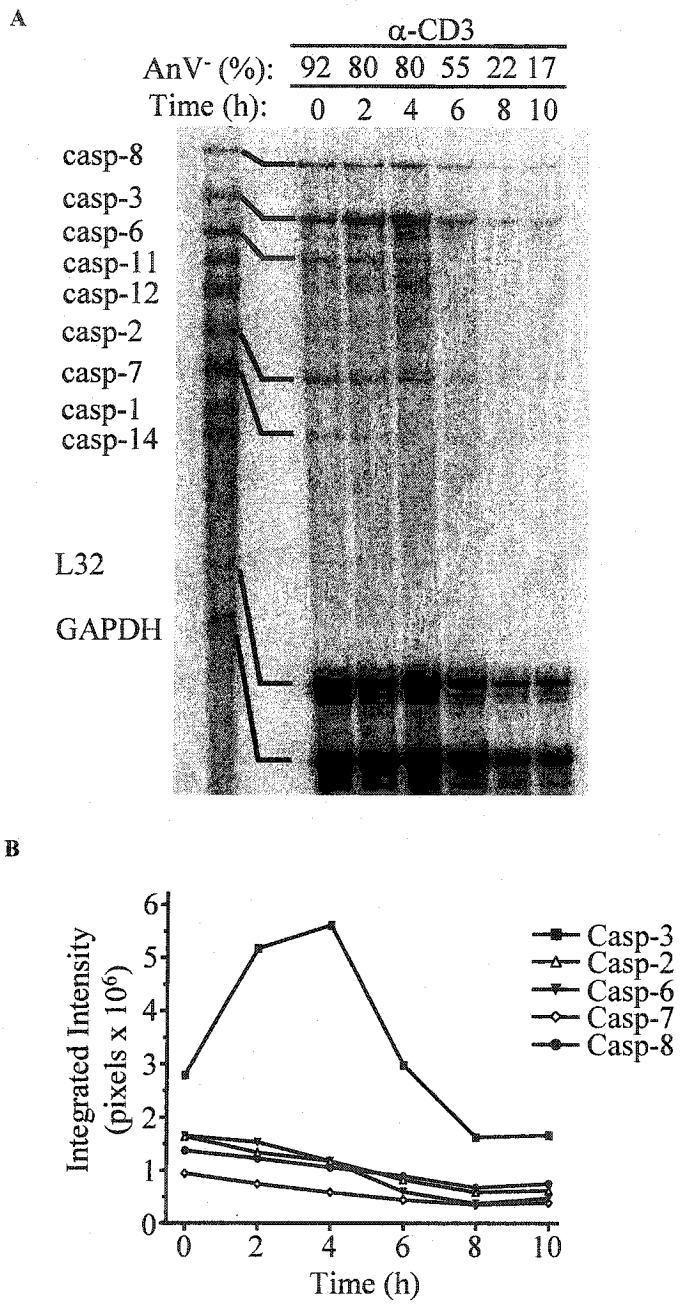


Figure 2-3. Selective increase in caspase-3 mRNA levels precedes the onset of apoptosis (A) KOX-14 cells were cultured in the presence of anti-CD3 (α-CD3). RPA was performed using probes corresponding to murine caspases. The percentage of AnV^- cells was assessed by AnV / PI staining. p, undigested free probe.(B) Densitometry analysis for caspase-2, -3, -6, -7 and -8 of the RPA gel presented in (A). Data is presented as the signal intensity of stimulated samples standardized to the L32 signal. Results shown are representative of four independent experiments.

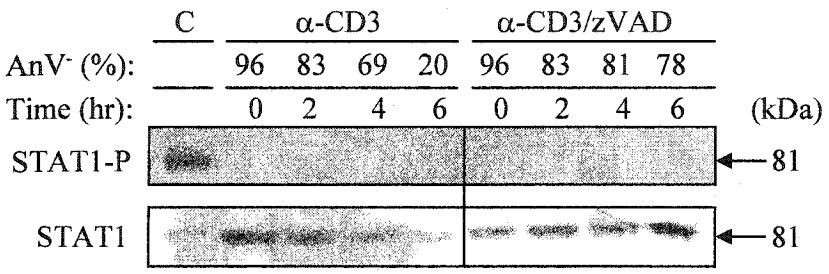


Figure 2-4. Induction of caspase-3 expression is independent of STAT1 activation
KOX14 cells were left untreated (Time 0) or cultured on anti-CD3-coated (α-CD3) plates in the presence or absence of zVAD-fmk (zVAD). At each time-point, the percentage of live cells (AnV^-) was determined by AnV/PI staining. Cell lysates were subjected to Western blot analysis using an anti-Phospho-Tyr701-STAT1 antibody. The same membranes were stripped and reblotted with an anti-STAT1 antibody. (C, Lysates of IFN-γ-treated HeLa cells; STAT1-P, phosphorylated STAT1).

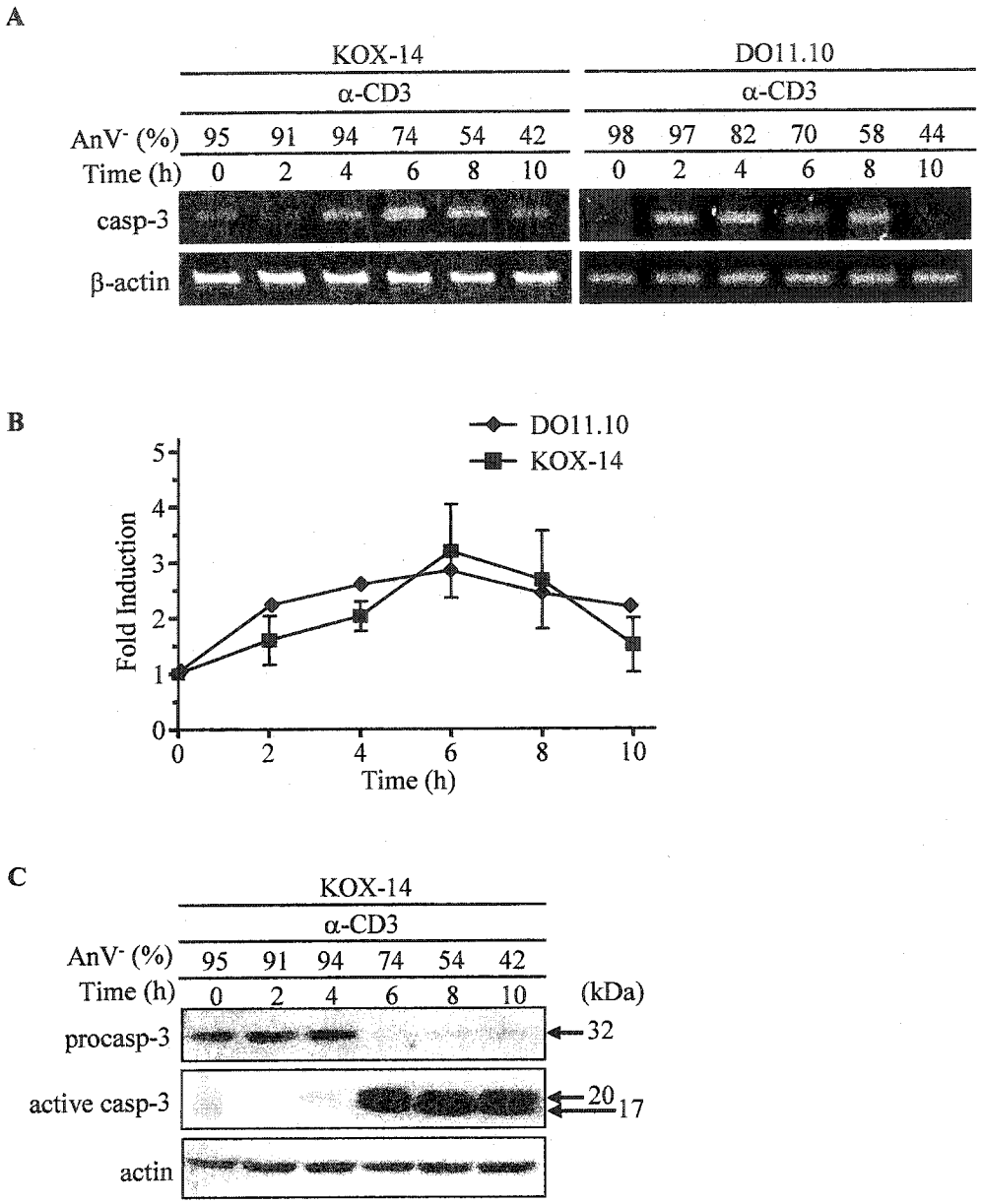
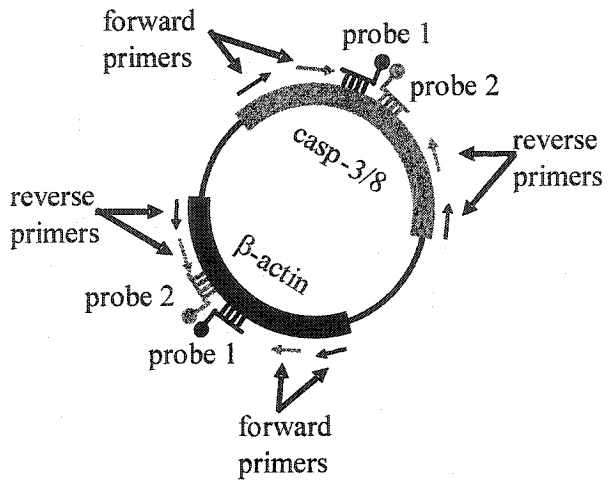
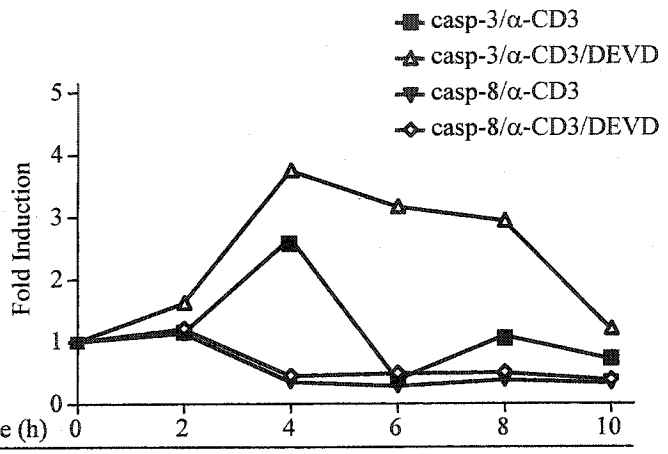


Figure 2-5. Caspase-3 gene expression is increased following TCR crosslinking
(A) KOX-14 and DO11.10 cells were cultured on anti-CD3 coated (α-CD3) plates for 10 hours. An aliquot was taken every two hours for AnV/PI staining. Total RNA was extracted and RT-PCR was performed using primers specific for the murine caspase-3 and β-actin genes. (B) Densitometry analysis was performed to determine the fold induction in caspase-3 mRNA levels. Data is presented as the signal intensity of caspase-3 standardized to the β-actin signal relative to the non-stimulated sample at time 0 hour (n=3 for KOX-14). (C) Cell lysates were prepared for KOX-14 cells stimulated with anti-CD3 at each indicated time of culture, and analyzed by Western blot for the cleavage of caspase-3. The percentage of live cells (AnV^-) was assessed by AnV/PI staining. Results shown are representative of three independent experiments.

A



B



Stimulus	Percentage of AnV$^-$ cells					
α-CD3	98	87	89	60	34	25
α-CD3/DEVD	98	90	93	92	87	79

Figure 2-6. Induction of caspase-3 gene expression as determined by real-time RT-PCR (A) A portion of the caspase-3 or -8 cDNA was cloned within the same plasmid containing a portion of the β-actin cDNA to generate the standard curve for quantification of gene expression by real-time PCR. The external primers are used to perform the RT-PCR and internal primers are then used for the nested real-time PCR. Sequence specific oligonucleotides labeled with fluorescent dyes are also shown and designated as probes 1 and 2. (B) KOX-14 cells were stimulated with immobilized anti-CD3 (α-CD3) for 10 hours, in the presence or absence of zDEVD-fmk (DEVD), and total RNA was isolated at two hour intervals. Caspase-3 and -8 mRNA levels were measured by real-time RT-PCR. The percentage of live cells (AnV$^-$) was determined by AnV/PI staining (n=2).

Chapter 3: The selective increase in caspase-3 expression in effector but not memory T cells allows susceptibility to apoptosis

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Preface

In chapter 2 we demonstrated that caspase-3 levels are selectively induced during T cell activation in KOX-14 cells. However, the increase in mRNA levels occurred independently of IFN-γ signaling and in the absence of apoptosis suggesting that the increase in caspase-3 mRNA levels occurs early during the activation phase. Therefore in this chapter we investigated the role of T cell activation on caspase-3 expression in different models and in different T cell subsets isolated from mice.

Abstract

Caspases play a central role in both T lymphocyte activation and death. We have previously demonstrated that caspase-3, which is critical for activation-induced cell death (AICD), is processed following T cell activation in the absence of apoptosis. The renewal of the procaspase-3 pool was investigated in activated live T cells. We report here that caspase-3 mRNA levels were selectively increased in peripheral T cells (13-fold), following antigen receptor-mediated T cell activation. The upregulation of caspase-3 mRNA levels was confined to cells in the early phases of the cell cycle (G0/G1) and was independent of IL-2 signaling, implying a critical role for early T cell receptor mediated signals. This increase led to the renewal of the procaspase-3 pool as evidenced by a 6-fold upregulation of the zymogen in non-apoptotic stimulated T cells. Activated T lymphocytes isolated from caspase-3 heterozygous and knockout mice were significantly more resistant to etoposide- and IL-2-withdrawal-induced apoptosis, when compared to caspase-3 wild-type mice. More importantly, the increase of mRNA levels and both the zymogen and the cleaved forms of caspase-3 was observed in *in vivo* stimulated antigen specific effector and not memory T cells, thereby providing a mechanism for the enhanced susceptibility of effector T cells to apoptosis during AICD. These findings indicate that the selective upregulation of caspase-3 transcription is required to reach a threshold in the expression levels of this protease which controls the efficient initiation of AICD and homeostasis of the immune response.

3.1 Introduction

Mature T lymphocytes circulate through the blood and peripheral lymphoid organs in a resting state until they encounter an antigen-presenting cell bearing a specific peptide presented by an MHC molecule [1]. Engagement of the TCR/CD3 complex following a response to a pathogen results in T cell activation, an event characterized by the highly regulated expression of a large number of activation specific genes, involved in cell cycle progression, proliferation and apoptosis [2;3]. Following clearance of the pathogen, the majority of activated effector T cells are eliminated by activation-induced cell death (AICD), a mechanism which results from the interaction of death receptors with their specific ligands [4]. Binding of these ligands to death receptors results in the recruitment of the Fas-associated death domain (FADD) adapter molecule. The adaptor FADD binds to the intracellular death domain of Fas via its own death domain and recruits procaspase-8 through the interaction of death-effector domains found in both molecules [5-8]. Aggregation of all three molecules (Fas, FADD and procaspase-8) leads to the formation of the death-inducing signaling complex and subsequent cleavage of procaspase-8 to its active form (caspase-8) [9;10]. The active form of caspase-8 then initiates the proteolytic cleavage of caspase-3, which in turn cleaves caspase-6 and -7 and a multitude of cellular substrates leading to apoptosis [11].

Caspase-3 is also the point of convergence of an intrinsic apoptotic pathway initiated through the mitochondria, which further illustrates its central role in apoptosis. Following mitochondrial outer membrane permeabilization and loss of the $\Delta\Psi m$, cytochrome *c* is released from the intermembrane space and binds Apaf-1 [12]. The interaction between Apaf-1, dATP, cytochrome *c* and procaspase-9 in the cytoplasm leads to the formation of a multiprotein complex referred to as the apoptosome [13]. These series of events trigger the processing and activation of caspase-9, which then efficiently cleaves and activates caspase-3. Similar to apoptosis initiated through death receptors, the active form of caspase-3 then cleaves cellular substrates involved in cellular integrity and metabolism.

Surprisingly, cleavage of caspase-3 occurs in activated T lymphocytes in the absence of apoptosis and is required for T cells to enter the cell cycle [14-17]. Indeed, inhibition of caspase-3 activity results in defective T cell proliferation following stimulation of naïve T cells through the TCR, although the initial steps of T cell activation remain intact [16]. Cleavage of caspase-3 and caspase-8 thus appears to be an integral component of the T cell activation process. The strength of TCR signaling also regulates caspase activation during T cell proliferation [18;19]. It was demonstrated that stimulation of naïve CD4 T cells with high affinity ligands leads to caspase activation whereas low affinity ligands failed to show any caspase activity [19].

The mechanisms behind the selective elimination of effector T cells during AICD are not fully understood, although it has been clearly shown that members of the bcl-2 family are upregulated in memory T cells while these levels are downregulated in effector T cells [20-23]. Levels of expression of caspases could furthermore contribute to differences in the susceptibility of distinct T cell subsets to apoptosis. However, experimental evidence is accumulating showing that both the mRNA and protein levels of caspase-3 have a profound effect on the onset of apoptosis. For example, the reduction in caspase-1 and -3 basal expression observed in STAT-1 null cells leads to resistance to TNF-α-induced apoptosis [24]. The lack of induction of caspase-2 and -3 gene expression in tumor cell lines correlates with resistance to etoposide-induced apoptosis [25]. In line with these findings, it also was found that a majority of tumor cells isolated from breast cancer patients lack caspase-3 mRNA and protein expression, suggesting that absence of caspase-3 could play a role in tumor development [26]. Finally, peripheral T lymphocytes isolated from mice, in which the caspase-3 gene has been inactivated by homologous recombination, are partially resistant to apoptosis following treatments with either anti-CD3 or Fas, suggesting that an intact procaspase-3 pool is critical for T lymphocytes homeostasis [27].

In this study, we have shown that mRNA levels of caspase-3 are modulated following TCR stimulation in order to maintain adequate levels of the procaspase-3 pool required for the onset of AICD. Our results show that T cell activation leads to a selective

increase in caspase-3 mRNA levels in effector T cells as well as a concomitant increase in the levels of its proenzyme. The selective upregulation of this executioner caspase in effector and not memory T cells and the increased sensitivity of these cells to apoptosis highlight the importance of maintaining adequate levels of caspase-3 through transcriptional regulation to ensure the death of effector T cells by AICD.

