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Generation of transgenic mice expressing a granzyme B-EGFP fusion protein

by

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Abstract

Cytotoxic T cells (CTLs) kill by inducing apoptosis in target cells through the exocytosis of cytotoxic granules. Granzyme B (grB) is a serine protease that is a major component of these granules, and is important in promoting target cell death. Although purified grB has been shown to be taken up by cells *in vitro*, this uptake has never been explicitly seen in more physiologically relevant conditions, as when CTLs kill a target cell.

We have generated a transgenic mouse that expresses grB fused to the enhanced green florescent protein (EGFP) on its carboxy terminus. These mice have grB-EGFP positive CTLs after *in vivo* infection. A CTL line has been generated from primary naïve splenocytes of these mice, and induce uptake of the grB-EGFP protein into target cells. Granzyme B uptake was also confirmed using antibody staining, demonstrating for the first time, the delivery of grB from CTLs to their targets.

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Abbreviations

α	anti
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocator
Apaf-1	apoptotic protease activating factor-1
APC	antigen presenting cell
ATP	adenosine triphosphate
BCR	B cell receptor
β-gal	beta-galactosidase
BSA	bovine serum albumin
^{o}C	degrees Celsius
Ca^{2+}	calcium ion
CAD	caspase-activated DNAse
Caspase	cysteinyl aspartate-specific proteinase
CD	cluster of differentiation
cDNA	complimentary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CI-MPR	cation-independent mannose-6-phosphate receptor
CO_2	carbon dioxide
Con A	concanavalin A
CRT	calreticulin
c-SMAC	central supramolecular activation complex

CTFR	cell trace far red
CTL	cytotoxic T lymphocyte
dATP	deoxyadenosine triphosphate
DED	death effector domain
DEPC	Diethylpyrocarbonate
DIABLO	direct inhibitor of apoptosis-binding protein with low pI
DISC	death inducing signalling complex
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
E/T	effector/target
FACS	fluorescent activated cell sorter
FADD	Fas-associated death domain
FasL	Fas ligand
FCS	fetal calf serum
FITC	fluorescein
gr	granzyme
gtBid	granzyme B-truncated Bid
GvHD	graft versus host disease
HBSS	Hank's balanced salt solution

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O	water
HS	hypersensitivity site
HtrA2	high temperature requirement A2
IAP	inhibitor of apoptosis protein
ICAD	inhibitor of caspase-activated DNAse
IL	interleukin
IL-2	interleukin-2
IL-2 Rα	IL–2 receptor α chain
i.p.	intraperitoneal
Kb	kilobase
$KHCO_3$	potassium bicarbonate
KO	knockout
LAK	lymphokine-activated killer
LCMV	lymphocytic choriomeningitis virus
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
MOPS	3-(N-morpholino)propanesulfonic acid
MPR	mannose-6-phosphate receptor
mRNA	messenger RNA
NaCl	sodium chloride
Na_2EDTA	sodium EDTA
NF-IL6	nuclear factor of interleukin-6

NFAT	nuclear factor of activated T cells
NH_4Cl	ammonium chloride
NK	natural killer
PARP	poly-(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PE	phycoerythrin
PEL	peritoneal exudate lymphocytes
PerCP	peridinin chlorophyll-a protein
PFU	plaque forming units
PS	phosphatidylserine
p-SMAC	protein supramolecular activation complex
PTP	permeability transition pore
RANTES	raised on activation, normal T-cell derived and secreted
RBC	red blood cell
RHFM	RPMI, HEPES, FCS, 2-mercaptoethanol
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SG	serglycin

Smac	second mitochondria-derived activator of caspase
SP	single positive
tBid	truncated Bid
TCR	T cell receptor
Tg	transgenic
Th	T helper effectors
TNFR	tumour necrosis factor receptor
Tris-Cl	trishydroxymethylaminomethane chloride
U/mL	enzymatic units per millilitre
VDAC	voltage-dependent anion channel
WT	wildtype

1. Introduction

1.1 Overview of the Immune System

The immune system has developed largely to defend organisms from pathogen invasion. In vertebrates this defence occurs through two distinct mechanisms; the innate and the adaptive immune response. Innate immunity is non-specific, and includes such anatomical features as the physical barriers of the skin and mucous membranes. Cells remove pathogens non-specifically when macrophages and monocytes perform phagocytosis, and when granulocytic cells such as eosinophils release cytotoxic substances. Natural killer (NK) cells are also considered part of innate immunity. These responses are extremely important for the successful removal of many pathogens and are also involved in the body's ability to inhibit tumourigenesis. However, the innate immune response is unable to adapt to changing pathogenic environments that may be encountered during the host's lifetime. This ability is called the adaptive immune response which is specifically activated by, and targeted against, the antigens expressed by the invading organism. Although this adaptive immune response requires a few days to develop after exposure to these antigens, it is able to eliminate the pathogen with minimum damage to non-infected tissues due to the nature of its specificity. An adaptive response is also able to retain memory of antigen exposure, and thus is able to mount a quicker and more powerful response when again presented with the same antigens. The

adaptive immune response therefore possesses two main advantages of specificity and memory that contributes to the success of pathogen removal.

The adaptive immune response is primarily mediated by two types of cells, the T lymphocytes (both $CD4^+$ and $CD8^+$ T single positive (SP) cells) and the antigen-producing B lymphocytes. Antigens are recognised by surface receptors on these cells, the T cell receptor (TCR) on T lymphocytes, and the B cell receptor (BCR) on B lymphocytes. During the maturation process of T and B cells, the variable regions of the TCR and BCR genes undergo rearrangement to produce a huge variety of TCR and BCR proteins that bind to and recognise a potentially unlimited number of antigens. Each unique TCR or BCR protein is expressed to exclusion on a single T or B cell, and during maturation T or B cells that express receptors that recognise self-reacting antigens are eliminated. The specificity of the response to infection occurs because of the clonal expansion of the individual T and B cells expressing the TCR and BCR proteins that recognise the specific antigens of an infecting pathogen. After an infection, this clonal population is reduced to few cells that remain circulating in the body to provide memory of the infecting pathogen, and enable a stronger and quicker response if the infection is re-introduced.

T and B lymphocytes each have distinct roles in the initiation of an immune response. When B cells are activated by antigen, they differentiate into two different effector phenotypes; plasma cells that secrete cytokines and antibodies, and B memory cells. Antibodies are soluble versions of the BCR, and they circulate the body until they

specifically recognise and bind the antigen that initiated the B cell activation. These antibodies can coat the invading pathogen to either neutralise it, or make it easier for macrophages and monocytes to recognise and phagocytose the infecting organism. The cytokines released by B cells increase the response and activation of other immune cells, including T lymphocytes. In turn, cytokines produced by T cells help to fully activate the B cell response to antigen.

Activation of precursor T cells induces the terminal differentiation into effector phenotype. The two SP phenotypes are stimulated by antigen presented when bound to surface receptors called the major histocompatibility complex (MHC). For $CD4^+$ T cells, this process begins when antigen presenting cells (APCs) endocytose and digest foreign proteins from pathogens. These digested particles are then brought to the cell surface bound to MHC class II molecules. The $CD4^+$ T cells recognise these antigens when presented in the context of MHC class II molecules via their TCR and the CD4 co-receptor. Thus, $CD4^+$ T cells are said to be restricted to class II MHC presentation, meaning these lymphocytes are stimulated primarily by foreign (non-self) antigen. This presentation activates these cells to terminally differentiate into two effector phenotypes, $CD4^+$ helper T (Th) lymphocytes, and memory cells. The Th effector cells produce cytokines that are needed for maintenance and amplification of both the B cell-mediated, and $CD8^+$ T cell-mediated immune responses. $CD8^+$ T cells are similarly stimulated through recognition of antigen bound to MHC class I protein again through the TCR and CD8 co-receptor to generate into two effector phenotypes the $CD8^+$ killer or cytotoxic T lymphocytes (CTLs) and memory cells. However, MHC class I molecules are not

restricted to so-called professional APCs, but are expressed by every cell in the body. Therefore, $CD8^+$T cells are activated when a cell of the body displays an altered-self protein via the MHC class I receptor, and these lymphocytes screen the health of all endogenous cells. The CTLs are responsible for the cell-mediated immune response of the adaptive immune system, and induce apoptosis or programmed cell death, in virally-infected cells or tumour cells within the body. The CTLs therefore primarily serve to destroy cells in the body that have been changed due to viral infection or tumourigenesis, but are also primarily responsible for allograft rejection and pathologically can destroy the body's own tissues in autoimmune disease. This lymphocyte activation can be duplicated *in vitro* with factors such as CD3 antibodies (αCD3) and concanavalin A (con A) in the presence of the cytokine interleukin-2 (IL-2).

1.2 CTL differentiation and formation of granules

When $CD8^+$ T cells differentiate from the naïve state into effector CTLs, a number of proteins are expressed that allow the CTL to perform their effector function, mainly the ability to induce apoptosis in target cells. Resting $CD8^+$ T lymphocytes do not contain cytotoxic granules, which are formed during the period of effector differentiation, when many effector proteins unique to CTLs and NK cells are first transcribed and translated. The following sections briefly describe the transcriptional control of CTL differentiation, and the biogenesis and secretory mechanisms of the cytotoxic granules in these cells.

1.2.1 Transcriptional regulation during effector CTL differentiation

Early events in effector differentiation have been studied extensively in $CD4^+$ T cells (Isakov and Altman, 2002; Mariat et al., 2005; Serfling et al., 2000) and to a lesser extent in $CD8^+$ T cells (Conze et al., 2002). Downstream signalling pathways are initiated almost immediately after TCR stimulation, and within hours induce the transcription of hallmarks of early T cell activation such as the IL-2 gene (Song et al., 2000). Many T cell-specific transcription factors important in controlling early gene expression have been identified, including the nuclear factor of activated T cells (NFAT) and nuclear factor of interleukin-6 (NF-IL6). However, late stages of effector CTL differentiation occur 3 to 5 days after activation, when the activity of these transcription factors have been downregulated (Ortiz et al., 1999; Ortiz et al., 1997). There are a number of CTL effector-specific genes, including the granzymes, granulysin, perforin, and RANTES (discussed in Sections 1.3.1 and 1.3.2) that are only upregulated at this time (Song et al., 2000) (Figure 1-1). For example substantial granzyme B (grB) gene transcription, an important granule constituent of the CTL (see Section 1.5.2.2), begins only after 48 hours of *in vitro* activation of primary murine $CD8^+$ T cells, and peaks after 5 days have passed (Babichuk et al., 1996; Kelso et al., 2002). Little is known about the processes inducing transcription of these late genes, many of which are important for the establishment of CTL cytotoxicity and are essential components of the granules that develop in the CTL at this time. Determining proteins that control transcription of these genes is vital in elucidating the processes involved in late stage CTL activation, and this knowledge could be applied in clinical situations where CTL activity is important. The inactivation of such

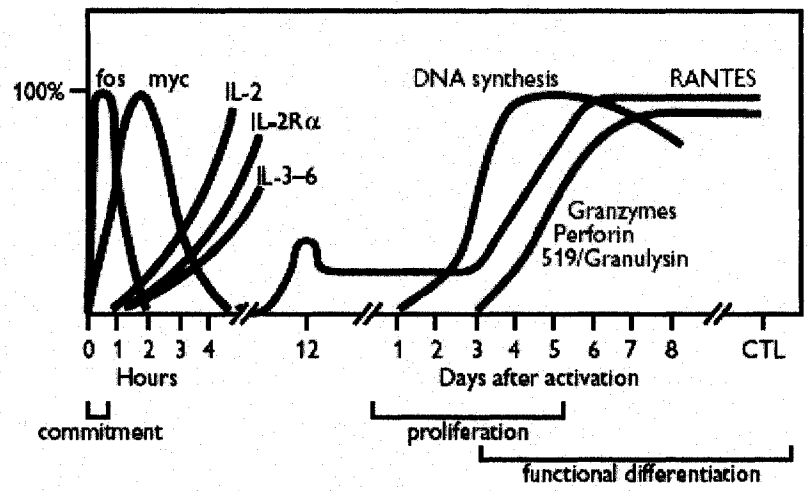


Figure 1-1. Kinetics of protein expression in activated cytotoxic T lymphocytes

This figure illustrates the late expression of the CTL effector proteins: the granzymes, perforin, granulysin, and RANTES. Please see Section 1.2.1 for more details on grB transcriptional regulation. Abbreviations: IL, interleukin, IL-2 Rα, IL-2 receptor α chain (Song et al., 2000).

transcription factors to decrease the killing efficiency of CTLs could be useful in treating autoimmune diseases or in graft versus host disease. Increasing the cytotoxicity of CTLs in a controlled manner could be used in the treatment of cancer or virus infection. The study of grB gene transcription in particular allows the description of an inducible, cell type-restricted gene.