3.2 Materials and Methods

3.2.1 Mice and Viral Infection

BALB/c (Charles River), C57BL/6 and B6.PL-Thy1a/Cy mice (Jackson Laboratory), the 2C αβ TCR transgenic mice (H-2K^b) and the wildtype, heterozygous and homozygous caspase-3 knockout mice [27] were used in our experiments. Lymphocytic choriomeningitis virus (LCMV) TCR specific P14 transgenic mice (H-2^b; Thy1.2$^+$) [22] were crossed onto B6.PL-Thy1a/Cy (H-2^b; Thy1.1$^+$) to generate Thy1.1$^+$ P14 transgenic mice. To generate acutely infected and immune animals, ~2 x 10^5 P14 splenocytes (Thy1.1$^+$) were adoptively transferred into normal (non-irradiated) C57BL/6 mice by i.v. injection. On the next day chimeric mice were infected with 2 x 10^5 pfu of LCMV-Armstrong i.p. For LCMV reinfection, LCMV immune animals containing memory Thy1.1$^+$ P14 CD8 T cells were infected with 2 x 10^6 pfu of LCMV-cl. 13 i.v. and the P14 CD8 T cells were examined four days later. All animal experiments were done with approved Institutional and Animal Care and Use Committee protocols.

3.2.2 Cell Preparation, Activation and Apoptosis Assay

Total lymphocytes were isolated from lymph nodes and thymii of mice, cultured in 6-well plates at 5 X 10^6 cells per well in the presence of 200 U/ml of IL-2 (National Institutes of Health AIDS Research and Reference Reagent Program) and pre-incubated with 300 μM L-mimosine (Calbiochem), when indicated. Cells were stimulated with 10 μg/ml of immobilized anti-CD3 antibody, (145-2C11, from Dr. M. Julius, University of Toronto, Ontario, Canada). Lymph node T cells were labeled with 0.5 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes) for 10 min at 37 °C in PBS. Labeling was quenched with FCS and cells were washed in DMEM 10% FCS and seeded at 10^6 cells in 24-well cell culture plates in the presence or absence of coated anti-CD3. Lymph node T cells from 2C mice were stimulated with 1 μM of the SYRGL peptide in the presence of 200 U/ml of IL-2 and antigen presenting cells (APCs). Activation was assessed by the levels of CD69 and CD25 surface expression

(Pharmingen) by flow cytometry. The number of live cells was determined by Annexin V (AnV, Biosource International) and Propidium Iodide (PI) staining and analyzed by flow cytometry. The PC61 antibody was used at a concentration of 50 μg/ml to block signaling through the IL-2 receptor. Following 48 hours of stimulation, T cells isolated from caspase-3 wild-type, heterozygous or knockout mice were incubated in the presence of 1 μM etoposide (Calbiochem) or 50 μg/ml PC61 antibody to induce apoptosis over the course of 10 hours. AnV^- cells were negatively sorted on the Auto-MACS™ using the Dead Cell Removal Kit (Miltenyi Biotec) and T cells were then isolated by positive selection using MACS CD90 (Thy1.2) MicroBeads (Miltenyi Biotec).

3.2.3 Real-Time RT-PCR assay

Reverse transcriptase reactions were performed on 400 ng of total RNA, using ThermoScript One-Step RT-PCR with Platinum *Taq* (Invitrogen). A construct encompassing nucleotide sequences from caspase-3 or -8 and β-actin was developed to generate a standard curve for real-time PCR using LightCycler™ technology (Roche Diagnostics). Following the first round of amplification, PCR products were diluted 10-fold prior to on-line, nested real-time PCR using fluorescent probes. All samples were normalized to the relative levels of β-actin, and results are expressed as the fold increase in the relative levels of caspase-3 or -8 in stimulated relative to non-stimulated cells.

3.2.4 Cell Cycle Analysis and FACS Cell Sorting

Cells were fixed and permeabilized in 70% ethanol, stained in 500 μl PBS containing 300 μg/ml PI and 100 μg/ml RNase for 30 min at 37°C and then analyzed by flow cytometry. When sorting for the different phases of the cell cycle (G0/G1 and S/G2/M), cells were stained with the Hoechst 33342 dye (Sigma) at a final concentration of 10 μg/ml for 2 hours at 37°C, washed with cold PBS and sorted on a FACS Vantage™ cell sorter (Becton-Dickinson). Splenocytes from naïve P14 mice or from P14 chimeric mice infected with LCMV, 8 (effector T cells) or greater than 40 days (memory T cells)

previously were stained with anti-CD8α and anti-Thy1.1 antibodies, filtered and sorted as described.

3.2.5 cRNA Synthesis and DNA Microarray Hybridization

Isolated total RNA from P14 naïve or day 8 and 40 P14 chimeric sorted cells was resuspended in 5μl DEPC water per 10^6 cells. cDNA was synthesized from total RNA of ~10^6 cells using SuperScript Choice cDNA synthesis kit (GIBCO/BRL Life Technologies) and an oligo(dT) primer containing a T7 promoter. Four hour *in vitro* transcription reactions using T7 RNA polymerase were used to amplify poly (A)+RNA (referred to as cRNA) from the cDNA using the MEGAscript T7 kit (Ambion). The cRNA was extracted and a second round of double-stranded cDNA was synthesized from the cRNA using random and T7-oligo (dT)24 primers. A second round of cRNA synthesis was performed using biotinylated ribonucleotides and 20 μg of biotinylated-cRNA was fragmented and hybridized to the Affymetrix U74A chips according to manufacturer's protocols, as previously described [28].

3.2.6 Detection of intracellular caspase-3 and flow cytometry

Mice adoptively transferred with Thy1.1$^+$ P14 CD8 T cells were infected with LCMV and on days 4, 6, 8 14 and 65 post infection or 4 days following LCMV reinfection, the splenocytes were harvested and cells were stained with anti-Thy1.1 antibodies in staining buffer (PBS, 1% FCS) on ice for 30 min. The cells were washed, fixed and permeabilized using the intracellular staining kit (Becton-Dickinson) as previously described [29]. The cells were incubated with anti-caspase-3 and anti-active caspase-3 antibodies (Cell Signaling Technology) at 1:100 dilution in permwash for 30 min on ice. The cells were washed several times and then stained with FITC-conjugated anti-rabbit fragmented antibody (Fab) (Caltag) for 30 min on ice. The cells were washed several times and analyzed using a Becton-Dickinson FACS Calibur™ to measure levels of

caspase-3 on a per cell basis. Dead cells were gated out on the basis of forward/side scatter. For each sample, 10^4 events were collected.

3.2.7 Western blotting

Cells were washed twice in cold PBS and lysed in Tris buffered saline containing 1mM EDTA, 1mM DTT, 0.2% triton, 0.1% SDS and the complete protease inhibitors cocktail (Roche). 30 μg of proteins were subjected to SDS-PAGE, and then transfered to PVDF membranes (Boehringer Mannheim). Membrane were probed with antibodies specific for caspase-3 (New England Biolabs), cleaved caspase-3 (New England Biolabs) or β-actin (Sigma) and incubated with the HRP-conjugated anti–rabbit Ig or anti-mouse Ig antibody. Signals were revealed with the enhanced chemiluminescence ECL kit (Amersham) and visualized by autoradiography.

3.3 Results

3.3.1 Selective increase in caspase-3 mRNA levels is an early event (G0/G1) during T cell activation

We investigated, using a real-time RT-PCR assay, the effect of T cell activation on the upregulation of caspase-3 mRNA levels to test the hypothesis that an upregulation is required to replenish the pool of procaspase-3, which is cleaved upon TCR triggering [14-17]. Thymocytes and lymph node T cells from BALB/c mice were activated by crosslinking the TCR with anti-CD3 in the presence of APCs for 48 hours and live (>90% AnV^-) T cells were sorted for quantification of mRNA levels by real-time RT-PCR. In three independent experiments performed on live-sorted T cells, caspase-3 mRNA levels increased as early as 6 hours post-TCR stimulation, peaking (13-fold) at 48 hours (Fig. 1 A). The increase occurred in two steps with an initial 6-fold increase reached between 24 and 36 hours followed by a subsequent 2.2-fold increase occurring at 48 hours. More than 90% of the cells showed the presence of the activation markers CD69 and CD25 at their cell surface, as determined by flow cytometry, demonstrating proper T cell activation (data not shown). This increase was selective to caspase-3 since caspase-8 mRNA levels remained unchanged. A similar albeit lower (3-fold) upregulation of caspase-3 mRNA levels was observed in sorted live (>90% AnV^-) thymocytes (Fig. 1 A) and in murine T cell hybridomas (Chapter 2).

The large majority of peripheral T cells are synchronized in the quiescent (G0) phase thus providing an opportunity to define the stages of the cell cycle in which caspase-3 mRNA levels are upregulated upon T cell activation. Results presented in Figure 1 A show that caspase-3 mRNA levels were already increased by 6-fold, after 24 hours of stimulation at a time when only 11% of cells had transited from the G1 to S phase of the cell cycle. At 48 hours, a further two-fold increase was reproducibly observed (n=3) in peripheral T cells, at a time when these cells were now actively dividing (43% S/G2/M) (Fig. 1 A). To further confirm that the increase in caspase-3 mRNA levels occurs before entry into the S phase of the cell cycle, T cell proliferation

was blocked using the late G1 cell cycle inhibitor, L-mimosine. Expression of early markers of T cell activation remained intact after 48 hours of stimulation in the presence of L-mimosine (>90% CD69+CD25+ cells) (Fig. 1 B) but led to a large decrease in the number of cells entering the S-phase of the cell cycle (9% S/G2/M) as compared to cells stimulated in the absence of the inhibitor (62% S/G2/M) (Fig. 1 C). Caspase-3 mRNA levels were upregulated by 11-fold, at 48 hours following anti-CD3 stimulation, a response which was slightly reduced to 7-fold in the presence of L-mimosine (n=3) (Fig. 1 C). Cells were then activated in the presence or absence of L-mimosine for 48 hours and sorted according to the G0/G1 and S/G2/M phases of the cell cycle. Sorted stimulated cells showed a 15-fold (G0/G1) and 4-fold (S/G2/M) induction in caspase-3 mRNA levels, relative to non-stimulated samples (Fig. 1 D). Activation of T cells in the presence of L-mimosine confirmed that the bulk of the increase occurred in the G0/G1 phases (10-fold increase). These results demonstrate that the majority of the increase in caspase-3 mRNA levels is mediated early during T cell activation in the G0/G1 phases of the cell cycle.

3.3.2 IL-2-independent increase in caspase-3 mRNA levels in activated T cells

The IL-2 cytokine plays an essential role in the early phases of T cell proliferation and in cell death at the termination of an immune response [30]. In order to determine if the observed increase in caspase-3 mRNA levels was dependent on IL-2 signaling, lymph node T cells were preincubated with the PC61 antibody directed against the IL-2 receptor α-chain (IL-2Rα) which blocks signaling through this receptor [31] and therefore proliferation (n=3). Initially we confirmed that at 48 hours after TCR engagement T cells had undergone one to three divisions. However, the presence of the IL-2R neutralizing antibody (PC61) completely blocked T cell proliferation as we could not observe any T cells which had altered their levels of CFSE (Fig. 2 A). The levels of caspase-3 mRNA were then determined by real-time RT-PCR (n=2). Following 48 hours of stimulation with anti-CD3, we observed a 10-fold increase in caspase-3 mRNA levels in cells treated with the PC61 antibody, compared to 15-fold in the absence of the IL-2Rα antibody (Fig. 2 B). These results show that activation of T cells by anti-CD3 in the absence of

exogenous IL-2 still results in a significant increase in caspase-3 mRNA levels formally demonstrating that the upregulation of caspase-3 mRNA is independent of IL-2 signaling and takes place very early during T cell activation. Overall, these results demonstrate that TCR engagement results in a selective and significant (at least 10-fold) increase in caspase-3 mRNA levels in peripheral T lymphocytes.

3.3.3 Ex vivo and in vivo antigen-specific induction of caspase-3 mRNA levels are selective to effector T cells

To determine the upregulation of caspase-3 mRNA expression *ex vivo* following the specific interaction of a TCR with its cognate peptide/MHC complex, lymph node T cells from 2C mice expressing a transgenic TCR with specificity for the SYRGL peptide restricted by the class I H-2K^b molecule, were activated with the SYRGL peptide. The levels of caspase-3 mRNA were monitored for 5 days following an initial stimulation with the SYRGL peptide, using the real-time RT-PCR assay. Transient upregulation of caspase-3 mRNA levels was observed, with a peak occurring within 48 hours after antigen-specific stimulation (15-fold increase) followed by a sharp drop and a return to steady state levels (3-fold relative to day 0) at 3 days post-stimulation (n=2, Fig. 3 A). In contrast, caspase-8 mRNA levels remained unchanged further confirming the selectivity of the increase in caspase-3 mRNA levels.

Experiments were also carried out *in vivo* to determine whether modulation of caspase-3 gene expression was selective to effector T cells, a subset destined to undergo AICD. P14 mice expressing a transgenic TCR with specificity for the LCMV glycoprotein restricted by the class I H-2D^b molecule were thus used to compare caspase-3 mRNA levels between naïve, effector and memory CD8 T cells [29;32;33]. Initially, Thy1.1+ P14 splenocytes were adoptively transferred into C57BL/6 (Thy1.2+) mice that were subsequently infected with LCMV-Armstrong [33]. LCMV-specific effector and memory CD8 T cells were then isolated 8 days (at the peak of the effector CD8 T cell response) and 40 days post infection, respectively. Naïve P14 CD8 T cells were obtained from the spleens of uninfected P14 mice. cDNA microarray analysis confirmed the

selective upregulation of caspase-3 mRNA levels, when compared to caspase-2,-6,-7,-8, and –9, in effector T cells. Caspase-3 mRNA levels were increased 3-fold in effector T cells relative to naïve T cells, while they remained unchanged in memory T cells (1.1-fold) (Fig. 3 B). Similar results were obtained using real-time RT-PCR on mRNA isolated from effector T cells. We observed a 3.2-fold increase in levels of caspase-3 mRNA levels in effector T cells and a 1.4-fold increase in memory T cells, relative to naïve T cells (Fig. 3 B). In contrast, the levels of caspase-8 mRNA were reduced in both effector and memory T cells as determined by cDNA microarray analysis (0.7-fold for effector T cells; 0.6-fold for memory T cells) which were later confirmed by real-time RT-PCR (0.5-fold for effector T cells; 0.6-fold for memory T cells).

3.3.4 Procaspase-3 and activated caspase-3 levels are increased in effector T cells

We next sought to determine whether the increase in caspase-3 mRNA levels, observed in LCMV-specific effector CD8 T cells directly *ex vivo*, translates to an increase in protein levels as we hypothesized, to maintain a constant pool of procaspase-3 required for effector T cells to rapidly undergo AICD. Mice were infected with LCMV and on days 4, 6, 8 and 65 after infection splenocytes were isolated and stained with a caspase-3 antibody that recognizes both the procaspase and cleaved forms. Early after infection, on days 4 and 6, the relative expression of caspase-3 in LCMV-specific effector cells had increased to nearly twice that found in naïve CD8 T cells (68 and 61 MF1 versus 25 MFI, respectively) or in LCMV-specific CD8 T cells at 8 or 65 days after infection (32 and 30 MFI, respectively) (Fig. 4 A). The amounts of caspase-3 in naïve or memory CD8 T cells (from day 65 p.i.) were near that of background based on staining with an isotype control antibody (Fig. 4 B). Thus, the expression of caspase-3 increases early during infection but returns to background levels after virus is cleared (day 8 p.i.) and this low level of expression is maintained in resting memory CD8 T cells found several months later (n=3). As seen in CD8 T cells activated *in vitro*, the increased expression of caspase-3 at days 4 and 6 p.i. most likely directly correlates with recent T cell receptor triggering because virus is present at these times but is cleared by day 8 p.i. Next, we examined whether caspase-3 expression increases upon secondary antigenic

stimulation of memory CD8 T cells. LCMV immune animals were reinfected with a highly virulent strain of LCMV, LCMV-cl. 13, and four days later we observed that the secondary effector CD8 T cells had increased amounts of caspase-3 (52 MFI versus 25 MFI in naïve T cells), similar to that found in the primary effector T cells at days 4-6 p.i. (Fig. 4 A and B). Thus, increased expression of caspase-3 in CD8 T cells normally occurs following T cell activation and effector T cell differentiation.

Next, we examined whether effector T cells demonstrated increased levels of the active forms of caspase-3 (p20 and p17), since they represent the T cell subset that is the most susceptible to apoptosis in contrast to memory T cells. Therefore, as described above, at days 4, 6, 8, 14 and 65 post LCMV infection we stained LCMV-specific CD8 T cells with an antibody that specifically recognizes the cleaved forms of caspase-3. The expression pattern of cleaved caspase-3 was very similar to that observed for total caspase-3. The majority of effector CD8 T cells found early during infection, at days 4 and 6 p.i., contained higher amounts of cleaved caspase-3 (137 and 90 MFI, respectively) than those found at days 8, 14 and 65 p.i. (65, 48 and 45 MFI, respectively) (Fig. 4 C and D). And, when memory CD8 T cells were reactivated by reinfecting LCMV immune animals with LCMV-cl. 13, the amount of cleaved caspase-3 increased in the secondary effector T cell population (96 MFI versus 53 MFI in naïve T cells) (Fig. 4 C). Interestingly, around days 6 and 8 p.i. we observed the accumulation of a small subset of T cells (~1%) that expressed very high levels of cleaved caspase-3, referred to as cleaved caspase-3^{hi} effector T cells, and these cells are most likely actively undergoing apoptosis [23]. This population of cleaved caspase-3^{hi} effector cells peaks between days 8-10 p.i. (data not shown and [23]) when the majority of effector CD8 T cell contraction occurs. Detection of apoptosing effector CD8 T cells directly *ex vivo* is difficult and generally underestimates the actual number of dying cells because these cells are rapidly engulfed by macrophages *in vivo* [34;35]. These results demonstrate that the upregulation of caspase-3 expression (mRNA and protein) is confined to effector T cells, a subset destined to undergo AICD, suggesting that this selective increase is part of a homeostatic response critical for the regulation of cell numbers.

We further confirmed the increase, both the full length and cleaved forms of caspase-3, in activated T cells by Western blot. Procaspase-3 levels were increased 6-fold in live (AnV^-) activated mature T cells 36 hours after TCR stimulation (Fig. 5 A). Consistent with several findings [14-17], caspase-3 was cleaved to its p20 and p17 form after 36 hours in non-apoptotic mature T cells (85% AnV^- cells) (Fig. 5 B). In contrast, there was no increase in procaspase-3 levels and no processing to its active form in live (88% AnV^- cells) activated thymocytes, which had demonstrated a lower induction in caspase-3 mRNA levels (3-fold) when compared to mature T cells (12-fold) (Fig. 5 A and B). These results support the hypothesis that the pool of procaspase-3 is increased and maintained following activation and expansion of effector T cells in response to TCR crosslinking.