1.2.2 Cytotoxic granule biogenesis and secretion

Cytotoxic granules are defined as secretory lysosomes, containing a dense core of pro-apoptotic proteins required for granule-mediated target cell death and surrounded by a multilamellar region similar to lysosomes (Burkhardt et al., 1990). These granules are found both in effector CTLs and NK cells, but for the scope of this thesis, their formation and structure will be described in the context of the CTL. Secretory granules first appear as multivesicular structures that slowly acquire dense cores as newly translated secretory proteins accumulate within the organelles (Stinchcombe et al., 2000). These organelles are acidic, with a pH of 5.1-5.4 and act in the CTL both as lysosomal organelle, and as a secretory granule (Clark and Griffiths, 2003). The secretory lysosome differs from normal lysosomes in that it can fuse with the plasma membrane in a regulated fashion in response to stimulus, namely the activation of the TCR by a virally-infected or tumour cell. This stimulus initiates tyrosine kinase signalling cascades and calcium influx that redistributes adhesion and signalling molecules to the region of contact with the target cell, also called the synapse, which consists of two regions. The central area, called the central supramolecular activation complex (c-SMAC) contains the TCR and CD8

molecules, and is surrounded by a ring of adhesion proteins that makes the protein-SMAC, or p-SMAC. The secretory granules are transported by microtubule-dependent motor proteins to the synapse that is dependent on the calcium influx generated by TCR activation, and thus granule-mediated cytotoxicity can be inhibited by the chelation of calcium (Kupfer et al., 1983). The contents of the lytic granules are released in a secretory domain between the p-SMAC and c-SMAC (Stinchcombe et al., 2001). The tight synapse formed between target and effector cells ensures that the lytic proteins released from the CTL will be quickly taken up to the target cell, and therefore avoid damage to other neighbouring cells. This mechanism of targeted exocytosis of granules allows the CTL killing machinery to be directed very specifically to the target cell, and to release the contents of the cytotoxic granules in a very concentrated manner.

1.3 Granule Contents

The major proteins found in the granules of the CTL are chondroitin sulphate-rich proteoglycans, perforin, and granzymes (Peters et al., 1991). There are also other cytotoxic proteins found in granules, such as Fas ligand and some cytokines. Other proteins not exclusively found in CTLs and NK cells that are contained in these granules are calreticulin, and other proteins normally found in lysosomes. These proteins, and the mechanisms of their processing, targeting and storage in granules, are discussed in the following sections.

1.3.1 Granzymes and perforin

The lytic proteins, perforin and the granzymes, are found to be associated with proteoglycans both in the acidic environment of the granule and during exocytosis (Metkar et al., 2002; Raja et al., 2002). The proteoglycans are thought to be important in packaging of these proteins into granules, and to protect these proteins from inhibitors present in the extracellular environment. In the absence of these proteoglycans the cytotoxic granule does not properly mature (Grujic et al., 2005).

The lytic proteins are targeted to the granule through specific mechanisms. The granzymes become mannose-6-phosphorylated in the golgi apparatus, and are consequently sorted into the granule by the mannose-6-phosphate receptor (MPR) (Burkhardt et al., 1989; Griffiths and Isaaz, 1993). This modification has become important in models of CTL killing, since it has been proposed that uptake of granzymes is dependent on binding to the cation independent MPR (CI-MPR) on the surface of target cells (Motyka et al., 2000; Veugelers et al., 2006). Perforin, a pore-forming molecule essential to CTL granular killing, is sorted by an independent unknown mechanism into lytic granules and in this acidic environment it is proteolytically activated into a mature form (Uellner et al., 1997). The participation of these important cytolytic enzymes in granule-mediated apoptosis will be further discussed in a later section (see Section 1.5.2).

1.3.2 Other cytotoxic granule proteins

An important cytolytic protein present in granules is Fas ligand (FasL), and is initiates a distinct pathway of apoptosis in target cells, to be discussed in a later section (see Section 1.5.1). This protein was first thought to be present exclusively on the surface of CTLs, but has now been shown to also be stored in cytotoxic granules (Bossi and Griffiths, 1999; Kojima et al., 2002). Unlike granzymes, perforin, and granulysin, which must be internalised by the target cell to induce apoptotic pathways, FasL activates cell death at the plasma membrane (see Section 1.5.1), and the stored FasL translocates in a polarised manner to the plamsa membrane where the synapse with the target cell has formed. This allows FasL-induced apoptosis to occur in a directed manner to the target cell, and avoids non-specific bystander killing (Kojima et al., 1994). FasL is delivered to the granules from the trans-golgi network following a distinct sorting mechanism (Bossi and Griffiths, 1999).

Chemokines, such as RANTES and macrophage inflammatory protein (MIP-1α), have also been found in cytotoxic granules and are important in lysis of virion-producing cells in HIV-1 infection (Wagner et al., 1998). Another protein present in cytotoxic granules is granulysin (Clayberger and Krensky, 2003). Granulysin is a member of the saposin-like protein family (Munford et al., 1995) and is lytic against microbes and tumours (Gamen et al., 1998). There is no granulysin homologue in mice, and its mechanisms of action will not be discussed further.

1.3.3 Non-cytotoxic granule proteins

Calreticulin (CRT) is calcium storage protein and chaperone normally found in the endoplasmic reticulum, but has also been found in the cytotoxic granules of CTLs, in close interaction with perforin (Dupuis et al., 1993). CRT expression is upregulated upon CTL activation (Burns et al., 1992) and CRT-perforin interaction occurs only in the absence of calcium (Andrin et al., 1998), and inhibits perforin function (Fraser et al., 1998). The exact role for CRT in lytic granules is not fully understood although CTLs derived from knockout mice possess functional lytic machinery, demonstrating that CRT is not essential either as a chaperone protein for perforin, to protect CTLs from the perforin they express, or to allow proper sorting of perforin or other granule components (Sipione et al., 2005).

Lysosomal hydrolases are found in the periphery of the cytolytic granules, such as cathepsin B, C, D and cathepsin A-like protective protein, acid phosphatase, arylsulphatase and α-glucosidase. The cysteine proteinase cathepsin C is responsible for the proteolytic maturation of the enzymes granzyme A and B (Pham and Ley, 1999). Lysosomal membrane proteins are also present on the membrane surface of cytotoxic granules, such as Lamp-1, Lamp-2 and CD63.

1.4 Cell death

The death of a cell in a multicellular organism is sometimes necessary for normal development and homeostasis, and will be discussed in the following sections. Cell death occurs via apoptosis or necrosis and are each associated with distinct descriptive cellular morphology (Uchiyama, 1995). The mechanisms of apoptosis have been studied extensively, and can be initiated through a variety of pathways. A family of proteases, termed caspases, have been shown as major effectors of the apoptotic process, as well as a number of proteins normally found within the mitochondria. Programmed cell death has also found to occur using a variety of other pathways in cells, involving other organelles such as the endoplasmic reticulum and lysosomes. The mechanisms of cell death will be discussed in the following sections.

1.4.1 Apoptosis and Necrosis

Necrosis was originally thought to be a passive process, an "accidental" form of cell death resulting from extreme environmental pressure. Necrosis begins in the cell with organelle swelling and is accompanied by early disruption of the cell membrane resulting in the release of cellular contents that leads to inflammation of surrounding tissues. Apoptosis, in contrast, is a quiet form of cell death where cellular contents remain neatly packaged in membrane-enclosed apoptotic bodies to facilitate easy clean-up by macrophages. Although necrosis is a messier form of death and invokes a response from the body, it is now seen as a normal event in many body systems, such as embryogenesis,

and immune responses (Assuncao Guimaraes and Linden, 2004; Proskuryakov et al., 2003). Apoptosis is a process definitely requiring the participation of the dying cell, and is appropriately referred to as cell suicide. This process can be initiated by intrinsic or extrinsic stimuli and is important in the immune system to eliminate dangerous cells from the body, such as virally-infected or tumour cells. Apoptosis of target cells is directed by the cytotoxic lymphocytes of the immune system, namely CTLs and NK cells. These cells induce death in their targets through two methods, the activation of death receptors present on the surface of susceptible cells (see Section 1.5.1), or by the endocytosis of cytotoxic granules (see Section 1.5.2).

1.4.2 Hallmarks of Apoptosis

Cells undergoing apoptosis can be recognised by a variety of distinct morphological and biochemical changes. Morphologically, these cells detach and shrink while maintaining organelle integrity. There is characteristic blebbing of the plasma membrane, and condensation and fragmentation of the chromatin. The cell breaks up into small apoptotic bodies that are cleaned up efficiently by macrophages (Kerr et al., 1972). There are a number of biochemical markers that accompany this process. The apoptotic bodies are phagocytosed so efficiently because of the characteristic externalisation of phosphatidylserine (PS) on the outer membrane. In healthy cells, PS is exclusively found on the inner leaflet of the plasma membrane, because of the actions of flipase enzymes that maintain the asymmetrical distribution of this phospholipid in an energy dependent process (Tang et al., 1996). During apoptosis, the flipase activity is inhibited while a PS

scramblase is activated, to create a symmetrical distribution of PS, thus exposing PS to the outer leaflet of the plasma membrane. PS exposure is an "eat me" signal for phagocytic cells like macrophages and dendritic cells (Fadok et al., 1992), allowing apoptotic cells to be removed and disposed of in the body with minimal inflammation (Fadok et al., 1998). Another important biochemical marker of apoptosis is the loss of mitochondrial membrane potential and the release of pro-apoptotic proteins such as cytochrome c from the mitochondrial intermembrane space into the cytoplasm of the cell (discussed in Section 1.4.4). Finally, one of the most striking biochemical changes during apoptosis is the characteristic internucleosomal degradation of chromosomal DNA (Figure 1-2), creating a "ladder" of oligonucleosomal fragments when apoptotic DNA is visualised by agarose gel electrophoresis (Skalka et al., 1976; Wyllie, 1980). This specific degradation occurs when caspase-activated DNAse, or CAD, is activated in the apoptotic process. The caspase enzymes will be discussed in the next section (Section 1.4.3).