3.3.5 Sensitivity to apoptosis correlates with the levels of caspase-3

The increase in caspase-3 expression during T cell activation suggests that caspase-3 plays a critical role in T cell homeostasis. Caspase-3 deficient peripheral T cells are less susceptible to AICD [27]. Therefore, it is likely that the increased expression of caspase-3 reported here is important for sensitizing activated T cells to apoptosis. To confirm this hypothesis, T cells were isolated from the lymph nodes of caspase-3 wildtype, heterozygous and knockout mice and put in culture for 2 days in the absence or presence of anti-CD3. After sorting for live (AnV^-) cells, the sensitivity of T cells to apoptosis was determined by treating the cells with etoposide or the PC61 antibody which lead respectively to DNA damage- or IL-2 withdrawal-induced cell death (Fig. 6).

Western blot analysis using lysates from activated T cells isolated from either wildtype, heterozygous or caspase-3 knockout mice confirmed the relative abundance of the proenzyme (Fig. 6 A). We next determined the sensitivity of activated T cells isolated from wildtype, heterozygous or caspase-3 knockout mice to etoposide- or IL-2 withdrawal-induced apoptosis. T lymphocytes isolated from caspase-3 knockout or heterozygous mice were significantly ($p<0.05$) more resistant to etoposide-induced apoptosis (70% ± 4 and 69% ± 2 AnV^- cells, respectively) when compared to T cells

isolated from wildtype mice (45% ± 7 AnV^- cells). Similar results were obtained following IL-2 withdrawal-induced apoptosis (n=3, Fig. 6 B). Furthermore, non-stimulated samples, where caspase-3 expression is not increased, were more resistant to etoposide- or IL-2 withdrawal-induced apoptosis when compared to anti-CD3 stimulated T cells (Fig. 6 B). These results demonstrate that the relative levels of caspase-3 in T cells correlate with their sensitivity to apoptotic stimuli and that this protease plays an important role in controlling cell numbers by eliminating the expanded population of effector T cells at the termination of an immune response.

3.4 Discussion

The selective increase in caspase-3 mRNA levels is a general consequence of TCR interaction with its cognate MHC/peptide complex as our findings were reproduced in three different strains of mice, several murine T cell hybridomas, in live polyclonally activated T cells and in live antigen-specific cells activated *ex vivo* and *in vivo*. The increase of caspase-3 expression (mRNA and protein) was specifically observed in effector T cells and not in memory T cells. In contrast, we did not observe any change in caspase-8 mRNA levels which highlights the importance of caspase-3 in the maintenance of T cell homeostasis. We have also shown an increase and processing of procaspase-3 in sorted live (AnV^-) activated T cells. Furthermore, *in vitro* activated T cells isolated from caspase-3 knockout mice were significantly more resistant than activated T cell isolated from wild-type mice, when cultured under conditions leading to apoptosis. These findings clearly demonstrate the importance of maintaining minimal caspase-3 levels to ensure efficient apoptosis in peripheral T cells, more specifically the elimination of effector T cells which are the targets of AICD.

The differences observed in the upregulation of caspase-3 following T cell activation in the absence or presence of L-mimosine (11-fold vs 7-fold) or PC61 (15-fold vs 10-fold) can be explained by the fact that both inhibitors prevent T cells from dividing and further engaging their TCRs. Figure 1 A clearly demonstrates a two step process in the upregulation of caspase-3 mRNA levels. The initial increase (6-fold) occurred when 89% of the cells were in the G0/G1 phase of the cell cycle (Fig. 1 A). Once T cells were cycling following stimulation (43% S/G2/M at 36 hours) and had further crosslinked their TCRs, a second upregulation in caspase-3 mRNA levels (2.2-fold) was observed. The use of L-mimosine and the PC61 antibody, which both arrest T cells at the G0/G1 phase of the cell cycle, confirmed that the initial increase in caspase-3 mRNA levels occurs very early following TCR engagement and prior to the engagement of the IL-2 receptor (Fig. 1 and 2). Overall, our results suggest that the increase in the levels of caspase-3 prior to cell division ensures that each effector T cell will have sufficient amounts of the proenzyme to rapidly undergo apoptosis in the event of an extracellular and/or intracellular signal

since caspase-3 is at the crossroad of both the extrinsic (death receptors) and intrinsic (mitochondria) pathways of apoptosis [36;37]. Indeed, our study using T cells from caspase-3 knockout mice shows a clear correlation between the levels of proenzyme and sensitivity to apoptosis (Fig. 6).

The upregulation in caspase-3 gene expression following TCR triggering in thymocytes was less important than in peripheral T cells and showed only a 3-fold increase after 36 hours of stimulation (Fig. 1 A). When thymocytes were incubated with anti-CD3 in the presence of IL-2, only 20% of the cells were viable (AnV^-) after 48 hours of stimulation (data not shown) as previously shown [38]. The live population showed surface expression of the early activation markers, CD69 and CD25, which indicated that the cells were indeed activated but are destined to undergo apoptosis rather than proliferation. Alam *et al.* have shown specific activation of caspase-3 during negative selection of T cells in the thymus and that the broad range caspase inhibitor zVAD-CH2F prevented apoptosis of thymocytes following TCR triggering demonstrating a caspase-dependent cell death [38;39]. However, caspase-3 does not appear to be essential for T cell development since there were normal CD4 and CD8 thymocyte populations from caspase-3 knockout mice [27;40].

Thymocytes from caspase-3 knockout mice and wild-type mice were equally sensitive to induction of apoptosis by anti-Fas, anti-CD3, ceramide, staurosporin and dexamethasone [40]. In contrast, peripheral T cells from caspase-3 knockout mice were less susceptible to AICD, anti-CD3-, anti-Fas-induced apoptosis [27], and etoposide- and IL-2 withdrawal-induced cell death (Fig. 6 B). Since thymocytes undergo apoptosis following the first stimulation through the TCR, these findings suggest that there is no requirement for the upregulation in caspase-3 levels in thymocytes to insure cell death. However, lymph node T cells demonstrated a 13-fold increase in caspase-3 mRNA levels and proliferated in response to TCR stimulation, demonstrating a requirement for the upregulation in caspase-3 levels in mature T cells to replenish the cleaved caspase-3 following TCR triggering (Fig. 1 A). Furthermore, Western blot analysis demonstrated that the levels of procaspase-3 were higher in thymocytes when compared to peripheral T

cells, further demonstrating that the levels of proenzyme determine the fate of a cell (Fig. 5 A). Since the cleaved forms (p20 and p17) of caspase-3 disappear rapidly through XIAP- and cIAP2-mediated proteosomal degradation [41;42], the transcriptional upregulation of caspase-3 is most probably responsible for the replenishment and maintenance of adequate levels of the procaspase-3 pool.

Tight regulation of the number of activated T cells is important for preventing autoimmunity and disease due to excessive inflammation. In response to an acute LCMV infection, virus-specific CD8 T cells undergo massive expansion, but following viral clearance ~90-95% of the antigen-specific T cells die. Therefore, the increase in caspase-3 expression observed in LCMV-specific effector CD8 T cells (Fig. 3 B and 4) may be an important determinant of the natural process of effector T cell elimination. Salvesen and Dixit suggested that a disruption in the balance of pro- and anti-apoptotic proteins constitutes a major factor in regulating an apoptotic threshold [43]. In agreement with this hypothesis the imbalance between high levels of caspase-3 (Fig. 3 B and 4) and the low levels of member of the bcl-2 family in effector T cells [20-23] is likely to be one of the major causes leading to their death by AICD. In contrast, antigen-specific memory CD8 T cells have elevated levels of bcl-2 [20;23] and reduced caspase-3 expression (this study), and this would contribute to the survival and persistence of memory T cells.

Based on the results presented in figure 4, it appears that the increased expression of cleaved caspase-3 occurs in two incremental steps— early after T cell activation, the expression of cleaved caspase-3 increases to an intermediate level in effector CD8 T cells, but then after several days of antigenic stimulation the expression of cleaved caspase-3 greatly increases in the effector T cells that are becoming apoptotic. The significance of the intermediate level of cleaved caspase-3 found in effector T cells early during infection is not clear, but perhaps a low level of caspase-3 activity is non-lethal and important for effector T cell function, expansion or differentiation as has been previously suggested [14-17;44]. Although other factors regulating apoptosis are involved in mediating death of activated T cells, our results support a model whereby the selective upregulation of caspase-3 levels upon engagement of the TCR contributes significantly in

tilting T cell homeostasis towards apoptosis during an immune response and the subsequent elimination of effector T cells.

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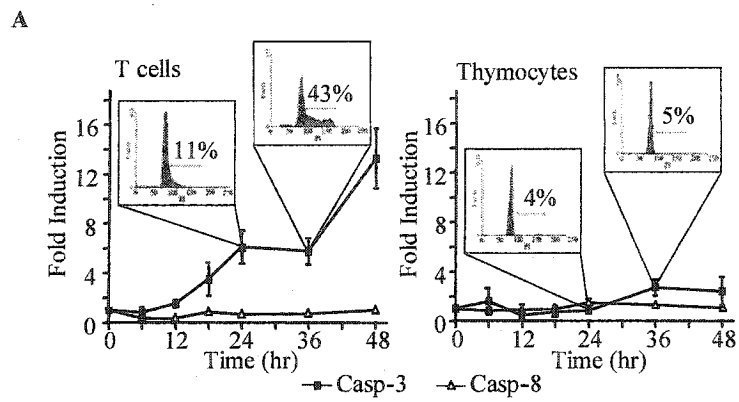
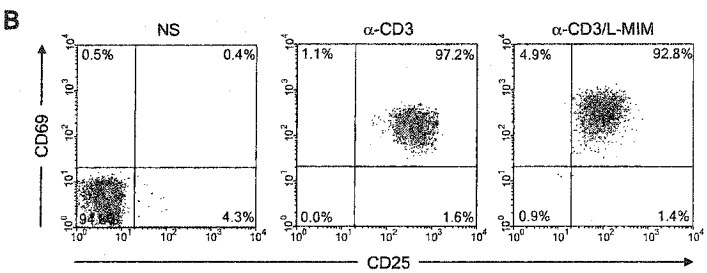
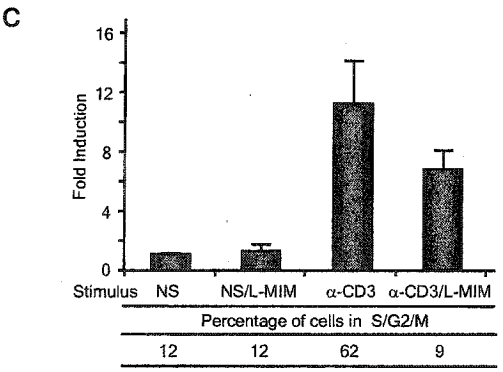

A
T cells
Thymocytes
Fold Induction
Time (hr)
11%
43%
4%
5%
Casp-3
Casp-8


B
NS
α-CD3
α-CD3/L-MIM
CD69
CD25
0.5%
0.4%
94.6%
4.3%
1.1%
97.2%
0.0%
1.6%
4.9%
92.8%
0.9%
1.4%


C
Fold Induction
Stimulus NS NS/L-MIM α-CD3 α-CD3/L-MIM
Percentage of cells in S/G2/M
12 12 62 9

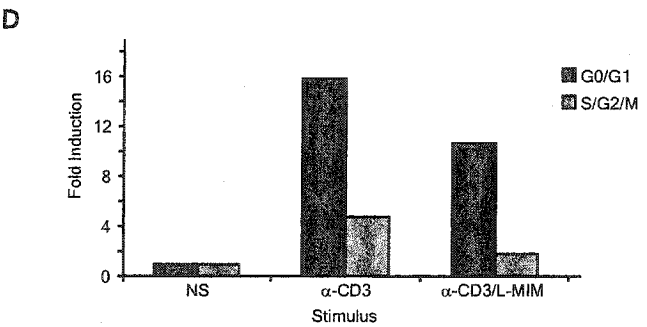

D
Fold Induction
G0/G1
S/G2/M
NS
α-CD3
α-CD3/L-MIM
Stimulus

Figure 3-1. Increase in caspase-3 mRNA levels in non-apoptotic activated T cells
(A) Peripheral T cells and thymocytes were stimulated with anti-CD3 in the presence of IL-2 for 48 hours. The fold induction of caspase-3 and -8 mRNA levels from live (>90% AnV^-) peripheral T cells and thymocytes, was determined by real-time RT-PCR (n=3). Proliferation was assessed by cell cycle analysis using PI. The percentage of cells in S/G2/M phase at 24 h and 36 h are representative of three independent experiments. (B) T cells were stimulated for 48 hours, in the presence or absence of L-mimosine (L-MIM) and activation was determined by surface expression of CD69 and CD25. (C) The fold induction in caspase-3 mRNA levels of live (AnV^-) activated T cells in the presence or absence of L-MIM was determined by real-time RT-PCR (n=3). The percentage of proliferating cells is shown as S/G2/M and is representative of three independent experiments. (D) T cells activated for 48 hours in the presence or absence of L-MIM were sorted for the G0/G1 and S/G2/M phases of the cell cycle, and the fold induction in caspase-3 mRNA levels was determined by real-time RT-PCR.

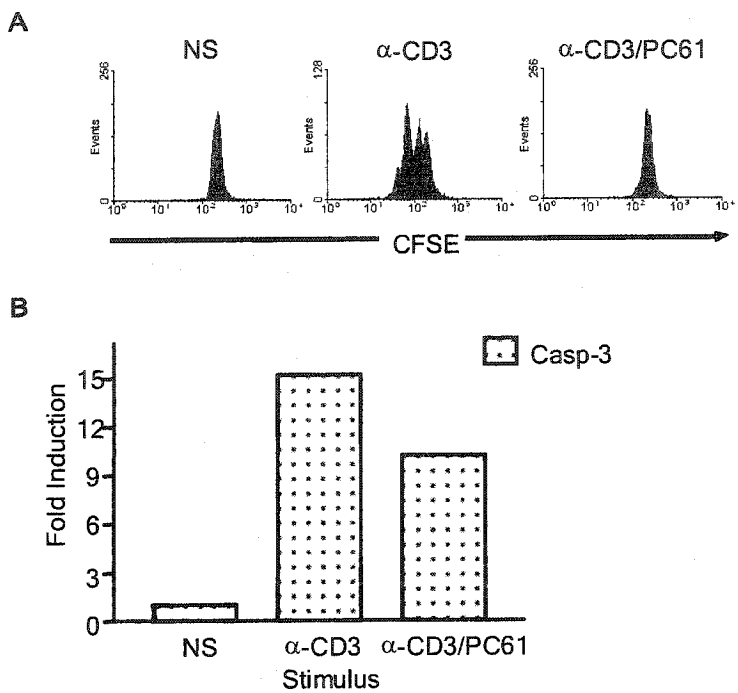
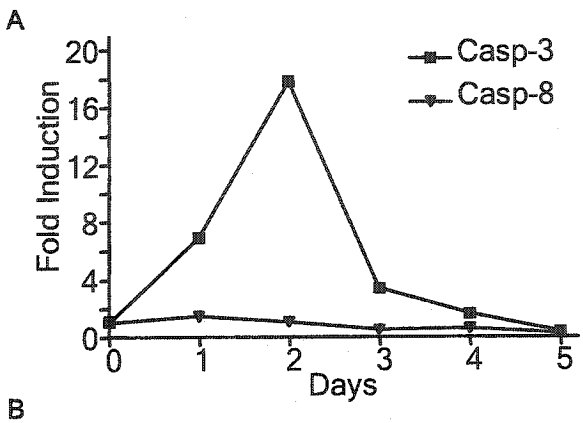
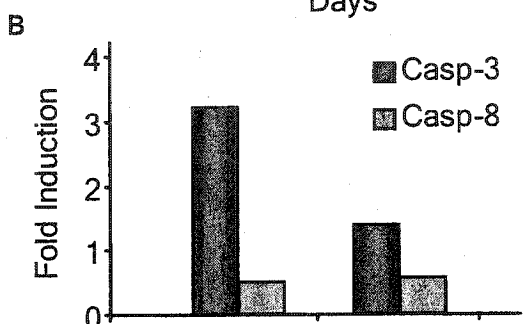


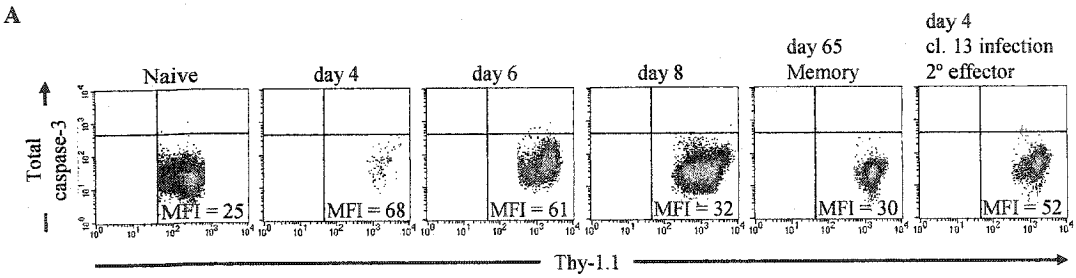
Figure 3-2. Induction in caspase-3 mRNA levels is independent of IL-2
(A) Lymph node T cells were stained with CFSE, incubated in the presence or absence of the PC61 antibody and stimulated for 48 hours with anti-CD3. The degree of cell proliferation was determined by flow cytometry analysis (n=3). (B) The fold induction of caspase-3 mRNA levels from peripheral T cells stimulated in A, was determined by real-time RT-PCR (n=2).