1.4.3 Caspases and apoptosis

Apoptosis is dependent on complex biochemical processes that occur in the target cell, and are very much dependent on the activation of a distinct family of proteases called caspases. Caspases (cysteinyl aspartate-specific proteinases) are proteases that have a cysteine residue in the active site, and cleave substrates on the carboxyl side of aspartate residues (Stennicke and Salvesen, 1999). These proteins are present in every cell as inactive pro-enzymes that contain an inhibitory domain at their amino-terminal, and a

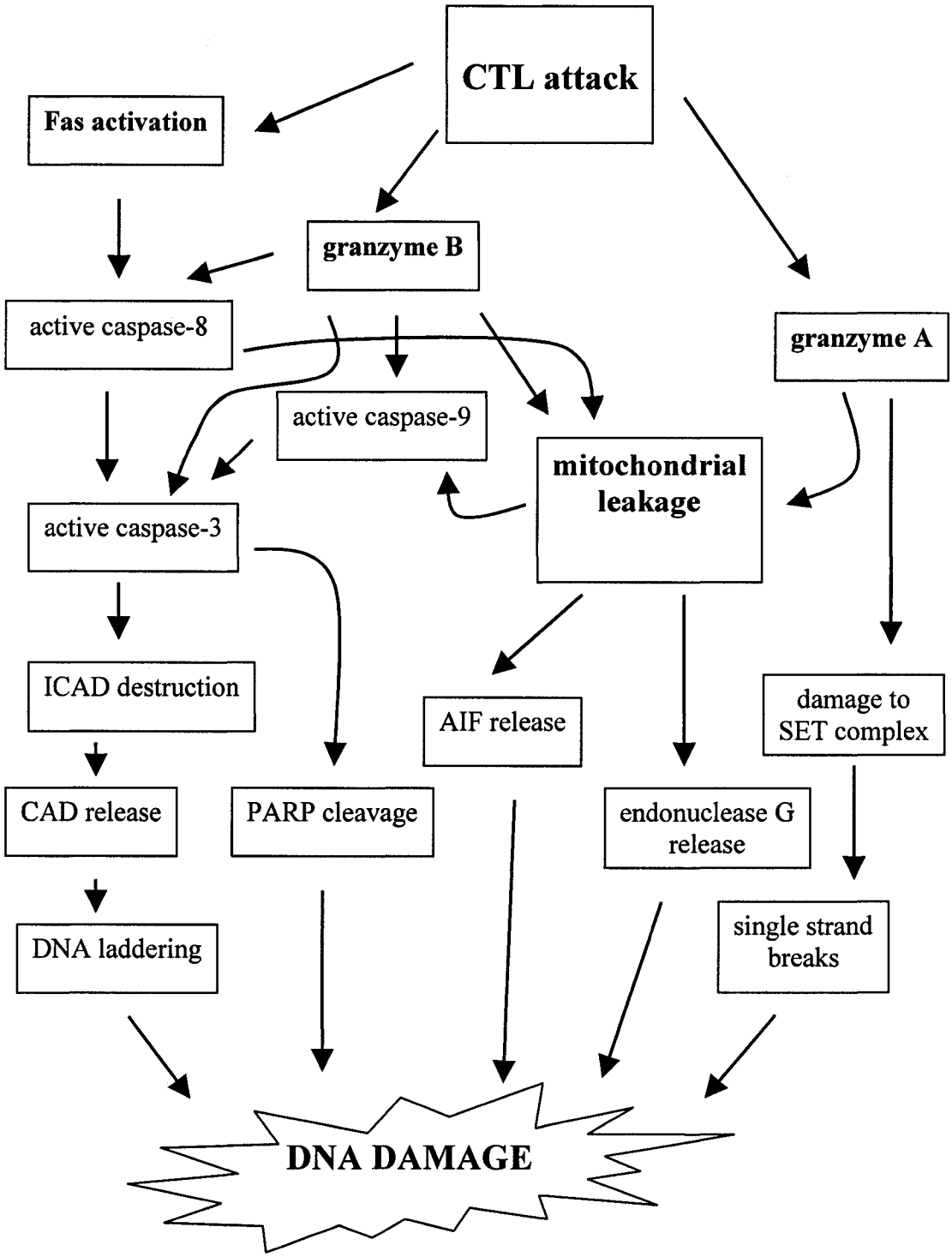


Figure 1-2. CTL-mediated DNA damage.

The molecular mechanisms leading to DNA damage during CTL-mediated apoptosis are illustrated. Included in this diagram are the pro-apoptotic proteins released by the loss of mitochondrial membrane integrity, and the DNA laddering that occurs after caspase-3 activation. Granzyme A causes DNA damage by inducing single-stranded breaks.

small and large subunit. The enzyme is activated when the pro-domain is cleaved from the rest of the protein, and the small and large subunits are proteolytically separated and associate to form a hetereodimer. The active caspases are tetramers formed from two heterodimers with two active sites (Walker et al., 1994). The linker regions between each of these domains contain caspase cleavage sites, and caspases are either activated by autocatalysis, or by other caspases. Caspases are thought to be either involved in cell death or in pro-inflammatory processes. The pro-apoptotic caspases are divided into two categories. Upstream or initiator caspases are activated at the beginning of apoptotic cascades, generally through auto-catalysis after pro-enzymes are recruited and clustered in response to an apoptotic signal, as during Fas-mediated cell death (see Section 1.5.1). Downstream or effector caspases are directly or indirectly activated by upstream caspases and can also be cleaved by the CTL protein granzyme B (see Section 1.5.2.2.2). These effector caspases, such as caspase-3, act as the executioners of the cell (Slee et al., 2001) and cleave substrates throughout the cell causing the hallmarks of programmed cell death (Ho and Hawkins, 2005). These substrates will be detailed in the following section.

1.4.3.1 Caspase substrates

One of the best characterised examples of caspase substrates is the PARP protein (poly-(ADP-ribose) polymerase, a DNA repair enzyme that is cleaved in the early stages of apoptosis (Duriez and Shah, 1997). Another important substrate is ICAD, or inhibitor of caspase-activated DNAse. This inhibitory protein is normally bound to CAD (caspase-

activated DNAse) in the cytoplasm of a healthy cell. When cleaved by an effector caspase, ICAD no longer inhibits the CAD enzyme, which translocates to the nucleus and is responsible for the internucleosomal degradation of the chromosomal DNA (Figure 1-2), producing the hallmark DNA "ladder" found during apoptosis (Enari et al., 1998; Liu et al., 1997). Caspases also cleave a number of structural proteins, such as lamins, actin, and fodrin, leading to cell shrinkage and cytoskeletal rearrangements (Martin et al., 1995; Mashima et al., 1995; Orth et al., 1996). The loss of mitochondrial potential and leakage of mitochondrial proteins can also be caused by caspase activity during apoptosis, such as during Fas-mediated cell death (discussed in Section 1.5.1). However, many other signals such as Ca^{2+} overload signal to the mitochondria to initiate an apoptotic cascade. Release of the mitochondrial protein cytochrome c has been shown as essential for the activation of caspase-9 and the so-called apoptosome, which will be discussed in a later section (Section 1.4.4.1).

1.4.4 Mitochondria in apoptosis

Apoptosis is almost always mediated at least partially through the mitochondria. These organelles are the powerhouses of the cell, supplying energy in the form of ATP. These organelles lose mitochondrial membrane potential early on in apoptosis, thus halting the energy production for the cell, as well as releasing many proteins involved in cell death from the inter-membrane space. The mitochondrial pathway of apoptosis can be initiated indirectly by caspases, directly from death signalling to the cell, or from intrinsic

signalling from the cell itself. The molecular pathway of mitochondrial dependent cell death will be described in the following sections (Figure 1-3).

1.4.4.1 The formation of the permeability transition pore (PTP)

Mitochondrial involvement in apoptosis begins with the loss of mitochondrial membrane potential and the release of proteins in the intermembrane space. These changes are the result of pore formation, called the permeability transition pore (PTP), spanning the inner and outer mitochondrial membranes (Bernardi et al., 2006; Crompton, 1999). This pore is thought to consist of the mitochondrial proteins adenine nucleotide translocator (ANT) in the inner membrane, the voltage-dependent anion channel (VDAC) in the outer membrane (Zamzami and Kroemer, 2001). The PTP causes depolarisation and swelling of the organelle, resulting in rupture of the outer membrane, and release of proteins residing in the intermembrane space. One of the first of these proteins found to be important in the apoptotic process was cytochrome c (Liu et al., 1996). This protein normally associated with the electron transport chain in the mitochondria but when released into the cytosol initiates formation of the apoptosome, a multi-protein complex containing two other proteins released from the mitochondria; apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9; together with dATP (Zou et al., 1997). The formation of this complex activates caspase-9, which can then activate the executioner caspase-3. However, caspase-9 can still be inhibited after mitochondrial release by cytosolic proteins called inhibitors of apoptosis (IAPs). This inhibition is removed by other pro-apoptotic proteins that are also released from the mitochondria,

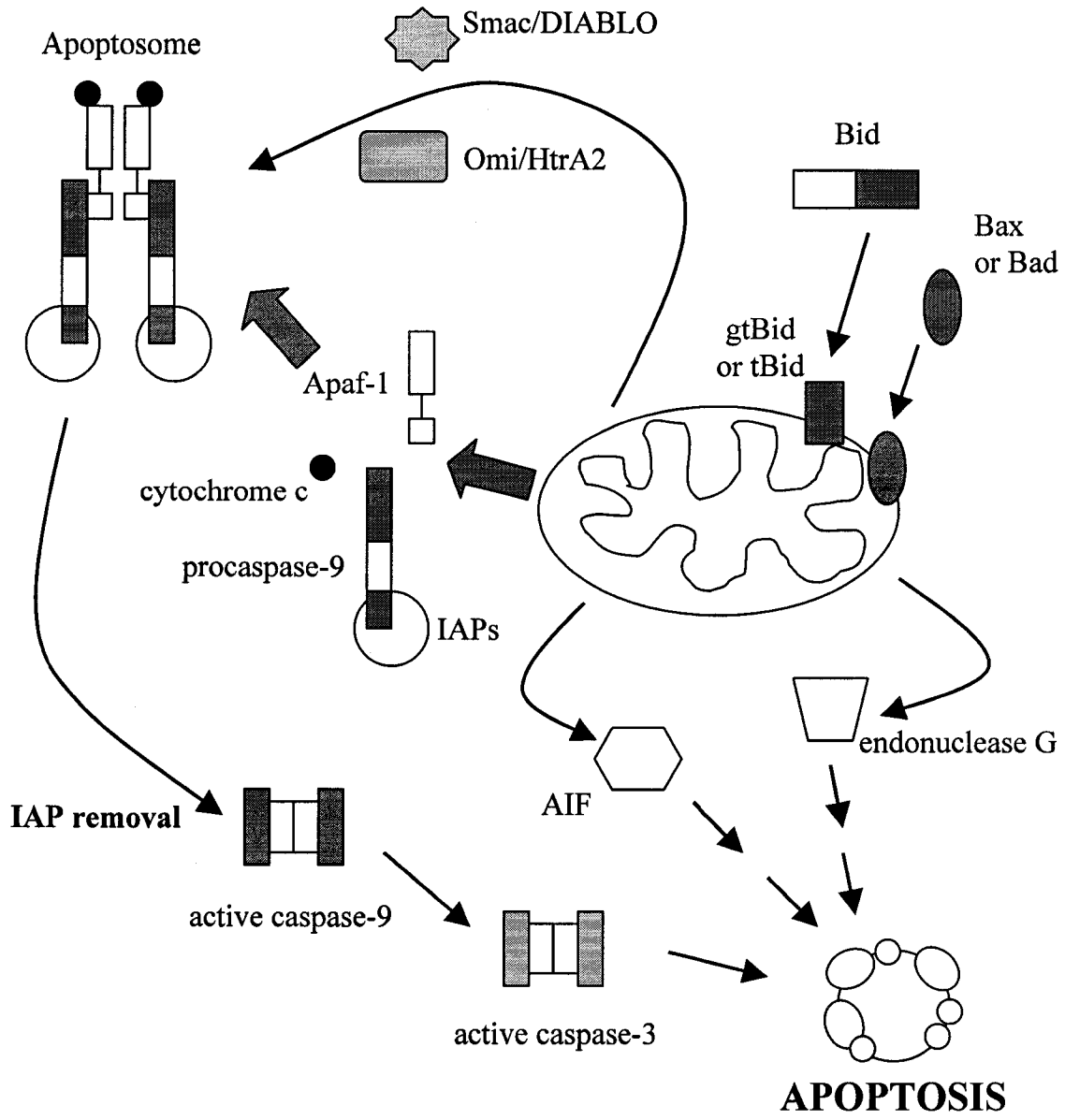


Figure 1-3. Mitochondrial pathway of apoptosis.

The molecular mechanisms of apoptosis resulting from loss of mitochondrial potential are illustrated, and release of pro-apoptotic proteins from the intermembrane space is shown. It is important to note that caspase-9 and caspase-3 activation is blocked by IAP binding, until the mitochondrial proteins Smac/DIABLO and Omi/HtrA2 release this inhibition. Please see Section 1.4.4 for more details.

called second mitochondria-derived activator of caspase (Smac)/Direct inhibitor of apoptosis-binding protein with low pI (DIABLO) (Du et al., 2000) and Omi/HtrA2 (Hegde et al., 2002). Other proteins similarly released, such as apoptosis-inducing factor or AIF (Zamzami et al., 1996) and endonuclease G (Li et al., 2001), are thought to contribute to caspase-independent apoptosis (see Section 1.4.5), including DNA damage (Figure 1-2).

1.4.4.2 Mitochondrial permeabilisation without formation of the PTP

The PTP is not the only method of releasing proteins from the mitochondria. In some cases, cytochrome c release precedes loss of membrane potential, which occurs during the formation of the PTP (Scorrano et al., 2002). This release is the result of the formation of another channel in the mitochondria, produced from the oligomerisation of the pro-apoptotic proteins Bak and Bax. Bax and Bak belong to a large family of proteins, the Bcl-2 family. These proteins can be divided into pro-apoptotic and anti-apoptotic members. The pro-apoptotic proteins Bak and Bax are known to oligomerise to form channels in liposomes and isolated mitochondria (Korsmeyer et al., 2000), and it is thought that these proteins can form channels that releases at least some of the cytochrome c from within mitochondria to induce apoptosis (Scorrano et al., 2002). Other pro-apoptotic members of this family such as Bid and Bim do not form pores themselves, but are thought to activate the pore-forming activity of Bak and Bax after Bim or Bid are activated through cleavage (Desagher et al., 1999). The proteins Bid and Bim are also able to initiate apoptosis by activating the formation of the PTP (Zamzami

and Kroemer, 2001). In CTL-mediated cell death, the protein Bid is cleaved by caspase-8 in Fas-mediated apoptosis (see Section 1.5.1), and by grB in granule-mediated apoptosis (see Section 1.5.2.2.2), which causes the truncated protein to move to the mitochondria to induce pore formation. The other group of proteins in the Bcl family is anti-apoptotic. These proteins, such as Bcl-2 and Bcl-XL, are thought to interfere with Bax or Bak pore formation although the mechanism for this protection is still unknown (Verma et al., 2006). In summary, mitochondria are important players in programmed cell death, and essential mediators of this pathway are the Bcl-2 family proteins.

1.4.5 Caspase-independent cell death

Over the years, it has become more recognised that dividing cell death into the two categories; apoptosis or programmed cell death, and necrosis or accidental cell death; is overly simplistic. Not only is necrosis now being see as a programmed event (see Section 1.4.1), but there is increasing evidence that there are other valid forms of programmed cell death (PCD) that do not follow that classical pathways of apoptosis. One such mechanism is caspase-independent cell death, which was first described when cell death was induced by over-expression of the pro-apoptotic protein Bax in the presence of *pan*-caspase inhibitors to stop the caspase cascades associated with apoptosis (Xiang et al., 1996). This treatment resulted in the cell losing mitochondrial potential and plasma membrane permeability, but this cell death did not have the classical hallmarks of apoptosis such as DNA laddering and PARP cleavage. More recently, other forms of PCD have been described, such as autophagy, a form of PCD that is thought to occur

when a cell undergoes "self-digestion" by its own lysosomal system in a caspase-independent manner (Gozuacik and Kimchi, 2004).

1.4.5.1 Organelles involved in caspase-independent cell death

There have been numerous forms of PCD defined, including "apoptosis-like PCD" and "necrosis-like PCD" (Clarke, 1990). These types of cell death can be initiated by numerous proteins found in both the mitochondria, the endoplasmic reticulum (ER) and the lysosome. In the mitochondria, numerous proteins released from the intermembrane mitochondrial space have been implicated in caspase-independent cell death. The protein Omi/HtrA 2, besides being involved in the classical apoptotic pathway (described in Section 14.4.1), can also act as an effector protein in necrosis-like PCD. Similarly, endonuclease G has also been shown to fragment DNA in isolated nuclei independent of caspase involvement (Li et al., 2001). Finally, the protein AIF has shown to initiate PCD when caspases are inhibited (Loeffler et al., 2001; Susin et al., 1999).

Cathepsin B and other lysosomal proteases have also been implicated in caspase-independent cell death (Broker et al., 2004; Guicciardi et al., 2004; Stoka et al., 2001). It is also thought that these proteins induce PCD indirectly by initiating release of mitochondrial proteins, either through the activation of Bid or Bax to form pores in the mitochondria (Bidere et al., 2003; Heinrich et al., 2004) or by cleaving caspases themselves (Vancompernolle et al., 1998). The ER is an important sensor of stress within the cell, and can signal PCD by release of calcium from within the ER or through the

unfolded protein response (Breckenridge et al., 2003). The ER is able to induce mitochondrial membrane permeabilisation through cytoplasmic calcium influx and the Bcl-2 family of proteins to induce conventional apoptotic signalling (Annis et al., 2004; Mattson and Chan, 2003). This ER-induced calcium release also activates calpains, cytosolic proteases that can contribute to PCD in the absence of caspases (Mathiasen et al., 2002). These caspase-independent pathways of PCD can also be initiated by CTLs, as when granzyme B induces mitochondrial protein release independent of caspase activation (see Section 1.5.2.2.2), thus ensuring target cell death even when caspase activation is inhibited.

1.5 Killing Mechanisms of CTLs

Effector CTLs induce apoptosis in target cells using two mechanisms, the Fas-mediated pathway, and the granule-mediated pathway. Fas-mediated cell death is thought to play a major role in thymocyte development, while granule-mediated cell death is thought to be the major mechanisms for elimination of virally-infected and tumour cells. However, recent findings have proved that these two mechanisms are not so easily separated. Fas ligand (FasL) has now been shown to be stored in cytotoxic granules (see Section 1.3.2), and perforin deficiency in humans show the importance of the granule-mediated pathway in the downregulation of the immune response (Stepp et al., 1999). The following sections will describe the mechanisms of these two important pathways.

1.5.1 Fas-mediated cell death

Death receptors of the tumour-necrosis factor receptor (TNFR) family, including the FasL protein, are expressed on the surface of effector CTLs (Figure 1-4). The FasL protein has found to induce Ca^{2+}-independent apoptosis in target cells expressing the Fas receptor, also known as CD95 (Rouvier et al., 1993). This receptor-ligand binding causes clustering of the receptor molecules, and recruitment of signalling molecules to form a multi-protein complex called the DISC, or death inducing signalling complex (Kischkel et al., 1995). The DISC consists of a docking protein FADD (Fas-associated death domain) and pro-caspase-8. The clustering of the Fas protein causes FADD proteins to bind to the intracellular tail of the Fas receptors via the presence of homologous death domain protein motifs in both FADD and Fas (Boldin et al., 1995; Chinnaiyan et al., 1995). This protein scaffold then recruits pro-caspase-8 molecules by binding of homologous death effector domains (DEDs) present in both FADD and pro-domain of caspase-8. The close association of a number of pro-caspase-8 proteins allows the slight activity of the pro-enzyme to trans-activate other caspase-8 molecules in close proximity (Medema et al., 1997; Muzio et al., 1998). Activated caspase-8 then initiates apoptosis in one of two ways, depending on the cell type (Scaffidi et al., 1998). In type I cells, the effector caspase-3 is activated directly by caspase-8, while in type II cells, caspase-8 cleaves Bid, a pro-apoptotic member of the Bcl family of proteins, to create a truncated form, or tBid (van Loo et al., 2002). This protein will move to the mitochondria to initiate the mitochondrial pathway of apoptosis (see Section 1.4.4.2) (Kuwana et al.,

2002). The second method used by CTLs to induce apoptosis is the granule-mediated pathway, to be discussed in the following sections.

1.5.2 Granule-mediated cell death

The granule exocytosis pathway has become well established as the major pathway of CTL-induced cell death. This pathway begins with the targeted secretion of granules that releases the granule contents into a target cell to induce both caspase-dependent, and caspase-independent cell death. The major mediators of granule-mediated cell death are the pore-forming protein perforin, and the family of serine proteases called granzymes. These proteins will be discussed in detail in the following sections.