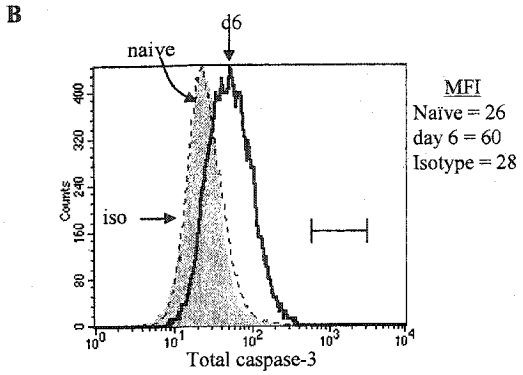


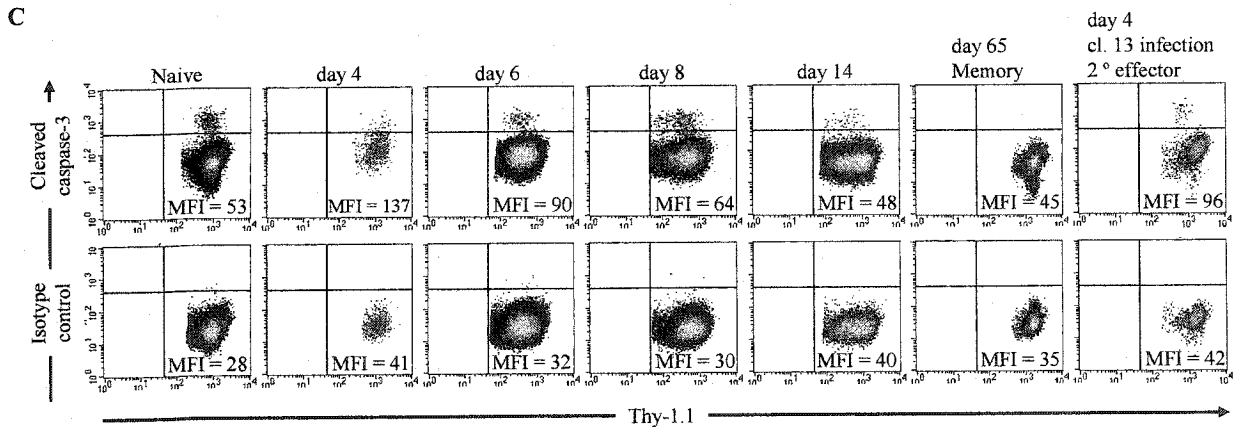


Casp	E/N	M/N
2	0.9	0.7
3	3.0	1.1
6	1.0	0.4
7	2.6	0.6
8	0.7	0.6
9	1.1	1.2

Figure 3-3. Effector T cells selectively upregulate caspase-3 mRNA
(A) Lymph node T cells from 2C mice were stimulated with the SYRGL peptide *ex vivo* for 5 days. The fold induction in caspase-3 and -8 mRNA levels was determined by real-time RT-PCR (n=2). (B) Total RNA was isolated from naïve, effector and memory P14 CD8 T cells and the fold induction in caspase-3 and -8 mRNA levels was determined by real-time PCR. mRNA levels of caspase-2, -3, -6, -7, -8 and -9 in effector (E) and memory (M) P14 CD8 T cells relative to naïve (N) P14 CD8 T cells, on cDNA microarrays from Affymetrix (U74A).


A
Naive
day 4
day 6
day 8
day 65
Memory
day 4
cl. 13 infection
2° effector
Total caspase-3
MFI = 25
MFI = 68
MFI = 61
MFI = 32
MFI = 30
MFI = 52
Thy-1.1


B
d6
naive
iso
Counts
Total caspase-3
MFI
Naïve = 26
day 6 = 60
Isotype = 28


C
Naive
day 4
day 6
day 8
day 14
day 65
Memory
day 4
cl. 13 infection
2 ° effector
Cleaved caspase-3
Isotype control
MFI = 53
MFI = 137
MFI = 90
MFI = 64
MFI = 48
MFI = 45
MFI = 96
MFI = 28
MFI = 41
MFI = 32
MFI = 30
MFI = 40
MFI = 35
MFI = 42
Thy-1.1

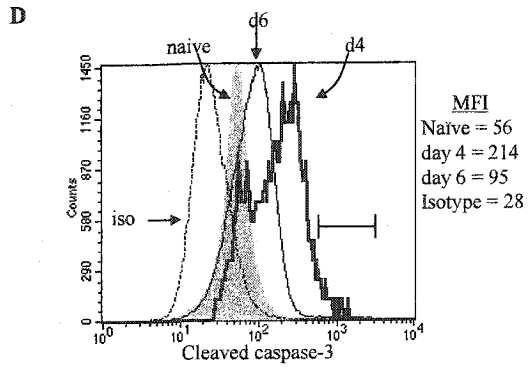

D
d6
naive
d4
iso
Counts
Cleaved caspase-3
MFI
Naïve = 56
day 4 = 214
day 6 = 95
Isotype = 28

Figure 3-4. Increased expression of procaspase-3 and active caspase-3 in early effector CD8 T cells during viral infection

Expression of total caspase-3 (A and B) and active caspase-3 (C and D) in LCMV-specific CD8 T cells during an acute viral infection. B6 mice containing Thy1.1$^+$ P14 CD8 T cells were infected with LCMV and on days 4, 6, 8, 14, and 65 after infection the level of caspase-3 and active caspase-3 expression in P14 CD8 T cells was examined. To analyze the expression of total caspase-3 and active caspase-3 during recall responses, LCMV immune animals were reinfected with LCMV-cl. 13 and four days later the secondary effector cell population was examined. (A and C) Dot plots showing total caspase-3 and active caspase-3 expression in Thy1.1$^+$ P14 CD8 T cells. The mean fluorescence intensity (MFI) for each cell population is shown. (B and D) Histograms displaying the total caspase-3 and active caspase-3 expression in Thy1.1$^+$ P14 CD8 T cells. Results shown are representative of three independent experiments.

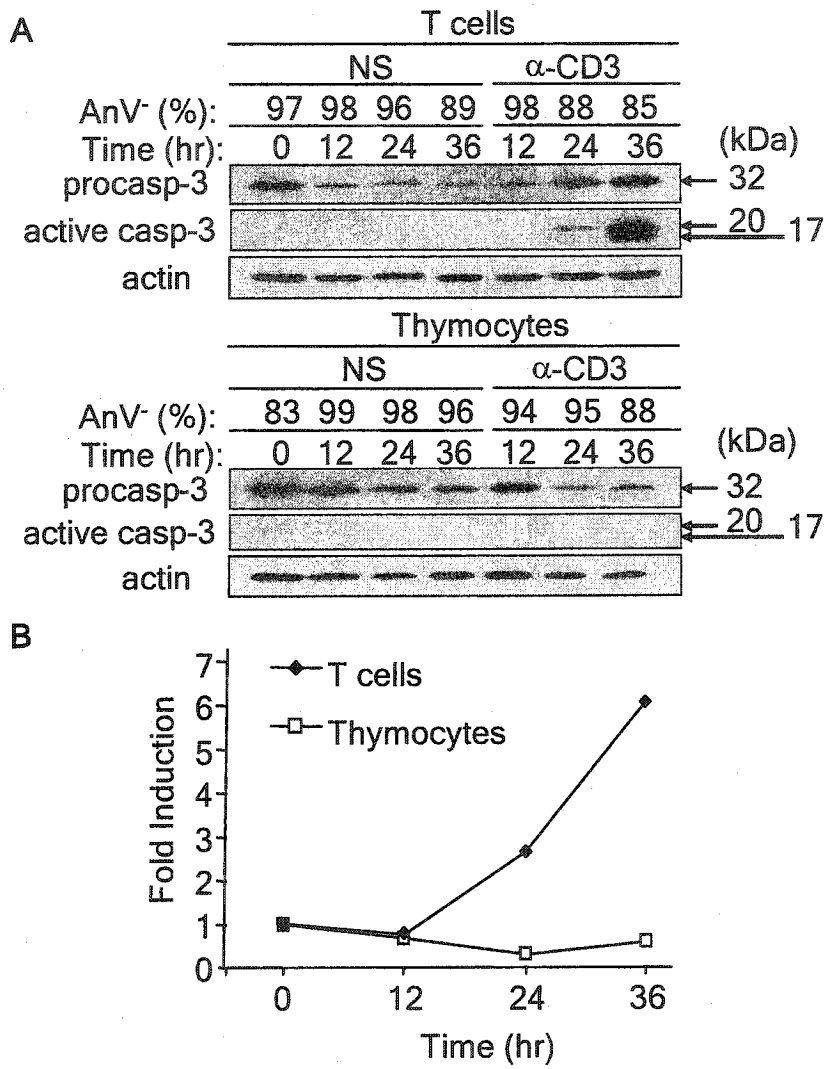


Figure 3-5. TCR stimulation leads to an increase in procaspase-3 levels in non-apoptotic activated T cells

(A) Cell lysates of non-stimulated (NS) and anti-CD3 (α-CD3) stimulated peripheral T cells and thymocytes, were analyzed by Western blot for the levels of procaspase-3 and the processing of procaspase-3 (n=2). The percentage of live cells (AnV^-) was assessed by AnV / PI staining. (B) Densitometry analysis was performed on the procaspase-3 Western blots presented in (A) and the fold induction was measured by standardizing sample loading to the actin signal and then normalizing to the NS sample for each corresponding time point.

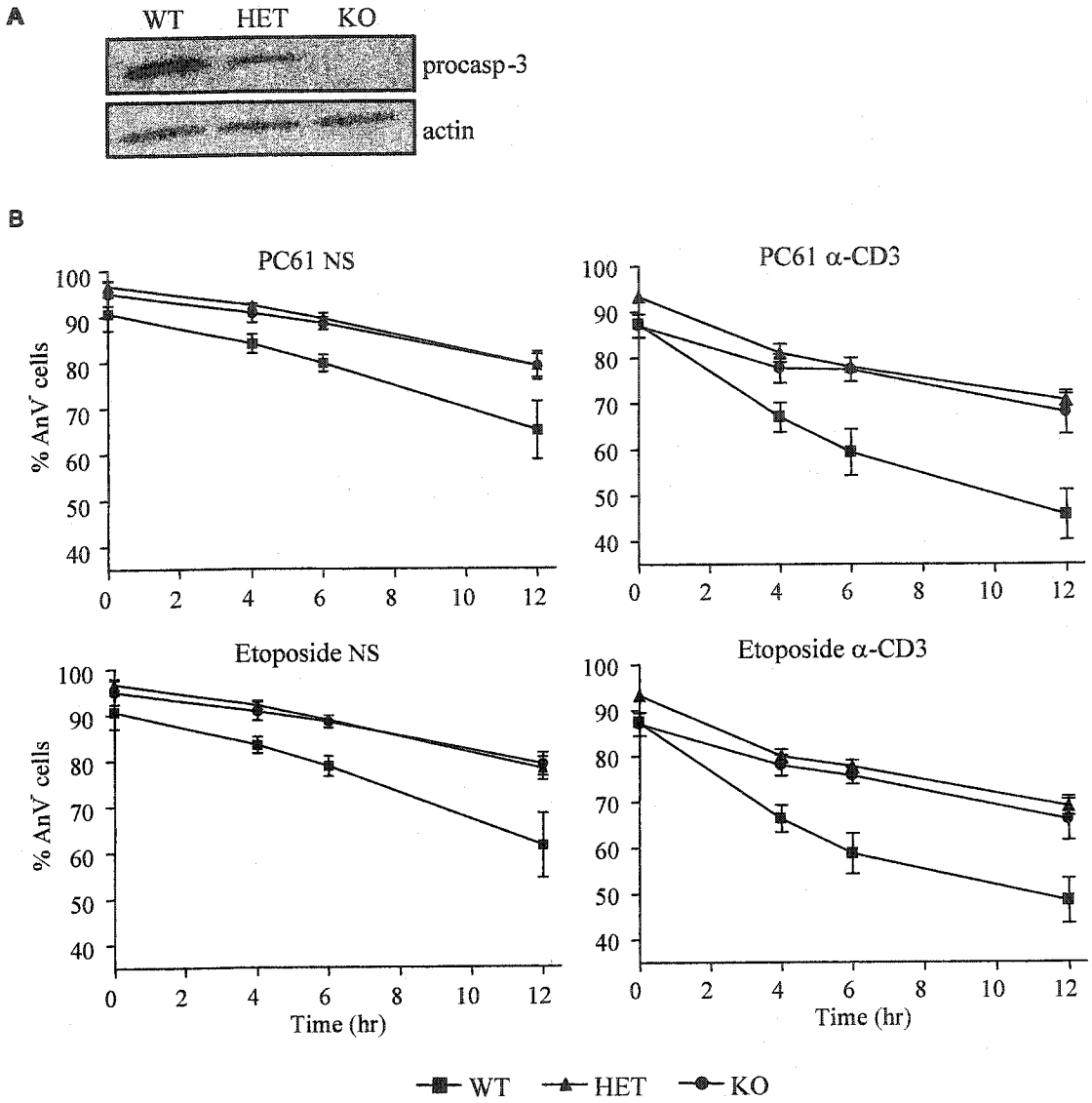


Figure 3-6. Caspase-3 expression correlates with sensitivity of T cells to apoptosis
(A) Cell lysates of activated T cells isolated from the lymph nodes of wildtype (WT), heterozygous (HET) and knockout (KO) caspase-3 mice were analyzed by Western blot for the levels of procaspase-3. (B) Annexin V negative cells of non-stimulated (NS) and anti-CD3 (α-CD3) stimulated peripheral T cells from WT, HET and KO mice were sorted after 2 days of culture. The cells were then treated with either 1 μM of etoposide or 50 μg/ml of the PC61 antibody in order to induce apoptosis. The percentage of AnV^- cells was assessed by AnV / PI staining (n=3).

Chapter 4: Cloning and functional characterization of the murine caspase-3 gene promoter during T cell activation

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Preface

In chapters 2 and 3 we demontrated that TCR crosslinking leads to the selective upregulation of caspase-3 mRNA and protein levels. Furthermore, the majority of the increase was confined to the G0/G1 phase of the cell cycle demonstrating that very early TCR signals are responsible for this increase. In this chapter we wanted to identify the transcription factors and regulatory regions within the caspase-3 promoter that are responsible for the observed increase in caspase-3 expression levels. Therefore, the caspase-3 promoter region was cloned, sequenced and characterized during T cell activation.

Abstract

We have previously shown that activation of T cells through the TCR leads to the upregulation of caspase-3 mRNA and protein levels which sensitized activated T cells to different apoptotic stimuli. These findings highlight the importance of regulating the expression of caspase-3 in order to prevent premature cell death. A segment of the 5' flanking region of the caspase-3 gene was cloned upstream of a luciferase reporter gene, in order to identify the transcription factors responsible for the induction in caspase-3 levels following T cell activation. Characterization of the -2245/+14 region of the caspase-3 promoter demonstrated that this fragment contained promoter activity. Higher luciferase expression was found with several of the deletion constructs in Jurkat T cells but not the mouse Neuro-2A neuroblastoma cell line, suggesting the presence of a T cell specific regulated region. Comparison of the mouse and human caspase-3 promoters revealed several highly conserved regions with putative transcription factor binding sites. Deletions of the promoter region lacking these sites were subcloned in an efficient retroviral reporter system and used to introduce these constructs in KOX-14 cells. Stimulation of these cells through the TCR did not show any change in reporter activity. These findings demonstrate that although the -2245/+14 region of the caspase-3 promoter shows constitutive levels of expression and that several regions appear to play a role in basal regulation, the sites responsible for increased expression in caspase-3 mRNA levels during T cell activation are not present within the sequences under investigation in this study. These regulatory elements could be present either upstream or downstream of the -2245/+14 fragment.

4.1 Introduction

Programmed cell death is a biological process that plays an important role in shaping the developing organism (Meier et al., 2000) and has been shown to participate in T cell homeostasis (Jameson, 2002). Disruption of several molecules involved in different apoptotic pathways lead to severe abnormalities, as was demonstrated by knockout mice models of Fas (Russell et al., 1993), FasL (Russell and Wang, 1993), caspase-3 (Kuida et al., 1996; Woo et al., 1998), caspase-9 (Hakem et al., 1998; Kuida et al., 1998) and Apaf-1 (Yoshida et al., 1998) and even lethal phenotypes in utero with gene targeting of cytochrome *c* (Li et al., 2000a), caspase-8 (Varfolomeev et al., 1998) and the adaptor protein FADD (Yeh et al., 1998). All these molecules are essential in mediating apoptosis either through TNF receptor family members (death receptors) or the mitochondria.

Caspases are the downstream effectors of apoptosis which are activated following trimerization of death receptors or loss of mitochondrial membrane potential. Interestingly, several studies have demonstrated an essential role of caspase-3 and -8 in activation induced cell death (AICD) of T cells and T cell homeostasis (Woo et al., 1998; Refaeli et al., 2002; Salmena et al., 2003; O'Neill et al., 2003). In addition to being involved in apoptosis, caspases have also been shown to be activated and required for T cell activation and proliferation in the absence of cell death (Miossec et al., 1997; Wilhelm et al., 1998; Alam et al., 1999; Kennedy et al., 1999; Boissonnas et al., 2002). These findings demonstrate a dual role for these proteases; they are implicated in expanding the T cell population to mount an efficient immune response as well as eliminating unwanted cells and controlling cell numbers (AICD).

The levels of caspases in different cell types have been shown to affect the fate of a cell and have been correlated in disease progression. Failure to induce the expression of caspase-3 in tumor cells treated with an apoptosis inducing agent lead to a decrease in sensitivity to cell death (Droin et al., 1998). Furthermore, it was demonstrated that breast cancer cells that were chemoresistant had downregulated their levels of caspase-3

(Devarajan et al., 2002). In contrast, increased levels of caspase-1 and -3 have been associated with Huntington disease (Chen et al., 2000) and amyotrophic lateral sclerosis (ALS) (Li et al., 2000b), in transgenic mouse models. Interestingly, an inherited genetic mutation of caspase-8, which renders it enzymatically inactive, resulted in defects in lymphocyte activation and human immunodeficiency (Chun et al., 2002). These reports highlight the importance of maintaining a minimal level of caspases to avoid the induction of pathologies.

Studies of several caspase promoters have identified transcription factors that are involved in the regulation of the rat caspase-3 (Liu et al., 2002) and the human caspase-8 genes (Liedtke et al., 2003). The SP-1 and Ets-like transcription factors were found to be responsible for sustained basal transcriptional activity of both the caspase-3 and -8 genes (Liu et al., 2002; Liedtke et al., 2003). Furthermore, the p53 transcription factor was absolutely required for the induction of caspase-8 promoter activity following adenoviral-induced apoptosis (Liedtke et al., 2003). However, E1A induced apoptosis was shown to increase the expression of caspase-3, -7, -8 and -9 but through a p53-independent mechanism (Nahle et al., 2002).

All prior studies demonstrating an increase in caspase-3 mRNA levels were shown in apoptotic cells. We have previously shown that caspase-3 is selectively increased during the activation of mature T cells in the absence of apoptosis. This increase appeared to be selective to caspase-3 since it was the only caspase to be induced in *in vitro* and *in vivo* stimulated naïve T cells. The significance of this increase was demonstrated in T cells isolated from caspase-3 knock-out mice which were considerably more resistant to different apoptotic stimuli following TCR stimulation. The levels of caspase-3 determine the fate of activated T cells since we observed higher levels of caspase-3 mRNA in effector T cells a subset destined to undergo AICD, in contrast to long lived memory T cells which had significantly less caspase-3 mRNA.