1.5.2.1 Perforin

Perforin was first identified as a lytic molecule found in cytotoxic granules (Millard et al., 1984; Podack et al., 1985). Perforin was found to have a region of strong homology to the pore-forming C9 protein of complement (Tschopp et al., 1986) and was consequently shown to polymerise and form pores in the membranes of red blood cells (Sauer et al., 1991). Perforin is inactive in the acidic environment of the cytotoxic granule, but forms pores when released into the neutral environment of the intermembrane space in the presence of Ca^{2+}. This lead to the proposition that CTLs caused death by releasing perforin to disrupt the plasma membrane of the target cell through pore formation (Henkart and Henkart, 1982). However, CTL-induced apoptosis

does not only result in cell lysis and membrane damage, but includes such markers of apoptosis as PS externalisation, cleavage of cellular molecules, and DNA fragmentation, suggesting additional factors besides perforin was involved in CTL-mediated cell death (Duke et al., 1983). These additional factors were later identified as a family of serine proteases, now called granzymes, which will be discussed in the next section (see Section 1.5.2) (Lobe et al., 1986; Redmond et al., 1987). The generation of perforin knockout mice showed the critical role of perforin for granule-mediated cytotoxicity of the CTL (Kagi et al., 1996), and to allow the delivery of granzymes into the cytoplasm of target cells. This delivery was originally visualised with granzymes simply entering into target cells through perforin pores formed in the plasma membrane. However, addition of sublytic concentrations of perforin that do not form pores in the plasma membrane is still sufficient to enable granzyme entry into target cells. Surprisingly, granzymes have also been shown to enter into target cells independently of perforin, but the proteases remain harmlessly confined to endocytic vesicles until perforin, or another permeabilising agent, releases them (Froelich et al., 1996; Pinkoski et al., 1998). Although the mechanisms remain unclear, it is now generally thought that perforin acts as an endosomolytic agent to release the granzymes from these endocytic compartments. Indeed, recent evidence has shown that endocytosis is absolutely required for *in vivo* uptake of the granzymes into target cells (Veugelers et al., 2004; Veugelers et al., 2006). Granzyme B entry into target cells will be discussed in more detail in a following section (Section 1.5.2.2.1).

1.5.2.2 Granzyme B

Granzyme B (grB) gene was first identified as being upregulated in CTL effectors (Lobe et al., 1986), and was later shown to induce DNA fragmentation when added with perforin to target cells (Nakajima et al., 1995; Shi et al., 1992). The generation of grB knockout mice confirmed the importance of grB in the induction of rapid DNA fragmentation in target cells (Heusel et al., 1994). The mechanisms of grB target cell entry and grB-induced cell death will be discussed in the following sections.

1.5.2.2.1 Granzyme B entry into target cells

As described previously (Section 1.5.2.1), grB and other granzymes have been shown to enter target cells into endocytic compartments in the absence of perforin, although subsequent addition of perforin is required to release the granzymes into the cytoplasm. These findings, along with studies demonstrating that grB binds to target cell surface in a saturable manner, suggests the presence of a receptor for grB on the surface of the target cell. This receptor was identified as the cation-independent mannose-6-phosphate receptor (CI-MPR) (Motyka et al., 2000). It was postulated that grB binding occurs through its post-translational modifications, namely mannose-6-phosphorylation (discussed in Section 1.3.1), and the protease enters the target cell through receptor-mediated endocytosis. Although this study showed grB entry into target cells as dependent on MPR expression, other work has suggested that this CI-MPR pathway can be circumvented in cells lacking MPR expression, and even enter cells independent of

dynamin-dependent endocytosis (Dressel et al., 2004; Trapani et al., 2003). However, conclusions were drawn from experiments in these studies that assumed granule-mediated cell death was exclusively grB dependent, when in fact FasL can also be released in granules (Bossi and Griffiths, 1999). Complicating matters is the use of recombinant grB in some uptake studies. Recent evidence suggests that grB is complexed with proteoglycans, specifically serglycin (SG) (also discussed in Section 1.3.1), and this large macromolecule (greater than 250 kDa) is the native form of grB *in vivo* (Metkar et al., 2002; Raja et al., 2002). The recombinant grB is not associated with these proteoglycans, is not mannose-6-phophorylated, is highly cationic and is now thought to bind non-specifically onto the surface of target cells because of its highly charged nature (Shi et al., 2005). Findings from experiments using this recombinant grB cannot be used to determine the mechanism of native grB uptake.

Further uptake studies using the physiologically relevant grB-SG complex demonstrated the requirement for dynamin-dependent endocytosis for grB-SG target cell entry (Veugelers et al., 2004), and that this entry requires the CI-MPR (Veugelers et al., 2006). Other recent studies have suggested that cell surface heparin sulfate is important for grB-SG uptake into target cells (Bird et al., 2005; Kurschus et al., 2005; Raja et al., 2005; Shi et al., 2005), and even that grB-SG may dissociate at the cell surface so that grB instead becomes bound with surface heparin sulfate (Raja et al., 2005). Intriguingly, these findings support the MPR uptake model, as interaction with heparin sulfate serves to increase interactions of grB-SG complex with the MPR (Veugelers et al., 2006). Indeed, heparin sulfate is an important cofactor in other receptor binding mechanisms (Cohen et

al., 1995; Lyon et al., 1997; Yayon et al., 1991; Zioncheck et al., 1995). It has been proposed that both perforin and granzymes are complexed into a large macromolecular entity with proteoglycans, and it is this large particle which perhaps binds to the target cell (Metkar et al., 2002). The discovery of CI-MPR as a receptor for grB-SG suggests that such a macromolecular entity would allow delivery of both the granzyme effector molecules and the endosomolytic perforin together into a single endocytic compartment inside the target cell, thus facilitating release of the effector molecules into the cytosol. Once released into the cytosol, the granzymes induce apoptosis through cleavage of substrates within the target cell. Substrates for granzyme B will be discussed in the next section, and the other granzymes will be reviewed in following sections.

1.5.2.2.2 Granzyme B substrates

Granzyme B cleaves substrates on the carboxyl side of aspartate residues (Caputo et al., 1994), which is a unique specificity among serine proteases, but is the same as the substrate specificity of caspases. The most important substrates of caspases are the proteases themselves, since caspases are either activated by autocatalysis or by other caspases (see Section 1.4.3). It was therefore postulated that grB induced cell death through its activation of these pro-apoptotic proteins. Indeed, grB was shown to directly cleave and activate caspase-3 both *in vitro* and *in vivo* (Atkinson et al., 1998; Darmon et al., 1996; Darmon et al., 1995). Caspase-3 is an executioner caspase, and its activation leads to apoptosis (see Section 1.4.3.1, Figure 1-4).

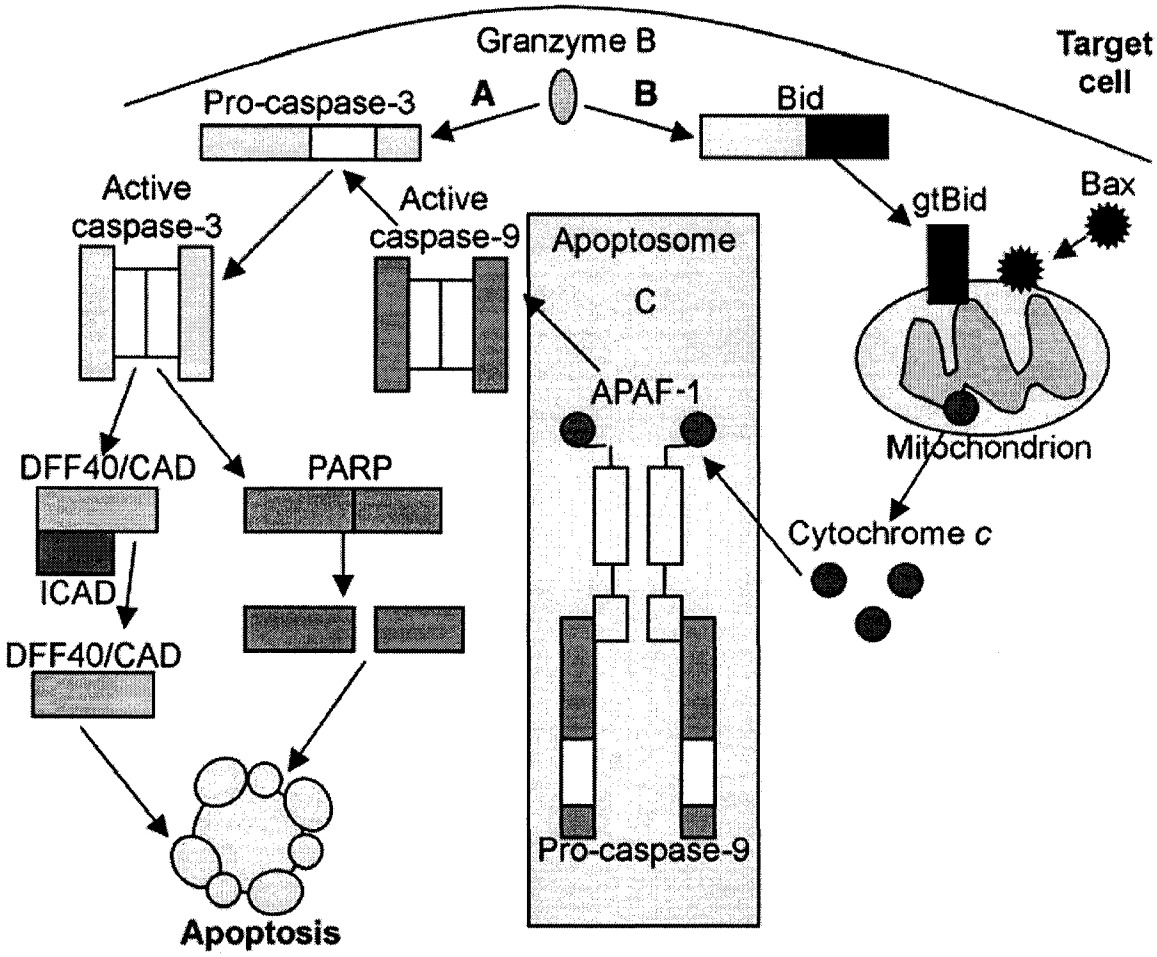


Figure 1-4. Granzyme B-mediated apoptosis.

The molecular events of the two major pathways of grB-mediated cell death are illustrated. In pathway **A**, grB directly cleaves and activates caspase-3. In pathway **B**, grB activates the mitochondrial pathways of apoptosis, leading to caspase-9 activation. **C** denotes the apoptosome. Adapted from (Lord et al., 2003). Please see Section 1.5.2.2.2 for more details.

Granzyme B has also been shown to initiate cell death independent of caspases (Sarin et al., 1997). It was determined that grB-induced cell death in the presence of caspase inhibitors did not stop loss of mitochondrial membrane potential or the release of cytochrome c from the mitochondrial intermembrane space (Heibein et al., 1999). Studies showed grB-induced mitochondrial damage was the result of grB cleavage of the pro-apoptotic protein Bid to form granzyme B-truncated Bid (gtBid) that initiates mitochondrial pore formation (see Section 1.4.4.2), thus inducing cytochrome c release (Barry et al., 2000; Heibein et al., 2000). Granzyme B had now been shown to initiate both a caspase-dependent (through cleavage of caspase-3) and caspase-independent (through cleavage of Bid) pathway leading to cell death. The importance of the mitochondrial pathway was soon determined, as complete caspase-3 activation did not occur without cytochrome c release (Pinkoski et al., 2001). This inhibition of caspase-3 was determined to be the result of IAP binding (see Section 1.4.4.1), and was relieved by the release of Smac/DIABLO from the mitochondria by gtBid (Goping et al., 2003). Granzyme B appears to cleave other substrates within the cell, multiple caspases (caspase-6, -7, -8, -9, -10) are substrates of grB *in vitro* (Lord et al., 2003). Granzyme B also cleaves other cellular proteins, such as α-tubulin (Goping et al., 2006); Hsp-70 (Loeb et al., 2006); and Hop (Hsp-70/Hsp-90 organising protein), an anti-apoptotic chaperone of Hsp70/Hsp-90 (Bredemeyer et al., 2004). The presence of multiple grB substrates within a cell may enhance apoptosis, but more importantly will provide alternate pathways of cell death in the event of immune evasion of a virally-infected or tumour cell.

1.5.2.3 Granzyme A

Granzyme A (grA) is the most abundant of the granzymes in the granules of CTLs (Pasternack and Eisen, 1985). Granzyme A induces caspase-independent apoptosis by inducing DNA damage by single stranded nicks (Beresford et al., 1999) and disrupting the mitochondrial membrane potential without the release of cytochrome c (Lieberman and Fan, 2003). Granzyme A cleaves proteins after arginine or lysine, and targets proteins found in the SET complex (Beresford et al., 2001; Fan et al., 2002; Fan et al., 2003b). This SET complex contains proteins involved with repairing DNA, modulating chromatin, and regulating transcription (Fan et al., 2003a), and is also thought to help repair DNA in response to oxidative damage (Fan et al., 2003b). The cleavage of the SET protein by grA releases the NM23-H1, a nucleoside diphosphate kinase responsible for the single-stranded nicking of the DNA in grA-induced apoptosis. The grA-dependent cleavage of Apel, a SET protein involved in DNA repair, also contributes to DNA damage. Granzyme A also induces nuclear destruction by cleaving nuclear structural proteins lamins (Zhang et al., 2001a) and histones, resulting in chromatin unfolding (Zhang et al., 2001b). The mechanism of grA-dependent mitochondrial disruption has not yet been elucidated.

1.5.2.4 Human and murine orphan granzymes

There are numerous other granzymes of unknown function, so-called "orphan" granzymes, found in human and murine cytotoxic granules, predominantly in LAK and

NK cells (Grossman et al., 2003). It is unclear if many of these granzymes are important in the apoptotic mechanisms of CTLs or NK cells; however three of these granzymes, namely the murine granzyme C, and the human and murine granzymes K and M; have been shown to induce apoptosis and will be discussed briefly in the following sections.

1.5.2.4.1 Granzyme C

Granzyme C is a granzyme found in mice but not humans (Pham et al., 1996), and closely resembles the human granzyme H. The murine grC gene is found immediately downstream of the grB gene, and is thought to have arisen from an early grB gene duplication. The human grH gene is found downstream of the human grB gene, and is thought to have arisen in a similar manner. Granzyme C has been shown to cause apoptosis by inducing caspase-independent mitochondrial depolarisation, nuclear collapse, and single-stranded DNA nicking (Johnson et al., 2003). Furthermore, CTLs derived from grB knockout mice with accompanying reduction of grC and granzyme F expression (the murine grF gene is immediately downstream of grC gene) have a more severe cytotoxic defect than grB knockout CTLs that do not lack grC and grF expression, suggesting the importance of grC or grF in apoptosis (Revell et al., 2005). The mechanisms of grC-induced apoptosis are still unknown, and human grH has not been shown to induce apoptosis (Edwards et al., 1999).

1.5.2.4.2 Granzyme K

The grK gene is found downstream of grA in both the human and mouse genome, and is thought to have arisen from an early gene duplication of the grA gene. Granzyme K, like grA, is a tryptase and the two granzymes are thought to have similar substrate specificity. Granzyme K has been shown to induce target cell death similar to grA-induced apoptosis (Shi et al., 1992). However grK does not induce mitochondrial depolarisation, as seen in grA-induced apoptosis, and so may induce cell death by a unique mechanism (MacDonald et al., 1999).

1.5.2.4.3 Granzyme M

The grM gene is present in both human and mouse genomes, and grM is expressed highly in NK cells, and not in T cells (Sayers et al., 2001). Granzyme M preferentially cleaves after methionine, leucine, or norleucine residues (Smyth et al., 1992). Recent studies have shown that grM induces PCD in target cells, although the mechanism of this death remains controversial (Kelly et al., 2004; Lu et al., 2006). The presence of another pro-apoptotic protein found in NK cells suggests diversity in which different cytotoxic cells induce cell death.

1.6 Thesis Objectives

Although the presence of grB in target cells is implied by the cleavage of target protein substrates, it has never been physically documented in target cells killed by CTLs. The CTL effector phenotype is characterized by the expression of grB, and this expression can be used as a marker for activated or stimulated $CD8^+$ T cells. Characterization of the processes involved in CTL activation *in vivo* can be used to develop methods for treatment of diseases associated with CTLs. We have labelled murine grB *in vivo*, by creating a grB-EGFP fusion protein expressed under the endogenous murine grB gene promoter in transgenic (Tg) mice. We have confirmed that CTLs generated from these Tg mice express grB-EGFP in activated CTLs, but not in resting $CD8^+$ T cells. These Tg CTLs function normally and they kill allogeneic target cells, and induce normal levels of target cell death. The generation of an *in vivo* marker of grB allows us to follow the kinetics of infection in the whole animal. We are also able to visualise the transfer of grB into target cells. My objectives for this thesis work include:

1) To determine if grB-EGFP expression can be used as a *in vivo* marker of CTL activation.
2) To show the physical transfer of endogenous grB from effector cells into target cells using grB-PE antibody staining.
3) To confirm the physical transfer of grB from effector cells into their target cells using the *in vivo* marker grB-EGFP.

2. Materials and Methods

2.1 Cloning, cell culture and transgenics

2.1.1 Generation of Reporter and Transgene Constructs

The pNC2 and pNC3 reporter constructs used in stable transfections were made as follows. Using the pEGFP-1 plasmid (Clonetech, Mountain View, CA) the EGFP cDNA was amplified by PCR. The PCR primers had an Xho I restriction site added to their 5' end. The upstream primer also contained 36 basepairs of coding sequence for the last 12 amino acids of the murine granzyme B gene, excluding the stop codon. The resulting PCR product contained the 3' end of the coding region of granzyme B, and the entire EGFP cDNA. The Xho I digested PCR fragment was inserted into the exon 5 Xho I site of the endogenous murine granzyme B gene. To confer G418 resistance in stable transfections, this granzyme B-EGFP fusion DNA fragment was then cloned back into a modified pEGFP-1 vector where the EGFP cDNA had been removed. G418 resistant clones containing the insert were identified, and the orientation of the EGFP cDNA was confirmed first by PCR and later by sequencing.

2.1.2 Cell culture

All cells were maintained in a humidified atmosphere of 5% CO_2 at a concentration of 2-5x10^5 cells/mL. The mouse lymphocytic leukemia L1210 cells (H-2^d), mouse lymphoma EL4 cells (H-2^b) and mouse T lymphocyte CTLL-R8 cells were obtained from American Type Culture Collection and were maintained in RHFM (RPMI 1640 medium supplemented with 20 mM HEPES, 1 mM sodium pyruvate, 50 U/mL penicillin, 50 μg/ml streptomycin, (Invitrogen Canada Inc., Burlington, ON), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich Ltd., St. Louis, MO), and 10% heat-inactivated fetal calf serum (HyClone, Logan, UT)). The CTLL-R8 cell media was also supplemented with 50 U/mL of recombinant human interleukin-2 (IL-2). CTL lines were grown in RHFM supplemented with 80 U/mL IL-2 (RHFM/IL-2 media). Cells were centrifuged in an Allegra 6R centrifuge (Beckman Coulter Inc., Fullerton, CA), typically at 1250 rpm for 4 minutes, and U-bottom 96-well plates (Falcon, BD Biosciences, Mississauga, ON) were centrifuged at 1450 rpm for 5 minutes.

2.1.3 Stable transfections of CTLL-R8 cells

CTLL-R8 cells were washed twice with centrifugation in RH (RPMI 1640 and 20 mM HEPES, Invitrogen Canada Inc., Burlington, ON) and resuspended in the same media at 1x10^7 cells/ml. Subsequently, 750 μl of cells were mixed with 10 μg of linear test plasmid. CTLL-R8 cells were electroporated by a single pulse at 260 V, 960 μF using a Gene Pulser (BioRad Laboratories, Hercules, CA). Cells were subsequently divided into

two 100 mm plates containing 12 mL RHFM. Forty-eight hours after transfection, 0.3 mg/ml G418 (Invitrogen Canada Inc., Burlington, ON) was added to the cells. After 7-14 days, bulk populations were analysed by an Aria Fluorescent Activated Cell Sorter (FACSAria) (BD Biociences, Mississauga, ON). The bulk populations containing the pNC2 and pNC3 constructs were sorted and selected EGFP-positive cells were allowed to grow in flat bottomed 96-well plates (Nalge Nunc International, Rochester, NY).

2.1.4 Generation of transgenic mice

The pNC2 construct was used to generate transgenic mice at the University of Alberta Transgenic Facility by pronuclear injection into fertilized oocytes from FVB/N mice (Taketo et al., 1991) then transferred into pseudopregnant females. Litters were screened for the presence of the transgene by PCR analysis of genomic tail DNA. Founder mice were propagated by serial backcrosses with FVB/N mice. C57Bl/6 wildtype (WT) mice, and granzyme B knockout (GrB KO) mice on a C57Bl/6 background were purchased from Jackson Laboratory (The Jackson Laboratory, Bar Harbor, ME). All mice used in this study were generated and maintained at the Health Sciences Laboratory Animal Services at the University of Alberta under specific pathogen-free conditions.

2.1.5 Generation of CTL lines

On day 0, spleens from transgenic (Tg) or WT littermate FVB/N ($H\text{-}2^q$) mice were dissected and the tissues were ground through a fine wire mesh in cold RHFM and the

cells pelleted. Red blood cells (RBCs) were lysed by resuspending the cell pellet in buffered ammonium chloride lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$ and 0.1 mM Na_2EDTA) and incubating samples at room temperature for 2 minutes. The RBC lysis buffer was then washed out by twice centrifuging cells and resuspending in RHFM. Resulting primary splenocytes were grown for 3 days in RHFM supplemented with 80 U/mL IL-2 (RHFM/IL-2) with an equal amount of gamma-irradiated stimulator splenocytes from Balb/c ($H\text{-}2^d$) mice in a mixed lymphocyte culture. Irradiated stimulators were removed by 20 minute centrifugation of samples (at 2100 rpm) that were layered over 5 mL of Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) on day 3, and CTLs were then resuspended and grown in RHFM/IL-2 as before. One more stimulation with irradiated Balb/c splenocytes was performed at day 7, at a ratio of 1:15 (CTLs:stimulators). On day 10 stimulators were again removed as described on day 3, and the EGFP-positive CTLs were sorted using a FACSAria (BD Biosciences, Mississauga, ON). These green Tg CTLs were used in subsequent killing assays, and the WT CTLs of FVB/N background as controls in these experiments. C57Bl/6 ($H\text{-}2^b$) mice were also used to generate wild type (GrB WT) and $grB^{-/-}$ (GrB KO) CTL lines using the same stimulators, and these lines were maintained for up to 10 cycles of stimulation and used for experimentation during that time.

2.2 Analysis of Tg CTLs

2.2.1 Confocal Microscopy

Primary murine splenocytes were derived as described previously, and activated with soluble αCD3 antibody (clone 145-2C11, 1/500 dilution of hybridoma supernatant) for 48 hours in RHFM/IL-2 media. Morphology of live activated cells was assessed on a LSM510 laser scanning confocal microscope mounted on a Zeiss Axiovert 100 M microscope (Carl Zeiss Canada Ltd., Toronto ON). EGFP-positive cells were visualized by excitation at 488 nm, with the fluorescence signal collected using a 505–530 nm band pass filter.