In order to identify the transcription factors responsible for the increase in caspase-3 levels during the activation phase of T cells through the TCR, approximately

2.2 Kb of the 5'-flanking region of the murine caspase-3 gene was cloned upstream of the luciferase reporter gene. Bioinformatics analysis revealed the presence of several putative transcription factor binding sites within the promote region that are responsive to T cell activation. Serial deletions from the 5'-end of the promoter demonstrated regions that may be responsible for basal regulation of caspase-3 mRNA levels. Furthermore, deletions of two specific regions within the promoter lead to significant changes in luciferase expression in a T cell line but not in neuroblastoma cells suggesting the presence of elements that are regulated in T cells only. We have previously shown an induction in caspase-3 mRNA levels in the KOX-14 murine T cell hybridoma following anti-CD3 stimulations. However, when the different deletions of the caspase-3 promoter were introduced into the KOX-14 cells no changes in reporter activity were observed. Although the 2.2 Kb region of the caspase-3 promoter is constitutively active, elements required for the induction in caspase-3 mRNA levels upon T cell activation may involve elements outside the 5'-regulatory region.

4.2 Materials and Methods

4.2.1 Cell Cultures

The human T cell leukemia cell line, Jurkat clone E6.1, was grown in RPMI medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate adjusted to contain 10 mM HEPES, 1.0 mM sodium pyruvate, and 10 % fetal calf serum (FCS). The mouse neuroblastoma cell line, Neuro-2A, was grown in minimum essential medium adjusted to contain 1.0 mM sodium pyruvate, 2 mM L-glutamine, and 10 % FCS.

4.2.2 Screening of the genomic library

A 350 bp fragment of the murine caspase-3 gene, amplified from mouse cDNA using rat specific and degenerate caspase-3 primers (5'-GGGATCCAAGCACTGGAATGTCAGCTCGCAATGG-3' and 5'-GGGATCCACG(AG)CA(ATCG)GC(TC)TG(ATG)AT(ATG)AT(ATG)A-3') was used as a probe for screening a lambda GEM12 murine ES DNA library (kindly provided by Dr. Christophe Benoit) and yielded clone I (Fig. 1A). In order to identify a genomic clone containing exon 1 and upstream regulatory sequences (clone II; Fig. 1A), another PCR product was generated spanning the murine caspase-3 exon 1 and part of intron 1 (5'-CTCTAGATGGAAGAAGTTGGGTCCACAAGAGGTG-3' and 5'-CTCTAGATGGTGGGATCAAAGCGCAGTGTCCTCG-3') and used to screen the same library. In order to complete the sequence between exon 1 and exon 2, we used nested PCR on genomic DNA from ES cells with the following primers: first PCR 5'-TCAAACCTCGCAACACATTCAC-3' and 5'-AGTTTGTTATCATCACGGCG-3'; nested PCR 5'-ATAAGAATGCGGCCGCCATCTGACTGTCTGAGGCCA-3' and 5'-TATCTAAGCGGCCGCAATCAAGAAGGCAGCAAGGA-3'. All PCR products were cloned into pBluescript and confirmed by sequencing before using as probes.

4.2.3 Primer extension assays

Primer extension assays were performed according to Current Protocols in Molecular Biology on 10 μg of total RNA from KOX-14 cells using the following primer: 5'-AGGACACTGCGCTTTGATCCCACCAGCACC-3'. The products of reverse transcription reactions were resolved on a 6% denaturing polyacrylamide gel. Sequencing reactions were used as ladders.

4.2.4 RNase protection assays (RPA)

A DNA fragment containing exon 1 and the putative promoter region was generated by PCR and introduced into the Ecl136 II site of pBluescript creating pBST7F1MB1.1. To generate the anti-sense probes, 1 μg of pBST7F1MB1.1 linearized with Eag I and pBSCasp3-T7 linearized with EcoR I (which contains the complete open reading frame of the murine caspase-3 gene) were used with the appropriate polymerase (T3 or T7). The actin probe was obtained from Pharmingen. RPAs were performed as recommended by Pharmingen. The protected fragments were resolved on a 6% denaturing polyacrylamide gel. Sequencing reactions were used as ladders.

4.2.5 Generation of murine caspase-3 promoter deletions

Initialy, the -2245/+14 construct was cloned upstream of the luciferase reporter gene in the pXP2 vector. A double digest with Spe I and Sac I restriction endonucleases was performed on 10 μg of purified DNA generating 5'- and 3'-overhangs. The digested sample was then treated with 100 U of exonulcease III (NEB), removing samples at selected time intervals and placing the aliquots on dry ice. Tubes are then heated at 68°C for 15 min and then placed on ice for 5 min. Then 3 U of mung bean nuclease (NEB) was added to the deletion products and incubated at 30°C for 30 min. Finally, the deleted clones were religated, transformed into *E. coli* DH5α, and screened for appropriate sized deletion products. All purification procedures were done by phenol/chloroform extraction.

4.2.6 Transient transfections

The QIAGEN Superfect transfection reagent was used for transient transfection of Jurkat and Neuro-2A cells which were all performed in 6 well plates. Exponentially growing Jurkat cells (5×10^6) were washed with 1X PBS and resuspended in 4 ml of supplemented RPMI medium (see above). 5 μg of reporter construct and 1 μg of the pRSV-βgal standardizing vector were used with 10 μl of Superfect reagent. Neuro-2A cells (2×10^4) were seeded a day before, and the next day transfected with 2 μg of reporter construct and 200 ng of the pRSV-βgal standardizing vector with 4 μl of Superfect reagent. Luciferase and β-galactosidase activities were assessed 48 hours post-transfections.

4.2.7 Luciferase and β-galactosidase reporter assays

After removing the growth medium and washing transfected cells with 1X PBS, Jurkat and Neuro-2A cells were lysed with 100 μl and 200 μl of 1X reporter lysis buffer (Promega), respectively. A freeze/thaw cycle at -80°C was performed to insure proper lysis. For the determination of luciferase activity, 20 μl of room temperature cell extract was mixed with 100 μl of room temperature Luciferase Assay Reagent (Promega) and luminescence was then measured for 10 sec in a luminometer. For the determination of β-galactosidase assay, 30 μl of cell extract was added to a solution of 100X Mg solution (0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol), 1X ONPG (4 mg/ml) and 0.1 M sodium phosphate (ph 7.5). The β-galactosidase reaction was performed at 37°C for 1 hr for Jurkat cells and 10 min at room temperature for Neuro-2A cells. Reactions were stopped by adding 500 μl of 1 M sodium carbonate solution. Optical densities of each sample were determined at a wavelength of 420 nm.

4.2.8 Retroviral reporter system

The hCD4-pA-GFP-IL-2p reporter construct (IL-2 control vector) was kindly provided by Dr. Kenneth M. Murphy (Washington University School of Medicine, St. Louis, MO) (Zhu et al., 2001). The RV10-x caspase-3 retroviral-based reporter constructs were constructed as follows. The pMSCVneo vector (Clontech) was digested with BamH I / Xho I, treated with Klenow and religated, eliminating the PGK-neomycin insert and generating pMSCV. Then an insert consisting of the first two domains of human CD4 (hCD4) fused to a glycine-glycine-serine-serine spacer and the transmembrane and cytoplasmic domains of hemagglutinin with the 3'-untranslated region of the rabbit β-globin gene (kindly provided by Dr. Nathalie Labrecque, Hopital Maisonneuve Rosemont, Montreal, Quebec, Canada) was introduced into the EcoR I site of pMSCV in the same orientation as the 5'-LTR, generating pMSCVhCD4. A cassette consisting of different caspase-3 promoter constructs upstream of the d2EYFP gene (from pd2EYFP-1 from Clontech) were digested with Sph I / Sal I and introduced on the bottom strand of pMSCVhCD4 digested with Hpa I / Sal I, generating RV10-x.

4.2.9 Infection of KOX-14 cells and T cell activation

Vesicular stomatitis virus envelope G glycoprotein (VSV-G) pseudotyped retroviruses were generated by co-transfecting BOSC 23 cells (Pear et al., 1993) with 20 μg of retroviral reporter vector and 20 μg of VSV-G expression plasmid SVCMV-VSV-G (Yao et al., 1998) by the calcium phosphate precipitation method. Retrovirus containing BOSC cell supernatant was collected 48 and 72 hours after transfection, supplemented with 10 μg/ml of polybrene and 0.01M HEPES pH 7 and used to spin infect KOX-14 cells (two rounds 24 hours apart) at 2200 rpm for 90 minutes at room temperature. The levels of infections were determined by staining cells for hCD4 surface expression with a PE-conjugated anti-human CD4 antibody (Pharmingen). KOX-14 cells were cultured in 6-well plates at 5 X 10^6 cells per well and stimulated with 1 μg/ml of immobilized anti-CD3 antibody. Reporter expression (GFP or d2EYFP) of stimulated and unstimulated KOX-14 cells was analyzed on a FACSCalibur (BD Biosciences).

4.3 Results

4.3.1 Cloning of the mouse caspase-3 gene and 5'-flanking sequences

PCR-based screening of a lambda GEM12 murine ES cDNA library resulted in the isolation of one positive clone (Fig. 1A). The isolated clone contained a portion of the first intron and exons 2 to 7 of the murine caspase-3 gene. In order to identify 5'-flanking sequences of the caspase-3 gene further PCR analysis, on the same cDNA library, was performed using primers specific for exon 1 and intron 1 which identified a second independent clone (II). Additional screening by PCR of the genomic library was required to complete the sequence between the two isolated clones which corresponded to approximately 5 Kb of intron 1 (Fig. 1A). All PCR amplified fragments were cloned into pBSKS+ and sequenced. The genomic organization of the murine caspase-3 gene revealed that it consists of seven exons divided by six introns with the ATG start codon mapping to exon 2. A 12 Kb intron separates the first non-coding exon and exon 2 and the whole gene spans approximately 28 Kb (Fig. 1B).

4.3.2 Identification of the caspase-3 transcriptional start site and organization of the 5' region of the caspase-3 gene

To identify the transcriptional start site in the caspase-3 gene, we employed both primer extension and RNase protection assays using mRNA isolated from the KOX-14 murine T cell hybridoma. The primer extension reaction gave rise to a product corresponding to a sequence that starts at the guanine nucleotide 18 bp within exon 1 extending toward the 5'-flanking region of the caspse-3 gene. However, RNase protection assay gave rise to two transcriptional start sites corresponding to an adenine nucleotide 7 bp within exon 1 and a guanine nucleotide 36 bp within exon 1 (Fig. 2).

Several features of the nucleotide sequence upstream of the transcriptional start sites of the caspase-3 gene are notable. First, the putative promoter lacks an apparent TATA-box but contains the typical mammalian promoter consensus element CCAAT at -

114 (numbering based on the start site identified by primer extension as being +1). Second, the 5'-flanking region contains several putative binding sites for transcription factors such as nuclear factor κB (NF-κB), AP-1, and p53 among many other conserved sites (Fig. 2). Third, multiple GC box motifs that are bound by Sp-1 and related transcription factors have been identified which are found within a CpG island (Fig. 1C and 2). As was demonstrated with other promoters, CpG islands typically lack TATA core promoter elements which is consistent with our findings (Brandeis et al., 1994; Macleod et al., 1994; Delgado et al., 1998). Similar characteristics were observed with the rat caspase-3 core promoter region (Liu et al., 2002).

4.3.3 Functional analysis of the mouse caspase-3 5'-flanking region

In order to determine whether the DNA upstream of the transcriptional start site contained promoter activity we subcloned a 2259 bp fragment of 5'-flanking caspase-3 region into the pXP2 reporter plasmid encoding the firefly luciferase (Fig. 1C). This segment of DNA contains the transcriptional start site identified by primer extension (designated +1), a second upstream start site identified by RPA (at -11) and the region between positions -2245 and +14 of the putative caspase-3 promoter region (Fig. 3). Furthermore, serial deletion mutants of the 5'-flanking region were derived by 5'-exonuclease digestion, intra-molecular deletions using unique restriction sites within the putative promoter region and PCR amplification of products of different sizes thus generating deletion constructs spanning the 2259 bp fragment at approximately 100-200 bp intervals. Additionally, since promoters only function in the correct orientation the same 2259 bp fragment was cloned upstream of the luciferase reporter gene but in reverse orientation (-2245/+14R) as a control (Fig. 3).

The deletion constructs were then transiently transfected into human Jurkat T cells or mouse Neuro-2A cells, which normally express caspase-3. The full length construct (-2245/+14) demonstrated similar levels of luciferase reporter activity in both the Jurkat (4.7 ± 1.4) and Neuro-2A (3.7 ± 0.4) cell lines, when normalized to the activity of the minimal promoter construct -107/+14. The promotorless pXP2 vector and the -

2245/+14R control construct demonstrated almost no luciferase expression in both cell lines (Fig. 3). These findings demonstrate that the 2245 bp upstream of the caspase-3 transcriptional start site do indeed contain promoter activity.

Progressive deletions from the 5'-end of the -2245/+14 construct revealed that the essential regulatory element(s) necessary for basal luciferase expression is located within a 157 bp region between nucleotide -1275 and – 1118 (Fig. 3). Deletion of this region resulted in a decrease in the relative expression of luciferase which was below the levels of the shortest construct -107/+14. Further deletion of a region between nucleotide -1118 and -1072 led, however, to a moderate increase in promoter activity, suggesting the presence of a negative regulatory element(s) within the -1118/-1072 region and additional positive elements within the region between nucleotide -1072 and -267 (Fig. 3). These variations in luciferase expression upon deletion of the different putative regulatory regions were observed in both the Jurkat and Neuro-2A cell lines suggesting that some elements are present within these sequences for basal levels of expression of caspase-3 in various tissues.

4.3.4 The upstream caspase-3 region contains lymphoid-specific promoter activity

Although deletion of different regions within the caspase-3 promoter increased or decreased luciferase expression in both transiently transfected cell types, significant differences in the levels of promoter activity can be noted between the two cell lines. In addition to the region that appears to mediate negative regulatory functions on the caspase-3 promoter between nucleotide -1118 and -1072 in both cell types there is a second segment between nucleotide -1776 and -1695 that has similar effects on promoter activity but only in Jurkat cells. Deletion of this region increased luciferase expression from 4.7 ± 1.4 fold to 9.5 ± 1.3 fold in Jurkat cells relative to -107/+14. However, deletion of the same region between nucleotide -1776 and -1695 had no effect on promoter activity in Neuro-2A cells (Fig. 3).

The full length construct -2245/+14 had similar reporter activity in Jurkat and Neuro-2A cells. Besides -1118/+14, the different deletions showed levels of luciferase expression that varied between 2 to 5-fold relative to -107/+14 in the neuroblastoma cell line. In contrast, variations of 3 to 9.5-fold were observed in Jurkat T cells relative to -107/+14, with the deletions between -1776/+14 and -1368/+14 having significantly more promoter activity (5.5 to 9.5-fold) than Neuro-2A cells (1.5 to 3-fold) (Fig. 3). Deletion of the segment between nucleotide -1368 and -1275 reduced luciferase expression (4.2 ± 0.3) to about the same levels as the full length construct -2245/+14 (4.7 ± 1.4) in Jurkat cells with only minor differences observed in Neuro-2A cells.

Analysis of the region between nucleotide -1368 and -1275 revealed several putative consensus binding sites for transcription factors known to be activated in TCR stimulated T cells (Kuo and Leiden, 1999). Conserved sites for AP-1 (c-Jun/c-Fos) were identified along with a GATA consensus sequences and NF-κB sites. Furthermore, a STAT-1 and several NFAT binding sites were identified in the region between nucleotide -2245 and -871 which could explain why most of the constructs generated within this region always showed significantly more promoter activity in Jurkat cells than Neuro-2A cells (Fig. 3). These findings demonstrate the presence of a regulatory region within the caspase-3 promoter that appears to be specific to T cells.

4.3.5 Bottom-strand retroviral reporter system

We have previously shown that activation through the TCR induces a selective upregulation of caspase-3 mRNA levels in KOX-14 cells and in *in vitro* and *in vivo* activated mature T cells. Therefore, we wanted to identify the regulatory element(s) and transcription factors acting on the caspase-3 promoter during T cell activation, using the different luciferase reporter constructs. However, transient transfection experiments in the KOX-14 T cell hybridoma line did not yield satisfactory results since they were refractory to most transfection methods tested yielding very low transfection efficiencies and almost undetectable levels of luciferase expression.

To introduce the constructs into KOX-14 cells a bottom-strand reporter system based on delivery by recombinant retrovirus was used (Zhu et al., 2001). In contrast to the luciferase reporter system that requires lysis of the cells to determine the activity of promoter constructs, the retroviral vector that was designed allows a more convenient analysis at the single-cell level by FACS. The retrovirus expresses a portion of the extracellular domains of human CD4 (first and second domains) lacking the cytoplasmic signaling domain under the control of the murine stem cell virus (MSCV) long terminal repeat (LTR), while d2EYFP expression is controlled by different deletion constructs of the caspase-3 promoter inserted onto the retroviral bottom strand (Fig. 4A). The d2EYFP gene encodes a destabilized yellow-green variant of enhanced green fluorescent protein (EGFP) fused with residues of mouse ornithine decarboxylase which contain a PEST amino acid sequence that targets the protein for degradation and results in rapid protein turnover. Because of these modifications d2EYFP has a half-life of approximately two hours which provides a more accurate assessment of caspase-3 promoter activity *in vivo* over time.

As a positive control, a similar reporter system was used where the IL-2 promoter drives the expression of GFP on the retroviral bottom strand (hCD4-pA-GFP-IL-2p reporter construct) (Fig. 4B) (Zhu et al., 2001). The levels of GFP, determined by flow cytometry, are fairly low in unstimulated KOX-14 cells infected with the IL-2 control vector (KOX-14-IL-2-GFP cells) since the IL-2 promoter demonstrates very low activity under resting conditions. However, when KOX-14-IL-2-GFP cells are stimulated with anti-CD3 for 12 hours the mean fluorescence intensity (MFI) of GFP shifts from 10.48 to 125.20 MFI (Fig. 4B). These results demonstrate that this novel reporter system allows normal inducible promoter activation upon T cell activation through the TCR.

4.3.6 Conserved transcription factor binding sites between the mouse and human caspase-3 promoters

Rather than subclone all 17 deletion constructs of the caspase-3 promoter into the retroviral vector, bioinformatics analysis was performed to compare the human and

murine 5'-flanking sequences of the caspase-3 gene to identify regions that appear to be conserved in both promoters and which contain putative transcription factor binding sites. The human and mouse sequences were aligned using the VISTA software (www-gsd.lbl.gov/vista). Analysis of the regions showing high degrees of homology between the human and murine sequences revealed several highly conserved transcription factor binding sites that could potentially bind molecules regulating the promoter activity of the caspase-3 gene, using the web-based software TRANSFAC (transfac.gbf.de/TRANSFAC).