2.2.2 Northern blot analysis

Transgenic splenocytes were sorted for green fluorescence using a FACSAria and green fluorescent and non-fluorescent splenocytes were collected. RNA was collected from these cells and also from WT splenocytes and tissues using Trizol reagent (Invitrogen Canada Inc., Burlington, ON) and the method supplied by the manufacturer. The samples were resuspended in DEPC-treated H_2O and quantitated. One microgram of total RNA in 5.7 μL of water was mixed with 14.3 μL of sample buffer (1X MOPS, 3.7% formaldehyde, 41.5% (v/v) formamide 5% (v/v) glycerol, 0.1 mM EDTA), heated to 65°C for 10 minutes and then chilled for 5 minutes. Samples were then run on a denaturing 0.8% agarose gel (0.8% (w/v) agarose, 1X MOPS, 1.8% formaldehyde, 0.2

μg/mL ethidium bromide) for 2 hrs in 1X MOPS buffer at 50 V. The gel was washed for 20 minutes in DEPC H_2O, and transferred onto a nylon Hybond-XL membrane (Amersham Biosciences, Piscataway, NJ) overnight. The membrane was probed with 3 different α-^{32}P labelled probes: cDNA fragments of the murine grB and β-actin genes, and a cDNA fragment of EGFP sequence. Each time the blot was washed to remove unlabeled probe and positive signals were visualized by exposure to Kodak Biomax X-AR film (Kodak Graphic Communications Co. Canada, Toronto ON). The membrane was stripped of radiolabelled DNA in between incubation with each new probe by washing the blot with boiling 0.1% SDS for 10 minutes.

2.2.3 Western blot analysis

Cells were lysed directly into SDS-PAGE gel loading buffer (80 mM Tris-Cl pH 6.8; 10% (v/v) 2-mercaptoethanol; 2% (w/v) SDS; 10% (v/v) glycerin, 4 μg/mL bromophenol blue) and boiled for 10 minutes. The cell lysates from $2x10^5$ cells/lane were resolved on a 12% SDS-polyacrylimide gel under reducing conditions. Proteins were transferred to nitrocellulose (Micron Separations, Westboro MA) by use of a semi-dry transfer apparatus (Tyler Research Corp., Edmonton, AB) for 1 hour at 150 mA. Membranes were blocked in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) and 5% skimmed milk (PBS-T-milk) for 1 hour. EGFP was detected using a polyclonal αEGFP antibody (Chemicon International, Temecula, CA) at a dilution of 1:500. Granzyme B was detected using αgrB 16G6 antibody (eBiosciences San Diego, CA) at a dilution of 1 μg/mL. Membranes were washed in PBS-T three times and then incubated

for 1 hour with a horseradish peroxidase-conjugated secondary αmouse antibody at 1:3000 (Biorad, Hercules, CA) in PBS-T-milk, followed by five washes in PBS-T. Positive signals were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

2.2.4 *In vitro* enzymatic assay

To collect supernatants from CTLs, cells were incubated in 200 μL of phenol-red-free RHFM for 4 hours at 37°C, 5% CO_2 with beads coated with αCD3 antibody (clone 145-2C11; BD Biosciences, Mississauga, ON), or bovine serum albumin (BSA; Invitrogen Canada Inc., Burlington, ON) as a control. Cells were removed by centrifugation for 5 minutes at 1500 rpm and supernatants were stored at –80°C until use. Granzyme B activity in supernatant and total lysates was assayed as previously reported (Ewen et al., 2003). Briefly, 50 μL aliquots of incubation medium or cell lysates were dispensed into 96-well, flat-bottom tissue culture plates (Nalge Nunc International, Rochester, NY). Enzymatic reaction was conducted at 37°C in 50 mM HEPES (pH 7.5), 10% (w/v) sucrose, 0.05% (w/v) CHAPS, and 5 mM DTT containing 200 μM acetyl-Ile-Glu-Pro-Asp-paranitroanilide (Kamiya Biomedical Co., Seattle, WA) as substrate. Hydrolysis of acetyl-Ile-Glu-Pro-Asp-paranitroanilide was measured at 405 nm using a Multiskan Ascent spectrophotometer (Thermo Electron Corp., Milford, MA). The absorbance of a blank containing no proteins was subtracted from each sample.

2.3 *In vivo* generation of Tg CTLs

2.3.1 Lymphocytic choriomeningitis virus (LCMV) infection of mice

On day 0 of the infection experiment, 6 FVB/N mice (2 WT, 4 Tg) were i.p. injected with $2x10^5$ PFU of LCMV (Armstrong strain, kindly provided by Dr. Kevin Kane, University of Alberta, Canada).

2.3.2 Collection of LCMV-infected tissues

At day 7 of the infection experiment three mice (1 WT, 2 Tg) were sacrificed and tissues were extracted. The peritoneal exudate lymphocytes (PEL) were collected by 1 or 2 lavages of the peritoneum using 8 mL cold PBS each time. The lavages were centrifuged and resuspended in PBS supplemented with 2% (v/v) of fetal calf serum (2% FCS/PBS). The liver and lung samples were finely chopped and incubated in Hanks' balanced salt solution (HBSS; Invitrogen Canada Inc., Burlington, ON) supplemented with 1.3 mM EDTA for 1 hour in a 37°C shaking incubator. The samples were then centrifuged and resuspended in HBSS containing 5% (v/v) FCS and 150 U/mL collegenase (clostridiopeptidase A, EC number 3.4.2.4.3; Invitrogen Canada Inc., Burlington, ON) and incubated with shaking at 37°C for 1 hour. The samples were then passed through a fine wire mesh, and RBCs were lysed with buffered ammonium chloride lysis buffer as described in Section 2.1.5. The samples were resuspended in 5 mL HBSS, and cellular debris was removed by centrifugation of samples that were layered on 5 mL Lymphocyte

M media (Cedarlane Laboratories Ltd., Hornby, ON) and centrifuged at 2100 rpm for 20 minutes, and subsequently washed twice in 2% FCS/PBS. Splenocytes were extracted as previously described in Section 2.1.5. Cells from lymph nodes were collected by mincing nodes and passed through nylon 70 micron Falcon filters (Invitrogen Canada Inc., Burlington, ON).

2.4 FACS Analysis

2.4.1 Data collection

FACS data was obtained using the Calibur Fluorescent Activated Cell Sorter (FACSCalibur) machine, and data was collected in 4 channels (FL1 to FL4). Fluorochromes in the FL1 to FL3 channels were excited by a 488 nm argon laser, and data was collected using a 530 nm, a 585 nm and a >650 nm bandpass filter, respectively. Fluorochromes in the FL4 channel were excited by a 635 nm red diode laser and data was collected by a >670 nm bandpass filter.

2.4.2 Staining of target L1210 and EL4 cells with Cell Trace Far Red (CTFR) dye

Approximately 1 to $3x10^7$ target L1210 and EL4 cells were washed once in HBSS and resuspended in 5 mL HBSS and CellTrace Far Red (CTFR) (DDAO-SE; Molecular Probes, Invitrogen Canada Inc., Burlington, ON) at a concentration of 0.33 μg/mL for

L1210 cells, and 0.67 μg/mL for EL4 cells. After 30 minute incubation at 37°C and 5% CO_2 the cells were washed twice with RHFM to quench the dye and again incubated at 37°C and 5% CO_2 for at least an hour in RHFM to allow the cells to recover.

2.4.3 Intracellular antibody staining of cells for FACS analysis

Cells were stained intracellularly only after any surface staining and washing was completed. Cells were pelleted in a U-bottom 96-well plate (Falcon, BD Biosciences, Mississauga, ON) and resuspended in 200 μL of room temperature (RT) PBS containing 10 mM EDTA (pH 8) (EDTA/PBS) and incubated for 5 minutes at RT to disrupt conjugates between target and effector cells (Berke, 1980) and remove surface bound grB (Shi et al., 2005). The cells were washed twice in 2% (w/v) BSA in PBS (BSA/PBS), and resuspended in 100 μL of RT paraformaldehyde (PF) solution (2% (v/v) PF in BSA/PBS) and incubated at RT for 15 minutes to fix the cells. Cells were washed twice in cold BSA/PBS by centrifugation at 1600 rpm and resuspended in 100 μL cold BSA/PBS containing 0.3% (w/v) saponin (Sigma-Aldrich Ltd., St. Louis, MO) and 0.5 μL or 1 μL αgrB-Phycoerythrin (grB-PE) conjugated antibody (Clone GB12, Caltag Laboratories, Burlingame, CA) per well, and incubated for 30 minutes at 4°C in the dark. Cells were washed twice in cold BSA/PBS containing 0.3% saponin by centrifugation at 1600 rpm, and resuspended in 250 μL of cold BSA/PBS for analysis using the FACSCalibur machine.

2.4.4 FACS analysis of green fluorescent transfer into target cells from green Tg CTLs

Transgenic and WT CTLs and CTFR-stained target L1210 and EL4 cells were each resuspended in fresh RHFM/IL-2. The cells were counted and $2x10^5$ L1210 or EL4 target cells and $4x10^5$ WT or Tg CTLs (2:1 effector/target (E/T) ratio) were each added to a well of a U-bottom 96-well plate for a final volume of 200 μL of RHFM/IL-2 per well. The cells were incubated at 37°C, 5% CO_2 for the length of the killing assay, and then the plate was centrifuged and the wells were resuspended in 200 μL of RT EDTA/PBS, and incubated at RT for 5 minutes to disrupt conjugation between target and effector cells (Berke, 1980) and to remove surface-bound grB from target cells (Shi et al., 2005). The cells were pelleted and resuspended in 250 μL of cold BSA/PBS and read on a FACS Calibre machine (BD Biosciences, Mississauga, ON). The cell mixture was gated on live cells, and target cells were identified by their fluorescence due to the CTFR dye (FL4 channel). The gate for green fluorescence (FL1 channel) of target cells was set so that target cells alone had less than 1% positive cells, and this percent value was subtracted from the percent value found for each sample. Inhibition of the calcium-dependent degranulation of CTLs was achieved as described (Garner et al., 1994) by performing the killing assay in 200 μL of RHFM/IL-2 supplemented with 5 mM EGTA (pH 8) and 10 mM $MgCl_2$.

2.4.5 FACS analysis of grB-PE antibody staining of GrB KO CTLs

Granzyme B KO CTLs were counted, washed with centrifugation and resuspended in cold BSA/PBS at $5x10^6$ cells/mL. Cells were stained with 1 μL (0.2 μg) αCD8a-Fluorescein (CD8a-FITC) conjugated antibody (clone 53-6.7, BD Biosciences, Mississauga, ON) per 100 μL of cells and incubated for 30 minutes at 4°C in the dark. Cells were then washed twice in cold BSA/PBS, and $5x10^5$ GrB KO CTLs were added to $5x10^5$ GrB WT CTLs or to BSA/PBS alone in wells of a U-bottom 96-well plate. Samples were then stained intracellularly using 0.5 μL grB-PE antibody per well as described in Section 2.4.3, and samples were analysed using the FACSCalibur machine. Live cells were gated for, and the GrB KO CTLs were separately gated by their fluorescence in the FITC (FL1) channel and examined for grB-PE (FL2 channel) positive staining. GrB KO CTLs alone were compared to GrB KO CTLs that were incubated with GrB WT CTLs.