Within the -2245/+14 construct five putative NFAT binding sites, one E2F, Myc and STAT conserved regions and three possible p53 sites were identified and appear to be conserved between both the human and the mouse promoter regions (Table 1). The deletion consisting of the -1695/+14 construct does not contain the two most distal 5' NFAT binding sites, the -1472/+14 in addition lacks the E2F regulatory region, and the -871/+14 contains only three p53 and one NFAT binding sites within the promoter region of the murine caspase-3 gene (Table 1). All four of these deletion mutants were cloned upstream of the d2EYFP gene on the bottom strand of the retroviral vector (RV10-x, Fig. 4A). In addition, the -757/+14 and -107/+14 regions were introduced into the retroviral reporter vector as additional controls, since the former does not contain any conserved transcription factor binding sites between the human and the mouse caspase-3 promoter regions and the latter consists of the basal promoter that was used to normalize the transient transfections using the Luciferase reporter system (Table 1). Finally, a construct lacking any region of the caspase-3 promoter was also generated.

4.3.7 The -2245/+14 region of the caspase-3 promoter is not induced by TCR stimulation

All the retroviral reporter constructs were co-transfected with a vector expressing the glycoprotein of Vesicular Stomatitis Virus (VSV), which allows for more efficient infections (Yao et al., 1998), into the BOSC 23 packaging cell line. Viral supernatants were used to perform two rounds of infections to introduce the reporter system into the KOX-14 T cell hybridoma. The levels of GFP and d2EYFP were determined by FACS

following the stimulation of KOX-14-IL-2-GFP and KOX-14-RV10-x, respectively (Fig. 5A). As shown in figure 4B, the MFI of GFP increases in KOX-14-IL-2-GFP cells following T cell activation. Unlike the IL-2 promoter which shows very low levels of activity under resting conditions, the caspase-3 promoter is constitutively active as shown by the basal levels of d2EYFP in the absence of anti-CD3 (RV10-2245/+14). Furthermore, RT-PCR and Western blot analysis demonstrated the presence of mRNA and proenzyme in non-stimulated KOX-14 cells, respectively (data not shown).

In order to identify the TCR responsive element(s) within the caspase-3 promoter, KOX-14 cells infected with the different retroviral constructs were cultured in the presence or absence of anti-CD3 for 12 hours. In contrast to the positive control IL-2-GFP (Fig. 4B), the reporter constructs containing the full length and deletions within the caspase-3 promoter region did not demonstrate any changes in d2EYFP MFI 12 hours following TCR crosslinking (Fig. 5A). To rule out the possibility of an earlier activation of the caspase-3 promoter by TCR signaling during the stimulation, the levels of d2EYFP were determined at different time intervals. While the levels of GFP under the control of the IL-2 promoter started to increase between 2 to 4, there were no changes in the levels of d2EYFP under the control of the different caspase-3 promoter deletions (Fig. 5B). Although we have previously shown that T cell activation induces a selective upregulation in caspase-3 mRNA levels, the regulatory region responsive to TCR stimulation is not present within the -2245/+14 5'-region of the caspase-3 gene. In summary, the activity of the caspase-3 reporter construct shows constitutive activity that is unaffected by T cell activation.

4.4 Discussion

We have previously shown in KOX-14 cells and in live activated mature T cells isolated from the lymph nodes of mice that caspase-3 expression is selectively induced following TCR stimulation. Furthermore, an increase in the levels of procaspase-3 and subsequent activation of the protease sensitized activated T cells to different apoptotic stimuli. Earlier studies have also demonstrated up-regulation of caspase-3 gene expression under different conditions leading to apoptosis (Droin et al., 1998; Dai and Krantz, 1999; Chen et al., 1998; Ni et al., 1998; Kumar et al., 1997). In the present work, we isolated and functionally characterized the 5'-flanking region of the murine caspase-3 gene to elucidate the regulatory mechanisms responsible for the observed increase in caspase-3 gene expression during T cell activation.

Structural analysis revealed that the proximal 5'-flanking region of the caspase-3 gene lacked a TATA box but had a CpG island which is typical of TATA-less promoters (Delgado et al., 1998; Macleod et al., 1994). In addition, the core promoter included a CAAT-box and multiple putative transcription factor binding sites. Furthermore, a cluster of transcription start sites was identified and several Sp-1 binding sites were found, by bioinformatics analysis, upstream of the transcriptional initiation start sites within the CpG island (Fig. 2) (Brandeis et al., 1994). A similar organization was shown for the core promoter of the rat caspase-3 gene (Liu et al., 2002). The mouse and rat caspase-3 promoters are 83% identical and one study demonstrated an identity of 61.7% between the 5'-flanking sequence of the human gene and the rat gene regulatory region (Liu et al., 2002). The analysis we performed between the human and mouse promoter spanned a larger region of 5'-flanking sequences of the caspase-3 gene. Comparison of the mouse 2245/+14 promoter region with equivalent human sequences revealed multiple conserved regions. These sequences contained identical putative transcription factor binding sites, by bioinformatics analysis, besides the Sp-1 sites that were identified in all three promoters around the transcriptional start site (Table 1) (Liu et al., 2002). The similarities in the organization of the core promoter between these mammalian species

(i.e. all being TATA-less) and the presence of highly conserved clusters suggests that regulatory regions of the caspase-3 gene are conserved.

Cloning 2259 bp of the 5'-flanking region of the caspase-3 promoter upstream of the luciferase reporter gene demonstrated significant promoter activity relative to the minimal promoter (-107/+14) in both the Jurkat T cell leukemia line and the Neuro-2A neuroblastoma cell line (Figure 3). However, some notable differences were observed between the two cell lines. When the region between nucleotides -2245 and -1695 was deleted an increase in luciferase expression was only seen in Jurkat cells with no significant difference in promoter activity observed in Neuro-2A cells. Caspases have been shown to be expressed at different levels in various tissues and at different stages of developments (Denis et al., 1998). Although caspase-2, -3 and -7 are highly expressed in the developing brain, their levels are significantly decreased in the adult brain (Kumar et al., 1992; Kuida et al., 1996; Juan et al., 1996; Duan et al., 1996; Lippke et al., 1996; Krajewska et al., 1997). In contrast, caspases are expressed at considerable levels in mature lymphocytes and play a major role in AICD (Alam et al., 1999; Ding et al., 1998; Refaeli et al., 2002). The differences and the levels in promoter activity observed between Jurkat and Neuro-2A cells reflects the expression levels of caspase-3 observed in an adult organism.

Differences due to the presence of some regulatory elements could explain the different levels of expression in various tissues. Removal of the region between nucleotides -1368 and -1275 lead to a decrease in luciferase expression levels in Jurkat cells, similar to levels observed with the full length construct (-2245/+14) and in Neuro-2A (Figure 3). Bioinformatics analysis identified AP-1 (Kuo and Leiden, 1999), NF-κB (Baldwin, Jr., 1996; Ghosh et al., 1998), STAT1 (Leonard and O'Shea, 1998) and NFAT (Rao et al., 1997) transcription factor binding sites to be present in this region which are all activated in proliferating T cells (Kuo and Leiden, 1999). The presence of these regulatory regions could explain the differences in expression levels between Jurkat cells and Neuro-2A cells. Further functional characterization of the caspase-3 5'-flanking region revealed that sequences necessary for basal transcriptional activity are located

between nucleotides -1275 and -1118. In addition, deletion of the region between nucleotides -1118 and -1072 restored reporter activity to similar levels as the full length promoter construct -2245/+14 in both cell lines (Figure 3). Interestingly, comparable positive and negative regulatory regions were identified in the rat caspase-3 promoter (Liu et al., 2002), demonstrating that the basal transcriptional activity of both the rat and mouse promoter is similarly controlled.

Several reports have demonstrated a role of STAT signaling in the basal expression of caspases. STAT1 was found to be required for constitutive expression of caspase-1, -2 and -3 (Kumar et al., 1997). Furthermore, activation of STAT1 through the IFN-γ receptor lead to increased expression of caspase-1, -3 and -8 and sensitized cells to apoptosis (Dai and Krantz, 1999; Ossina et al., 1997; Chin et al., 1997; Fulda and Debatin, 2002). Our analysis of the caspase-3 promoter (-2245/+14) revealed a single putative STAT1 binding site which was mapped to the region between nucleotides -1472 and -1368. Conversely, deletion of this region did not alter luciferase expression levels (Figure 3) suggesting other sites may be present outside the region under study in this report. Moreover, IFN-γ has been recognized to be required for AICD of activated T cells by controlling T cell numbers at the termination of an immune response through the increase in caspase-3 and -8 gene expression (Refaeli et al., 2002). These findings further highlighting the importance of caspase regulation in T cell homeostasis. In addition, when NFAT is activated in the absence of AP-1 activation there is increased expression of anergy-associated genes and T cells become anergic (Macian et al., 2002). Interestingly, caspase-3 has been shown to belong to a group of genes (proteolytic pathway) which are upregulated under conditions leading to tolerant T cells (Macian et al., 2002). Within the -2245/+14 region of the caspase-3 promoter several NFAT consensus binding sites were identified which might be responsible for the increase in caspase-3 mRNA levels in T cells undergoing anergy. Nevertheless, the significance of the increase in caspase-3 expression in anergized T cells remains to be determined.

An efficient retroviral reporter system was used to introduce different deletion constructs of the caspase-3 promoter into KOX-14 cells (Figure 4), in which we have

previously shown to upregulate endogenous levels of caspase-3 mRNA upon TCR triggering. Unexpectedly, the -2245/+14 5'-flanking region of the caspase-3 gene did not shown an increase in reporter activity following anti-CD3 stimulation in KOX-14 cells (Fig. 5). These results demonstrate that the TCR responsive element is not present in the isolated fragment of the caspase-3 promoter and could be present outside the analyzed region. Overexpression of E2F transcription factors was shown to induce the expression of caspase genes (Muller et al., 2001; Nahle et al., 2002). An E2F binding site was identified, by bioinformatics analysis, between nucleotides -1695 and -1542. However, deletion of this region had no effect on reporter activity (Figure 4 and 5). Conversely, one study on caspase promoters identified, by bioinformatics analysis, a potential E2F binding site within the first intron of the murine caspase-3 gene (Nahle et al., 2002). These findings suggest that regulatory sites responsive to T cell activation could be present further downstream within an intronic region, which was not included in our constructs.

Besides the binding of transcription factors to specific regions within promoters, chromatin remodeling and DNA methylation may play important roles in caspase-3 gene expression (Wu, 1997; Brandeis et al., 1994). However, the role of chromatin remodeling in caspase expression is unclear. The retroviral promoter reporters are randomly integrated into the chromatin and the locus of the integrated promoter might already be opened, possibly explaining the uninduced activity of the caspase-3 promoter in response to TCR crosslinking. If this proves to be the case, then the chromatin containing the endogenous caspase-3 promoter might be closed in resting T cells, and upon T cell activation chromatin remodeling of the caspase-3 locus would allow regulation of caspase-3 expression. In conclusion, this study shows that the -2245/+14 region of the caspase-3 promoter is constitutively active but shows differences in basal regulation between cell lines. Finally, our results suggest that TCR induced regulation of the caspase-3 promoter may require *cis* regulatory elements located either downstream or >2245 bp upstream of the transcriptional start site.

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Table 4-1. Conserved transcription factor binding sites between the human and mouse caspase-3 promoter regions, identified by bioinformatics analysis.

Constructs	Conserved Transcription factor binding sites
-2245/+14	NFAT(2X), E2F, STAT/NFAT, MYC, NFAT, p53(3X), NFAT
-1695/+14	E2F, STAT/NFAT, MYC, NFAT, p53(3X), NFAT
-1472/+14	STAT/NFAT, MYC, NFAT, p53(3X), NFAT
-871/+14	p53(3X), NFAT
-757/+14	No conserved binding sites
-107/+14	Basal promoter

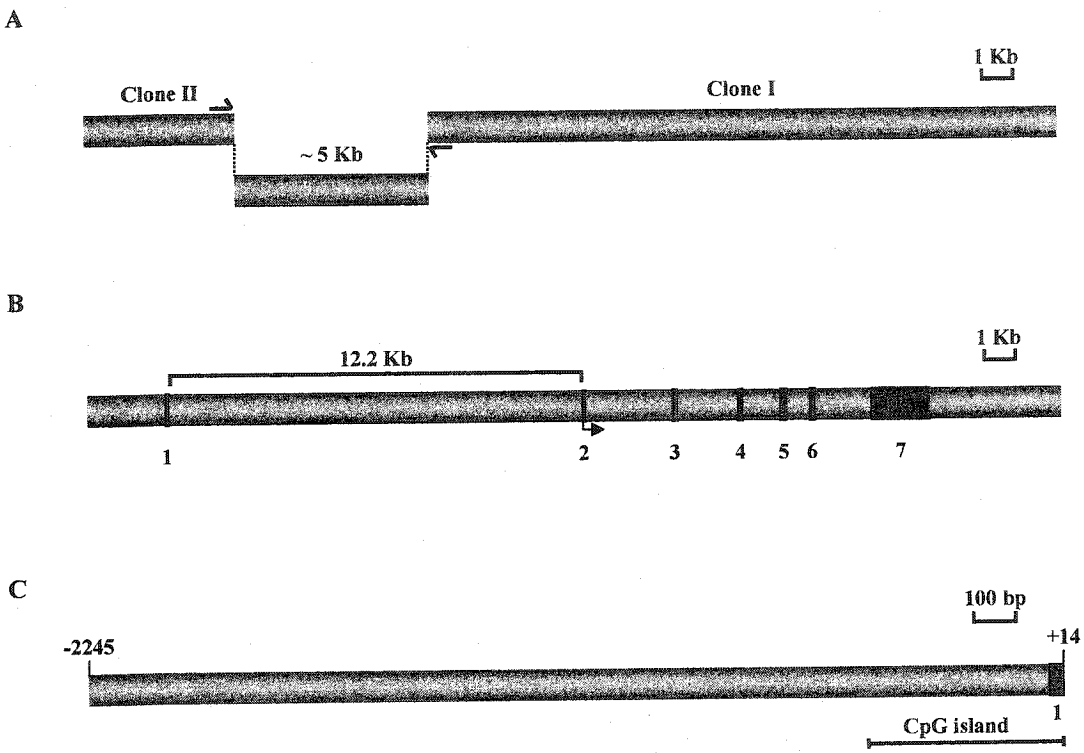


Figure 4-1. Schematic representation of the murine caspase-3 gene
(A) Two clones (I and II) were isolated by screening a lambda GEM12 murine ES cDNA library. A fragment of approximately 5 Kb was subsequently amplified by PCR and sequenced to complete the caspase-3 genomic sequence. (B) Structure of the murine caspase-3 gene showing seven exons, with the ATG start codon (represented by an arrow) mapping to exon 2. (C) Schematic showing the 2259 bp fragment from the 5' flanking region of the caspase-3 gene that was introduced upstream of reporter genes. A portion of exon 1 (31 bp) is included in the sequences. The region corresponding to the CpG island spanning the proximal promoter region is shown. Numbers are relative to the transcriptional start site identified by primer extension.

Figure 4-2. Nucleotide sequence of the caspase-3 gene promoter
The complete fragment of the caspase-3 promoter that was cloned upstream of reporter genes is shown (-2245/+14). A portion of the noncoding first exon sequences is underlined. The transcriptional start sites are shown in bold uppercase letters. The two sites that were identified by RPA are marked with an asterisks and the site identified by primer extension has been designated to be position +1. A CAAT-box and consensus sequences for SP1, AP-1, p53 and NF-κB are shaded.

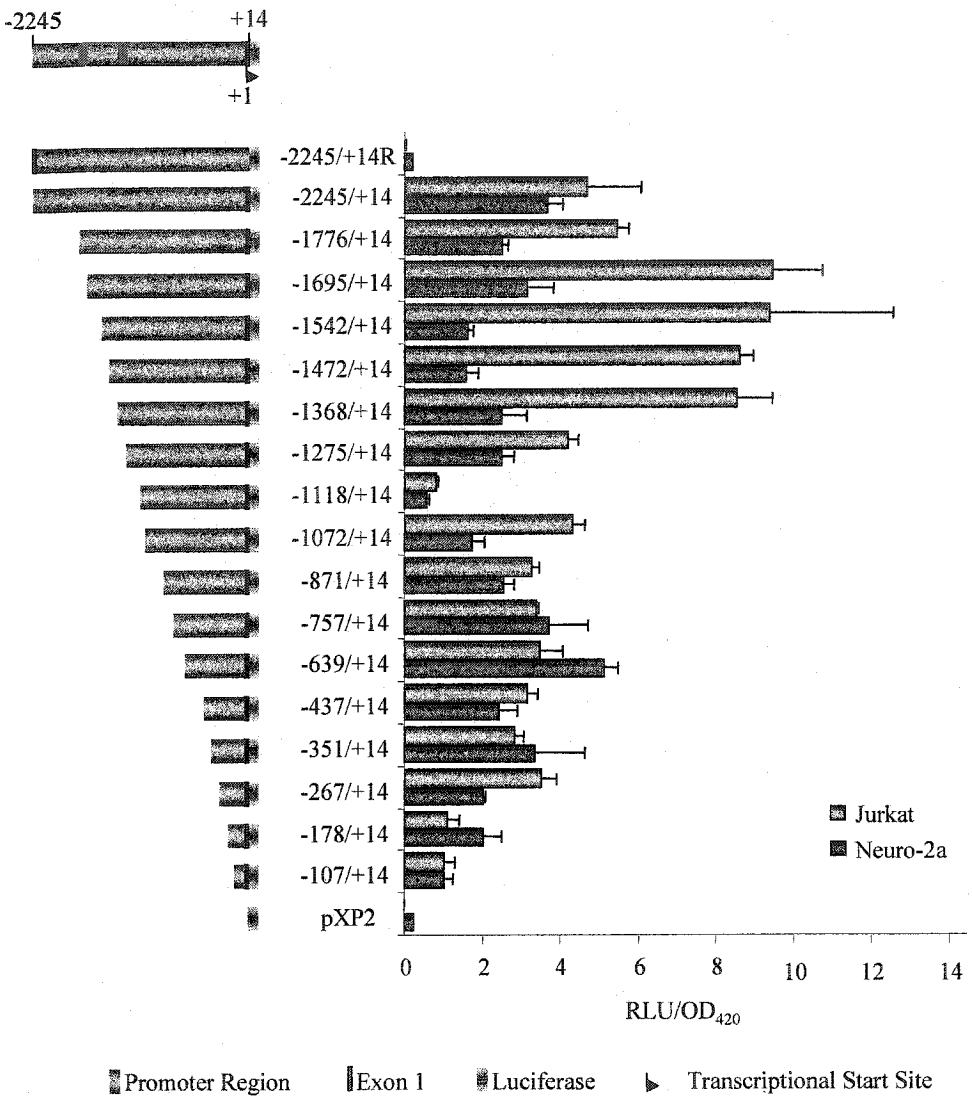


Figure 4-3. Deletion analysis of caspase-3 promoter activity in Jurkat and Neuro-2A cells

The schematics of the different deletion constructs are shown. The -2245/+14R reporter consists of the caspase-3 promoter in the reverse orientation. The pXP2 vector represents the promotorless vector. The 5'- and 3'-end points of each deletion construct are indicated. The basal promoter activity was determined following transient transfection of each construct into Jurkat and Neuro-2A cells. All transfection efficiencies were standardized to β-galactosidase activity and then normalized to the -107/+14 deletion. RLU/OD_{420}; relative luminescence units of luciferase activity/optical density at 420 nm representing β-galactosidase activity, n=3. The two boxes shown in the top construct represent regions which showed T cell specific regulation of reporter activity in Jurkat cells.

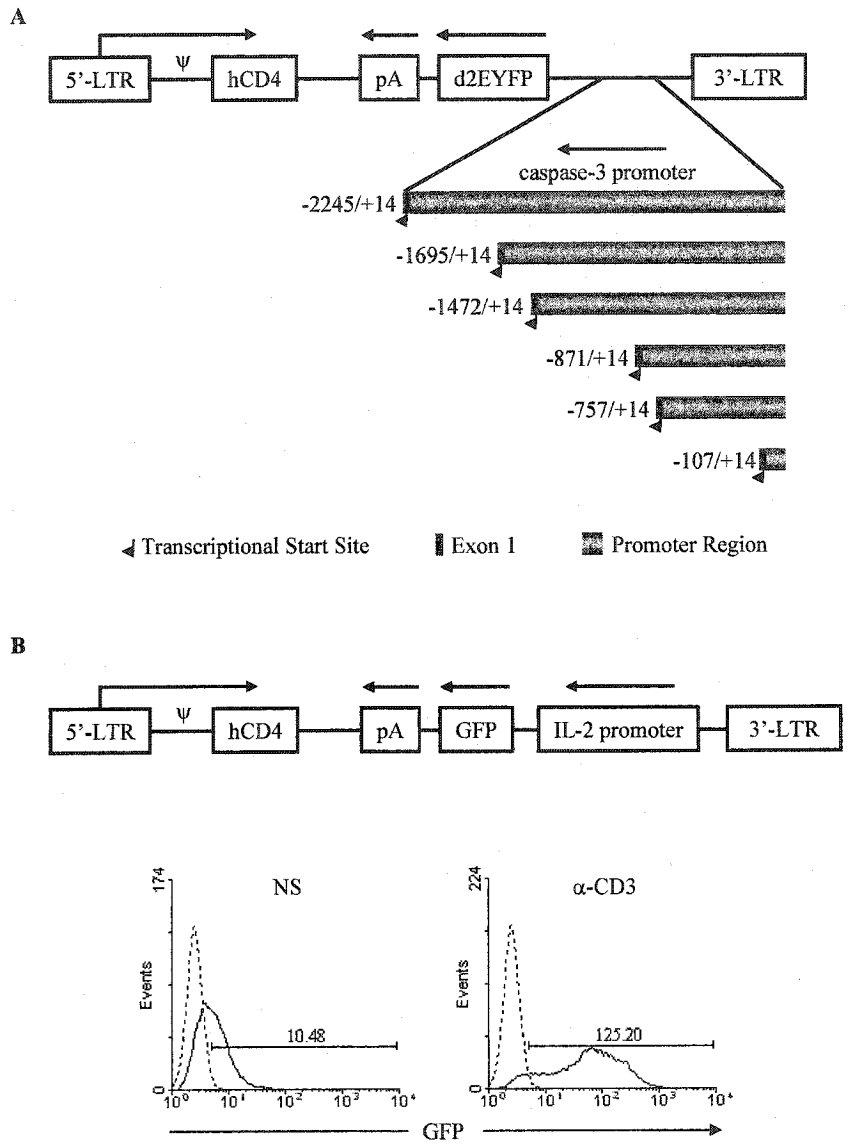
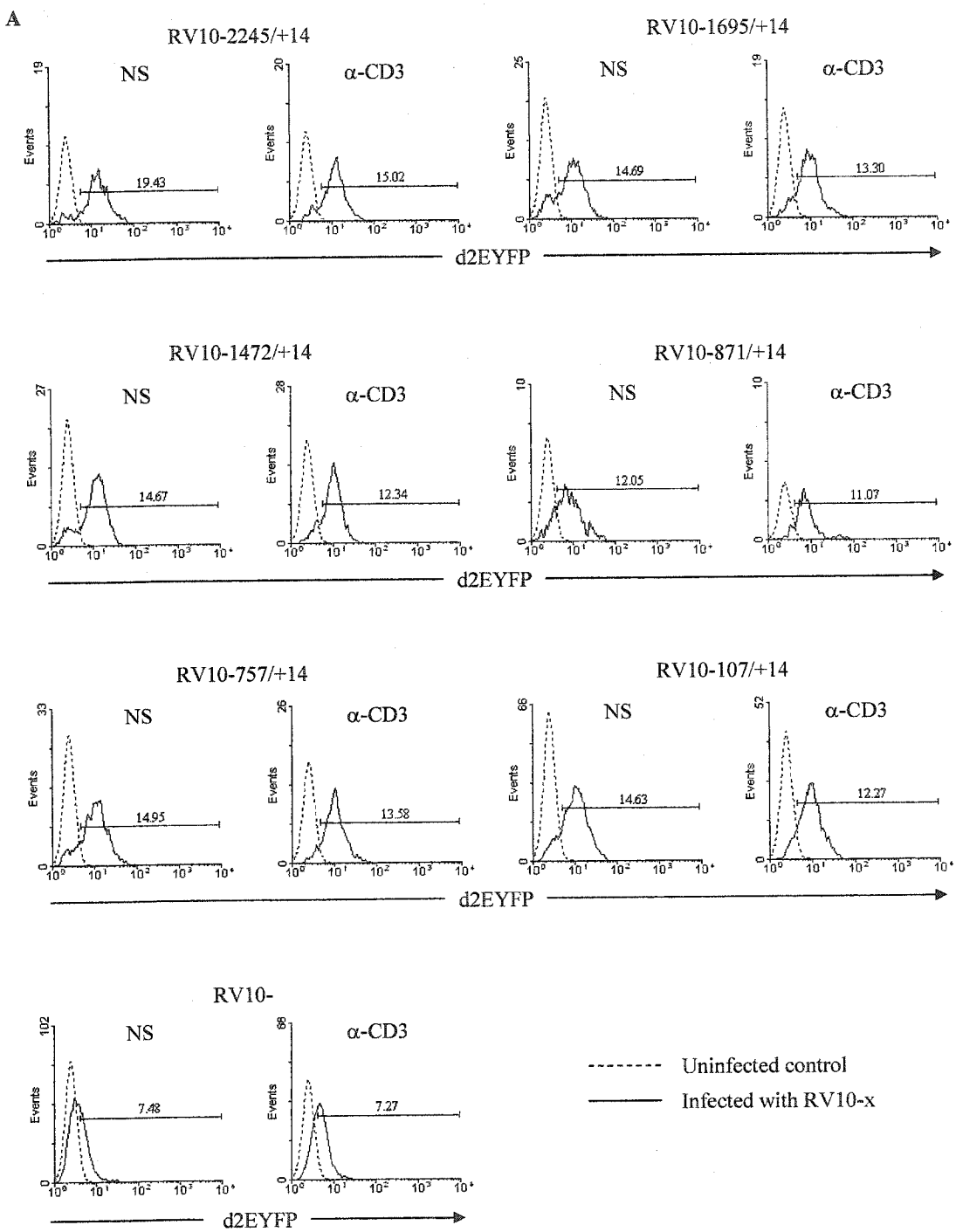


Figure 4-4. Caspase-3 retroviral reporter constructs
(A) Map of the RV10-x reporter constructs. A truncated form of human CD4 was used as a marker of infection and was under the control of the 5'-LTR, while d2EYFP expression was under the control of different caspase-3 promoter deletions. (B) Map of the hCD4-pA-GFP-IL-2p reporter construct. Human CD4 was also used as a marker of infection, while GFP was under the control of the IL-2 promoter (Zhu et al., 2001). The IL-2 control vector was introduced into KOX-14 cells and subsequently cultured in the presence (α-CD3) or absence (NS) of plate-bound anti-CD3 for 12 hours. Human $CD4^+$ cells were gated on and the mean fluorescence intensities (MFI) of GFP are shown. Results shown are representative of four independent experiments.


A
RV10-2245/+14
NS
α-CD3
19.43
15.02
RV10-1695/+14
14.69
13.30
Events
d2EYFP
RV10-1472/+14
14.67
12.34
RV10-871/+14
12.05
11.07
RV10-757/+14
14.95
13.58
RV10-107/+14
14.63
12.27
RV10-
7.48
7.27
Uninfected control
Infected with RV10-x

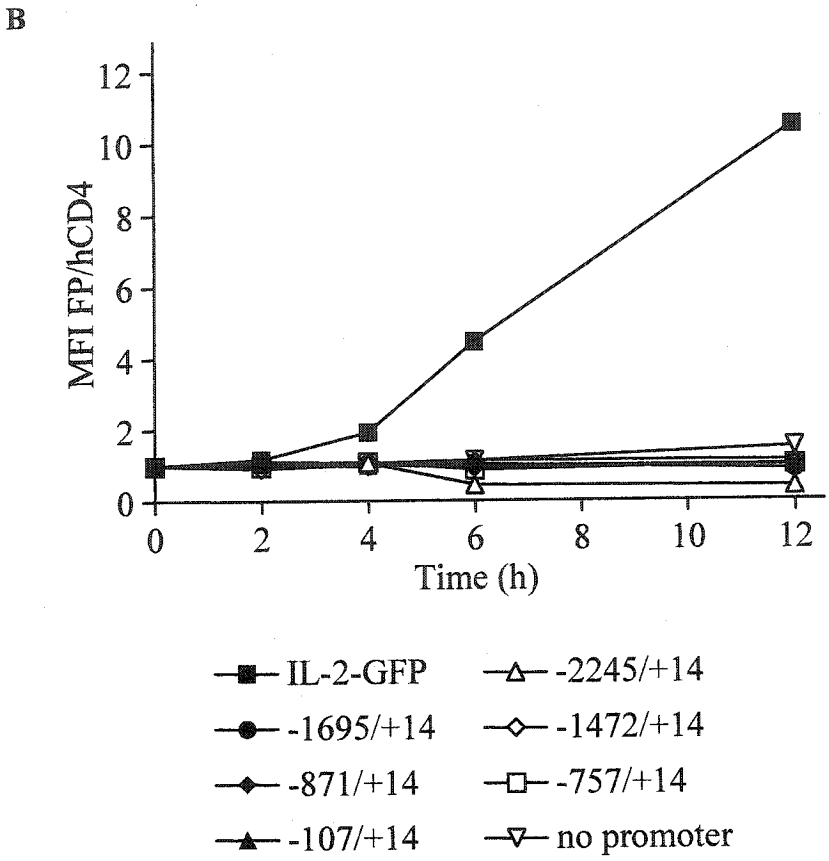


Figure 4-5. Expression of caspase-3 promoter in KOX-14 cells
(A) Different caspase-3 reporter constructs (RV10-x) were introduced into KOX-14 cells and subsequently cultured in the presence (α-CD3) or absence (NS) of plate-bound anti-CD3 for 12 hours. Human $CD4^+$ cells were gated on and the mean fluorescence intensities (MFI) of d2EYFP are shown. Results shown are representative of three independent experiments. The uninfected control represents uninfected KOX-14 cells. (B) KOX-14-RV10-x cells were cultured in the presence (α-CD3) or absence (NS) of plate-bound anti-CD3 over the course of 12 hours. The MFI of GFP or d2EYFP (FP) for each sample was normalized to the MFI of hCD4 expression, as a control for differences in infection efficiencies between the different constructs. Results shown are representative of two independent experiments.

Chapter 5: General Discussion

5.1 Caspases and development

Caspases are implicated in many biological processes during the course of development among different tissues. However, they are expressed at different levels during development. The requirement for caspases in brain development was clearly demonstrated in caspase-3 (Kuida et al., 1996; Woo et al., 1998) and -9 (Hakem et al., 1998; Kuida et al., 1998) knockout mice which showed severe neurological abnormalities. These mice had visible masses in their heads that represent ectopic masses of supernumerary cells which are normally eliminated by apoptosis forming pyknotic clusters during brain development (Kuida et al., 1996; Woo et al., 1998; Hakem et al., 1998; Kuida et al., 1998). These findings indicated decreased apoptosis in the absence of caspase-3 and -9 demonstrating a critical role of these proteases in shaping the developing mammalian brain. Conversely, immunohistochemical analysis on human adult tissues demonstrated almost no detectable levels of caspase-3 expression in the central nervous system (neurons in the spinal cord and brain) (Krajewska et al., 1997).

Interestingly, immunohistochemical studies on different human adult tissues showed clear differences in the expression of caspase-3 between short-lived and long-lived cell types (Krajewska et al., 1997). For example, high levels of caspase-3 were observed in mature neutrophils in contrast to myeloid progenitor cells which express lower levels. Furthermore, moderate to high levels of the protease was found in thymocytes whereas PBMCs demonstrated high immunostaining (Krajewska et al., 1997). Although it was shown that caspase-3 plays a role in thymocyte apoptosis (Alam et al., 1997; Clayton et al., 1997) it does not appear to be involved in T cell development since thymi and spleens from caspase-3 knockout mice contained normal CD4 and CD8 populations (Kuida et al., 1996; Woo et al., 1998). In addition, thymocytes isolated from caspase-3 deficient mice were sensitive to anti-CD3 crosslinking, anti-Fas and dexamethasone treatment.

While caspases are expressed at different levels in tissues during development and in an adult organism they don't appear to be required for all developmental processes. However, they are ubiquitously expressed which suggest a role for these proteases in a variety of cell types. It would be of interest to determine the mechanisms regulating the different expression levels of caspase-3 during development. One question that arises then is: are there elements or regions within the caspase-3 promoter that have the capacity to regulate the expression of this protease in a tissue-specific manner during the course of development? The study presented in chapter 4 on the caspase-3 promoter demonstrated differences in luciferase expression with several deletion mutants in transient transfection studies. A region within the caspase-3 promoter demonstrated higher basal promoter activity in Jurkat T cells in contrast to Neuro-2A neuroblastoma cells suggesting the presence of lymphoid specific regulatory elements within the 5'-flanking region of the caspase-3 gene. A more extensive analysis of putative transcription factor binding sites within the caspase-3 promoter region will allow a better understanding of the regulation of caspase-3 gene expression in various cell types.

5.2 Implications of caspase regulation in disease

In chapters 2 and 3 we have shown that the levels of caspase-3 determine the sensitivity of cells to different apoptotic stimuli and hence resolve the fate of a cell. Furthermore, based on our studies with activated T cells isolated from caspase-3 knockout, heterozygous and wild-type mice we have clearly demonstrated that a certain amount of proenzyme is required to induce efficient apoptosis. Although significant levels of procaspase-3 were observed in T cells isolated from mice heterozygous for the caspase-3 gene, these cells were as resistant to etoposide- and IL-2 withdrawal-induced apoptosis as T cells lacking the protease. In contrast, wild-type activated T cells were sensitive to both inducers of apoptosis.

Numerous reports have emerged demonstrating an implication of increased or decreased levels of caspase-3 expression in different models of disease. Could deregulation of caspase-3 expression be responsible for the pathologies observed in

several neurological disorders and cancer? If this statement proves to be true, then a better understanding of the regulation of caspase-3 expression would allow the development of new and more efficient therapies which would be based on modulating the levels of caspase-3 in order to correct the defects.

5.2.1 Neurological disorders

Several studies have provided evidence that apoptosis is not only required for normal development of the central nervous system, but it also is involved in several neurological pathologies, such as Alzheimer's disease and ischemic brain injury (Cotman and Anderson, 1995; Du et al., 1996). It has been suggested that prolonged transcriptional activation of caspase-3 after transient global ischemia may be responsible for the degeneration of CA1 pyramidal neurons (Ni et al., 1998; Chen et al., 1998). The induction in caspase-3 mRNA levels was observed in CA1 neurons that were committed to apoptosis but that were not yet dead (Ni et al., 1998). Furthermore, procaspase-3 protein levels were increased in ischemic hippocampus and caudate-putamen and showed the appearance of the active form of caspase-3 (p17) (Chen et al., 1998). These finding are in line with the data presented in chapter 3 where an increase in mRNA and protein levels of caspase-3 were observed which were followed by the appearance of both the p20 and p17 cleavage products of the proenzyme 36 hours after T cell activation.

The significance of increased caspase expression in the development of neurodegenerative disorders was clearly shown in transgenic mouse models of Huntington disease (Chen et al., 2000) and amyotrophic lateral sclerosis (ALS) (Li et al., 2000b). In addition, inhibition of caspase-1 and -3 expressions and activity delayed mortality in both mouse models (Li et al., 2000b; Chen et al., 2000). The authors suggested that inhibition of caspase function could prove to be a potential form of therapy in the earlier stages of ALS (Li et al., 2000b). Nonetheless, transcriptional modulation of caspase expression may provide a new target for treatment of neurodegenerative disorders. However, it remains to be determined whether the onset of illness is due to unregulated expression of caspases which appears to correlate with disease progression.

5.2.2 Cancer

The levels in caspase-3 have also been correlated with resistance of tumors to apoptosis-inducing agents. Human leukemic cell lines that survived treatment with a common cytotoxic drug used for various human tumors did not increase their levels of caspase-3 mRNA. In contrast, those that were sensitive induced the expression of caspase-3 mRNA (Droin et al., 1998). Similarly to our findings, the increase in caspase-3 mRNA in these tumor cell lines was followed by an induction in procaspase-3, which preceded the onset of apoptosis. Interestingly, another study demonstrated that 75% of a breast tumor tissue lacks caspase-3 mRNA and protein expression (Devarajan et al., 2002). These results suggest that a reduction in caspase-3 mRNA levels or the absence of caspase-3 expression contribute to the survival of tumors. Therefore, one can speculate that targeted enhancement of caspase-3 expression in tumors could prove to be an alternative or a supplement to today's therapies.

The data presented in chapter 4 on the murine caspase-3 promoter along with the report on the rat caspase-3 promoter (Liu et al., 2002) demonstrated that the regulatory regions of caspase-3 in both animals contained CpG islands. DNA methylation of CpG islands of multiple promoters has been previously shown in adult acute lymphocytic leukemia (Garcia-Manero et al., 2002). Furthermore, silencing of the caspase-8 gene has been described in childhood neuroblastomas (Teitz et al., 2000; Takita et al., 2001) and a possible role of methylation has been suggested (Banelli et al., 2002). Therefore, methylation of CpG islands within the caspase-3 promoter could potentially be responsible for reduced or undetectable levels of caspase-3 that have been described in certain tumors and might explain their resistance to cytotoxic agents used in chemotherapy (Droin et al., 1998; Devarajan et al., 2002).

In transient transfection studies we observed lower levels of caspase-3 promoter activity in Neuro-2A neuroblastoma cells relative to Jurkat T cells which could be due to DNA methylation of CpG islands within the promoter region. In addition, DNA methylation could be the mechanism that is responsible for the reduction in caspase-3

expression in the adult brain in contrast to the high levels observed in the developing brain of fetuses. Besides deregulation of caspase-3 expression, methylation of CpG islands as a mechanism of gene silencing leading to cancer should be another avenue worth investigating.

5.3 Caspase-3 regulation in the immune system

5.3.1 Caspase-3 function in AICD

Caspases are central to two pathways, both the death receptor (Fas and TNF-R) (Wallach et al., 1999) and mitochondrial (Wang, 2001) pathways of apoptosis. Furthermore, they play a crucial role in ensuring the elimination of expanded T cell populations at the termination of an immune response by AICD. Although several knockout models of caspases have demonstrated that T cell development is not affected the function of T cells is altered in the absence of certain members of the caspase family (Woo et al., 1998; Salmena et al., 2003). Caspase-3 deficient peripheral T cells were less susceptible to AICD-, anti-CD3- and anti-Fas-induced apoptosis (Woo et al., 1998). Furthermore, they showed higher and more persistent proliferation however this was attributed to decreased levels of apoptosis. These findings are in agreement with the results presented in chapter 2 and 3 demonstrating a requirement for maintaining a minimum level of expression of caspase-3 to ensure apoptosis.

The increase in caspase-3 mRNA levels was observed early on following T cell activation which was followed by an increase in protein levels and cleavage of the proenzyme. The activation of caspase-3 has been previously shown to be required for T cell proliferation (Alam et al., 1999). The selective increase in caspase-3 mRNA levels would then ensure a constant renewal of proenzyme which allows T cells to expand. However, maintaining caspase-3 levels are also required for efficient apoptosis and AICD at the termination of an immune response (Woo et al., 1998). In addition, results presented in chapter 3 demonstrated for the first time that maintaining caspase-3 expression levels is also required for apoptosis induced through mitochondria in caspase-

3 deficient T cells. These findings highlight the functional importance of caspase-3 as being a central mediator of cell death in the different pathways leading to apoptosis.

5.3.2 Caspase-3 and the generation of memory T cells

The factors that are involved in maintaining memory T cells in contrast to effector T cells (i.e. targets of AICD) are not well understood. Our work and that of others suggest that the fate of a cell lies in the expression levels of molecules with opposing effects (Salvesen and Dixit, 1999; Fesus, 1999). We have shown (chapter 3) that there are higher levels of caspase-3 mRNA in effector T cells in contrast to memory T cells. Effector T cells are destined to undergo apoptosis by AICD at the termination of an immune response whereas memory T cells are long-lived cells and persist for years in a host. Furthermore, it was previously shown that bcl-2 levels are higher in memory T cells in contrast to effector T cells (Grayson et al., 2000; Grayson et al., 2001). The balance between anti- and pro-apoptotic molecules would therefore determine the fate of cells.

It is well established now that T cell activation through the TCR leads to caspase-3 activation leading to proliferation in the absence of apoptosis (Miossec et al., 1997; Wilhelm et al., 1998; Alam et al., 1999; Kennedy et al., 1999; Boissonnas et al., 2002). In addition, one study used variant TCR ligands as well as a full agonist ligand to show that caspase activation was regulated by antigenic strength (Boissonnas et al., 2002). Caspase activity was associated with stimulations using high doses of agonist ligand whereas the expression of active caspases was not detected with low doses of the agonist peptide or partial agonist peptides. It would be of interest then to determine whether the strength of antigenic stimulation has any effect on caspase-3 expression levels. These studies can help determine the differentiation pathway leading to the generation of memory T cells.

If the extent of TCR stimulation leads to varying expression levels of caspase-3 then based on the data presented in chapter 3 we would observe populations of T cells with different degrees of sensitivity to apoptosis. Therefore, if naïve T cells are partially stimulated we could expect a lower fold induction in caspase-3 levels in the majority of

cells and the generation of a higher population of memory T cells than with stimulation using a strong agonist peptide. In contrast, if naïve T cell have been fully activated and contain the highest levels of caspase-3 the majority would be expected to eventually die by AICD. If this does prove to be true then the generation of memory T cells follows the dichotomic pathway of differentiation (Fig. 1A). Conversely, the strength of the initial stimulation may not alter caspase-3 expression levels. However, the number of subsequent stimulations might determine the outcome of a cell. Effector T cells that are constantly stimulated would maintain increased levels of caspase-3 expression and will be more susceptible to AICD compared to effector T cells that do not reencounter antigen and would therefore differentiate into memory T cells. This outcome would favor the linear pathway of differentiation for the generation of memory T cell (Fig. 1B).

Although the signaling pathways responsible for regulating expression levels of caspase-3 are not well understood, pro- and anti-apoptotic proteins appear to play crucial roles in the generation of different T cell subsets. However, the pathways of differentiation leading to the generation of memory T cells remain controversial. Several models have been proposed but no consensus has been reached to date.

5.4 Regulation of caspases

5.4.1 Function of IFN-γ signaling in caspase-3 regulation

IFN-γ has been shown to induce the upregulation of caspase-3 in different cell types. Following treatment of a human colon adenocarcinoma cell line (HT-29) (Ossina et al., 1997), human erythroid progenitor cells (Dai and Krantz, 1999) and T cells from spleens and lymph nodes of mice (Refaeli et al., 2002) with IFN-γ the expression levels of several caspases including caspase-3 were upregulated. To determine whether the induction in caspase expression was direct (requires no protein synthesis) or indirect (requires additional proteins such as transcriptional activators) cells were treated with IFN-γ in the presence or absence of the protein synthesis inhibitor, cycloheximide

(Ossina et al., 1997). Minimal or no increase in caspase-3 mRNA levels were observed indicating they were indirectly induced by IFN-γ.

Signal transducers and activators of transcription (STATs) enhance transcription of specific genes in response to IFN-γ. Treatment of cells with IFN-γ causes phosphorylation of STAT1 which subsequently form dimers and bind to specific sequences within promoters to activate the transcription of specific genes. Human fibroblasts lacking STAT1 demonstrated low constitutive levels of caspase-3 which lead to resistance to TNF-α-induced apoptosis (Kumar et al., 1997). However, it was further shown that the STAT1 homodimer was not the transcription factor required for constitutive caspase-3 expression since mutations disrupting dimer formation still maintained caspase expression. The authors suggest that STAT1 might form complex transcription factors by interacting with other protein (Kumar et al., 1997).

In our studies (chapter 4) we have identified a putative STAT binding site within the caspase-3 promoter region (-2245/+14). However, deletion of this site did not show any change in basal levels of expression demonstrating that this transcription factor might not be acting directly on the promoter region within the chromatin. Our findings are in agreement with the above study suggesting that STAT1 does not interact directly with caspase promoters but rather might bring other transcription factors to the regulatory region of the caspase-3 gene. However, we cannot rule out the possibility of other *cis*-acting regulatory region outside the -2245/+14 segment.

Furthermore, IFN-γ has been shown to be required for AICD. T cells lacking IFN-γ or STAT1 did not undergo apoptosis by AICD whereas wild-type cells did (Refaeli et al., 2002). However, the defect appeared to be downstream of Fas and FasL since their expression levels were not affected by the lack of both the cytokine and transcription factor. Assaying for the levels of RNA for several caspases demonstrated that expression levels of caspase-3, -6 and -8 were reduced in IFN-γ knockout T cells which were restored upon addition of IFN-γ (Refaeli et al., 2002). In contrast, in the absence of STAT1 supplementing the cultures with IFN-γ did not increase caspase expression to the

same levels as wild-type T cells. Although these findings strongly support a role for IFN-γ and downstream signaling components in the regulation of caspase-3 during AICD, the mechanism by which STAT1 regulates caspase-3 expression remains to be determined.

5.4.2 Cell cycle and caspase-3 expression

E2F-1 was another candidate transcription factor that was shown to increase the expression of caspase-3, under forced conditions of E2F-1 expression (Muller et al., 2001; Nahle et al., 2002). Although we have identified a putative E2F-1 binding site that is conserved between both the mouse and human promoters, deletion of this region did not alter expression levels in KOX-14 cells infected with the retroviral based reporter constructs. However one study identified a site within the first intron of the caspase-3 gene suggesting again potential sites outside the region we investigated (Nahle et al., 2002). However, E2F-1 binding to the caspase-3 promoter was never shown. Nevertheless, the caspase-3 promoter remains a potential target for E2F-1.

In chapter 3, we clearly demonstrated that the increase in caspase-3 occurred early following T cell activation, more specifically in the G0/G1 phase of the cell cycle. E2F-1 belongs to the family of activating E2Fs which are potent transcriptional activators of E2F-responsive genes (Trimarchi and Lees, 2002). Furthermore, E2F-1 is activated during the G1 phase of the cell cycle and induces cellular proliferation. In addition, it was demonstrated that over expression of E2F-1 induced approximately a 12-fold induction in caspase-3 mRNA levels (Muller et al., 2001). Interestingly, we observed a 13-fold induction in caspase-3 mRNA levels following T cell activation through the TCR. The fact that E2F-1 is a transcription factor that is activated early on upon cell cycle entry and the similarities in the magnitude in caspase-3 gene expression upon E2F-1 activation suggests that this transcription factor could potentially be responsible for the increase in caspase-3 mRNA levels following T cell activation.

Although E2F-1 induces cell cycle entry and proliferation it has been shown to be required for AICD. Introduction of a dominant-negative mutant E2F-1 protein into T

cells rendered them resistant to both DNA fragmentation and apoptosis (Lissy et al., 2000). Furthermore, E2F-1-null primary splenocytes failed to undergo apoptosis (Lissy et al., 2000). These observations show that E2F-1 is a specific mediator of AICD in T cells. It remains to be determined then whether the observed defect in AICD in T cells lacking a functional E2F-1 protein is due to a lack of induction in caspase-3 expression. Investigating the levels of caspase-3 expression in activated E2F-1 null T cells will be essential in determining whether E2F-1 is required for the induction of caspase-3 expression upon TCR crosslinking. These studies would also provide the link between E2F-1 and AICD.

5.5 Conclusion

While several studies have attempted to demonstrate the involvement of several transcription factors in the regulation of caspase-3 gene expression no one has yet provided definitive evidence of specific binding of a molecule to the promoter region of caspase-3. Based on the data present in the literature, regulation of caspase-3 mRNA levels by transcription factors appear to be a promising possibility. However, DNA methylation of CpG islands within the regulatory region of the caspase-3 gene remains to be explored. Methylation of the IFN-γ promoter region has been demonstrated to play a role in the differentiation between Th1 and Th2 cells (Yano et al., 2003). Therefore DNA methylation of the caspase-3 promoter could be the mechanism in reducing caspase-3 levels and lead to the differentiation of memory T cells. Furthermore, chromatin remodeling may prove to be another mechanism of regulation. Elucidating the mechanisms underlying the regulation of caspase-3 expression will be essential for understanding its role in disease progression. Finally, studying the functional importance of caspase-3 expression in the generation of memory T cells lies in the potential of developing new vaccine therapies based on enhancing immune responses by increasing the numbers of a T cell subset that is more efficient at responding to antigen.

Dissecting the molecular details of transcriptional regulation of caspases will help the scientific community at large in understanding the significance and importance of

regulating their expression to avert disease. This area of research has promising potential in developing new pharmaceutical approaches that would manipulate the expression of caspases to sensitize and destroy unwanted cells or to prevent the destruction and loss of indispensable cells required for proper functioning of an organism.

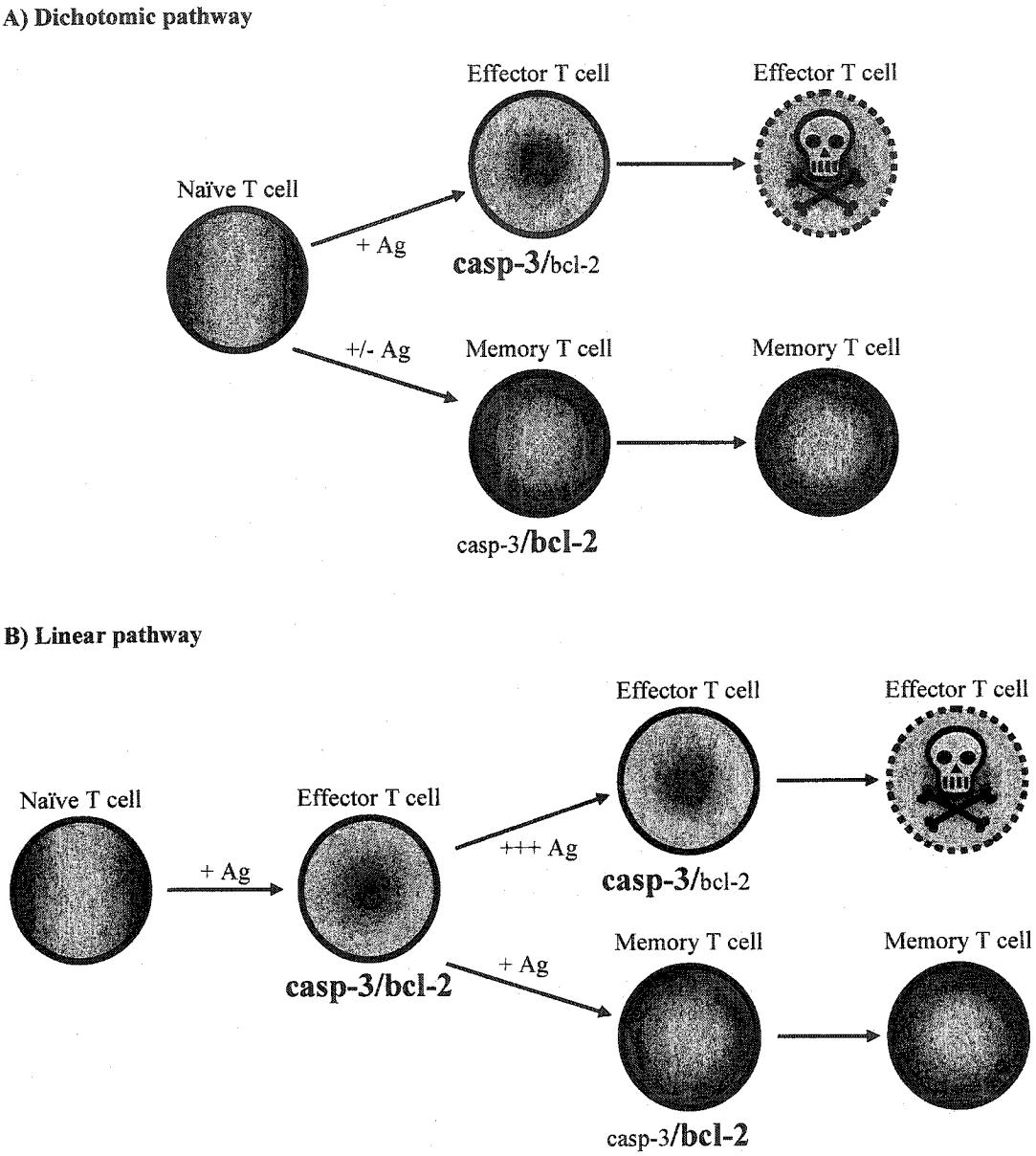


Figure 5-1. Determination of the pathway for the generation of memory T cells
A) The dichotomic pathway of memory T cell differentiation may be dependent on antigenic strength (+/-). B) The Linear pathway of memory T cell differentiation may be dependent on the number of secondary stimulations (+).

Original contribution to scientific knowledge

The results presented in this thesis contribute to scientific knowledge in several aspects.

The work presented in Chapter 2 is an original report on the transcriptional regulation of caspase-3 in an *in vitro* model of AICD. Although many studies have shown that the expression of caspases are induced in different cell types undergoing apoptosis this report demonstrates for the first time the selective increase in caspase-3 mRNA levels following T cell activation. Furthermore, the increase in caspase-3 gene expression occurred independently of caspse-3 activity. Finally, the increase in caspase-3 mRNA levels was observed early during T activation and in the absence of apoptosis. Chapter 2 also describes a novel quantitative real-time RT-PCR method that was used to determine quantitatively mRNA levels of caspase-3 and -8 in the KOX-14 T cell hybridoma.

The results presented in Chapter 3 support the requirement of maintaining minimal levels of caspase-3 in order to efficiently induce apoptosis. A selective increase in caspase-3 mRNA levels was demonstrated in T cells activated *in vitro* and *in vivo* in a model of infection using TCR transgenic mice. Interestingly, higher levels of caspase-3 mRNA and protein were observed in effector T cells when compared to memory T cells demonstrating for the first time a potential role of caspase-3 in the differentiation of memory T cells. In addition, the increase in caspase-3 expression was confined to the G0/G1 phase of the cell cycle and occurred in the absence of IL-2 suggesting that early TCR-mediated signalling is responsible for the increase. Furthermore, an increase in procaspase-3 levels and subsequent cleavage was observed in the absence of apoptosis. Activated T lymphocytes with reduced levels of caspase-3 (heterozygous mice) demonstrated a high degree of resistance to apoptosis similarly to caspase-3-deficient cells demonstrating that a significant increase in caspase-3 levels is required to induce apoptosis.

For the first time, the work presented in Chapter 4 described the functional characterization of the murine caspase-3 gene promoter in T lymphocytes. Several

sequences within the promoter region had positive and negative effects on the basal levels of reporter expression. In addition, one region demonstrated a potential specificity to lymphoid cells since reporter activity of several deletion constructs demonstrated higher levels of promoter activity in a T cell line in contrast to a neuroblastoma cell line. Bioinformatics analysis of the -2245/+14 region of the caspase-3 promoter region revealed the presence of several putative transcription factor binding sites several of which are conserved in the human caspase-3 promoter region. Using a novel retroviral based reporter system different deletion mutants were introduced into KOX-14 cells, which upregulate the levels of caspase-3 following T cell activation. Surprisingly, none of the promoter constructs induced reporter activity following TCR crosslinking. These results suggest that either other regulatory mechanisms are responsible for the induction in caspase-3 mRNA levels during T cell activation or the regulatory elements are present beyond the -2245/+14 region of the 5'-flanking region of the caspase-3 gene.

Altogether, the studies presented in this thesis provide evidence for the requirement of maintaining caspase-3 levels to ensure the elimination of expanded T cell population at the termination of an immune response. Although caspases have been mainly associated with apoptosis, part of the work presented herein brings into question the potential role of caspase-3 in the generation of T cell subsets, more importantly memory T cells. Finally, a better understanding of the regulation of caspase-3 expression and the mechanisms regulating its expression will be crucial in designing new therapies and different approaches to vaccine design.

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