2.4.6 FACS analysis of grB-PE staining of target L1210 cells without CTL removal

CellTrace Far Red-stained L1210 target cells and GrB WT and GrB KO CTLs were resuspended in fresh RHFM/IL-2. The cells were counted and $5x10^5$ L1210 target cells and either $1x10^6$, $5x10^5$ or $2.5x10^5$ GRB WT or GrB KO CTLs (to obtain 2:1, 1:1 or 0.5:1 E/T ratios, respectively) were added into wells of a U-bottom 96 well plate, and media was added to obtain a final volume of 200 μL of RHFM/IL-2 per well. The plate

was incubated at 37°C, 5% CO_2 for the time required for the killing assay. The cells were pelleted by centrifugation and the wells were resuspended in 200 μL of RT EDTA/PBS and incubated for 5 minutes. The plate was then washed twice with cold BSA/PBS using centrifugation and cells were resuspended in 100 μL of cold BSA/PBS with 1 μL αCD8a-Peridinin Chlorophyll-a Protein (CD8a-PerCP) conjugated antibody (clone 53-6.7, BD Biosciences, Mississauga, ON) and incubated for 30 minutes at 4°C in the dark. The wells were washed twice with cold BSA/PBS using centrifugation and the cells were stained intracellulary with 0.5 μL grB-PE conjugated antibody as described in Section 2.4.3. Cells were analysed using the FACSCalibur machine. Live cells were gated for, and L1210 target cells were identified due to CTFR fluorescence (FL4 channel), and lack of CD8a-PerCP staining (FL3 channel). These target cells were analysed for grB-PE (FL2 channel) fluorescence. The positive gate was set so that target cells incubated with GrB KO CTLs had less than 1% of positive cells, and this percent value was subtracted from the percent value found for each sample.

2.4.7 FACS analysis of grB-PE staining of target L1210 cells with CTL removal

The killing assay with CTFR-stained L1210 target cells and GrB WT or GrB KO CTLs was performed as in Section 2.4.6, and after the incubation period for the killing assay the cells were pelleted and the wells were resuspended in 200 μL of RT EDTA/PBS and incubated for 5 minutes at RT to disrupt conjugates. The cells were then washed once in cold BSA/PBS and resuspended in cold BSA/PBS containing 1 μL CD8a-PerCP

antibody per well. Magnetic Dynabeads coated with mouse αCD8 antibody (DYNAL Laboratories, Invitrogen Canada Inc., Burlington, ON) were added to each well at a volume depending on the amount of CTLs (18 μL for $1x10^6$, 9 μL for $5x10^5$, and 5 μL for $2.5x10^5$ CTLs respectively) for a final volume of 100 μL, and the wells were mixed thoroughly with pipetting. The plate was incubated on a nutator (BD Biosciences, Mississauga, ON) at 4°C for 30 minutes, and wells were mixed by pipetting after 15 minutes, and at the end of the incubation. The plate was then laid on a flat Dynal MPC 96B magnet (DYNAL Laboratories, Invitrogen Canada Inc., Burlington, ON) for 2 minutes to allow the CTLs bound to the αCD8-magnetic beads to pellet at the bottom of the wells. Seventy microlitres of supernatant was removed from the wells and was transferred to fresh unused wells in a new U-bottom 96-well plate using a multi-pipettor. The new plate was centrifuged and the cells were stained intracellulary using 1 μL of grB-PE as described in Section 2.4.3. The cells were analysed using the FACSCalibur machine. Live cells were gated for, and L1210 target cells were identified due to CTFR fluorescence (FL4 channel), and their lack of CD8a-PerCP staining (FL3 channel). These target cells were analysed for grB-PE (FL2 channel) fluorescence. The positive gate was set so that target cells incubated with GrB KO CTLs had less than 1% of positive cells, and this percent value was subtracted from the percent value found for each sample. Degranulation of CTLs was inhibited during killing assays by incubating both target and effector cells separately with RHFM/IL-2 supplemented with 20 mM colchecine (Sigma-Aldrich Ltd., St. Louis, MO) at 37°C, 5% CO_2 for 15 minutes. The cells were washed twice and resuspended in RHFM/IL-2 before beginning the killing assay and performing the rest of the experiment as outlined above.

2.5 Granzyme B enzymatic activity in target cells

2.5.1 Preparation cell lysates for enzymatic assay

L1210 and EL4 target cells were counted and $5x10^6$ target cells were each separately incubated with either $1x10^7$ GrB WT or GrB KO CTLs (2:1 E/T) or media alone at a total volume of 1 mL of RHFM/IL-2 for 30 minutes at 37°C, 5% CO_2. As controls, $1x10^7$ of GrB WT CTLs and GrB KO CTLs were similarly incubated alone in 1 mL media. Cells were pelleted, and incubated at RT in 1 mL PBS/EDTA for 5 minutes, and centrifuged again. Cells were resuspended in 1 mL cold PBS/BSA and 65 μL of magnetic Dynabeads coated with mouse αCD8 antibody (DYNAL Laboratories, Invitrogen Canada Inc., Burlington, ON) were added to each sample. Samples were placed on a tube rotator (Sepco Scientific Equipment Products, Baltimore, MD) at 4°C for 30 minutes. Tubes were placed in a 3-in-1 magnetic particle separator (CPG, PureBiotech LLC., Middlesex, NJ) for 2 minutes to allow the CTLs bound to the αCD8-magnetic beads to pellet at the side of the tube. Supernatant containing target cells was removed, centrifuged and resuspended in 1 mL RT EDTA/PBS for 5 minutes at RT. Samples were washed once in HBSS, and resuspended in 170 μL of lysis buffer (20 mM Tris-Cl (pH 7.2), 150 mM NaCl, and 1% Triton X-100) and incubating for 10 minutes on ice to prepare total cell lysates for the *in vitro* grB enzymatic assay. Lysates were centrifuged at 10,000 rpm for 10 min to remove cell debris and stored at -80°C until assayed as described earlier in Section 2.2.4. Lysates prepared from GrB WT CTLs alone

that were then depleted by the Dynabeads demonstrated the efficiency of the removal of the $CD8^+$ CTLs.

3. Creation and analysis of grB-EGFP Tg CTLs

3.1 Introduction

In this section of the results I will describe the construction of a plasmid expressing a grB-EGFP fusion protein under the control of the endogenous murine grB promoter. The grB gene was left intact so that regulatory elements that might exist in introns or other noncoding regions of the gene would remain to ensure normal transcriptional control of the transgene. Granzyme B gene transcription is upregulated at a late stage of CTL differentiation and is a hallmark of the CTL effector phenotype. Since the pathways controlling late stage activation of $CD8^+$ T cells are poorly understood, learning about grB transcriptional regulation will increase the understanding of these processes so important to the establishment of the cytotoxic phenotype. The characterization of a gene locus requires the identification of the regulatory elements needed for normal transcription *in vivo*. The presence of DNase I hypersensitive sites (HS) in chromosomal DNA is an indication of decreased condensation, typically associated with transcription factor binding. Earlier studies examined the sequence near the murine grB gene for areas of hypersensitivity. One HS, termed HS1, was located at the proximal promoter of the grB gene and occurred in $CD8^+$ T cells only after activation (Babichuk et al., 1996). Initial transcriptional regulation studies in CTL cell lines indicated that inclusion of HS1 in a reporter cDNA construct drove high level expression in transient transfection assays. However, the same construct was also incorrectly expressed in a mouse fibroblast cell

line. The HS1 site alone was therefore not sufficient to produce tissue-specific expression of the grB gene. Furthermore, the proximal promoter connected to the β-gal reporter cassette did not induce transcription in a transgenic mouse line, indicating that HS1 is not sufficient to induce normally regulated expression *in vivo*. Further investigation of the grB gene locus revealed another HS, termed HS2, approximately 3.8 Kb upstream of the grB gene transcription start site, and has been shown to be a conserved sequence between the human and murine gene loci. The grB-expressing CTLL-R8 cells were selected to study grB transcription because these cells are easily transfected, and the creation of the grB-EGFP fusion protein construct allowed transcriptional studies of the endogenous promoter to be easily analysed by measuring green fluorescence with FACS. The green fluorescence appeared in a punctate pattern in these cells, indicating normal trafficking of the fusion protein to the cytotoxic granules of the CTLL-R8 cells.

The same construct was then used to generate a transgenic mouse line to allow the study of this fusion protein in a *in vivo* model. The grB-EGFP transcript was not present in non-lymphoid tissues or in naïve splenocytes collected from Tg mice. Confocal microscopy of activated Tg splenocytes showed punctate green fluorescence within these cells, again suggesting normal trafficking of the fusion protein to the cytotoxic granules. A CTL line was derived from primary Tg splenocytes and analysed to determine that the Tg CTLs were functionally normal. The Tg CTLs transcribed and expressed a full length fusion transcript and protein as analysed by Northern and Western blotting. The Tg CTLs killed allogeneic targets only, and did not kill targets that were of a different haplotype when analysed using tritium and chromium release assays. An *in vitro* grB enzymatic

assay detected normal levels of degranulation of the Tg CTLs in response to αCD3 stimulus. These results indicated that the Tg CTL line, and therefore the primary CTLs in the Tg mouse, were functionally competent.

The establishment of this Tg mouse line has created mice with an "activation" tag to allow *in vivo* tracking of stimulated CTLs. This can elucidate, for example, the kinetics of homing and activation of these cells during an infection in the mouse with virus. Transgenic mice were infected systemically with LCMV to determine if grB-EGFP expression occurs in CTLs generated within Tg mice. On day 7 post-infection, the mice were sacrificed and tissues collected. A subset of $CD8^+$ and $CD4^+$ T cells in the Tg mouse showed an increase in green fluorescence when compared to WT littermate controls, demonstrating that the grB-EGFP transgene is also activated in *in vivo* generated CTLs.

3.2 Results

3.2.1 Creation of a murine grB-EGFP construct under endogenous transcriptional control

A 11 Kb grB-EGFP fusion construct was created termed pNC2, consisting of the endogenous grB locus from 5.3 Kb upstream of transcription start to 2 Kb after transcription end, to study the transcriptional effects of the DNase 1 hypersensitivity site (HS) region, termed HS2, *in vivo*. The EGFP cDNA was added on the last exon of the endogenous gene, thus allowing the grB-EGFP fusion protein to be transcriptionally controlled by the endogenous promoter of the murine grB gene. A further construct was made termed pNC3 that lacked the HS2 region (Figure 3-1). These constructs were used to derive stably expressing clones using the murine grB-expressing CTL line, CTLL-R8. When analysed by FACS, the pNC2 clones (n=63) showed statistically significant ($p<0.001$) greater EGFP fluorescence; when compared to mock transfected controls; than the pNC3 clones (n=54) (Figure 3-2). The pNC2 clones had a punctate pattern of green fluorescence when analysed by confocal microscopy (data not shown), suggesting that the grB-EGFP fusion protein was properly targeted to the cytotoxic granules of the cell.

The pNC2 construct was then used to generate a transgenic mouse line of FVB/N background. Northern blot analysis revealed an absence of transgenic mRNA found in non-lymphoid tissue samples of these mice, as well as in resting splenocytes (results not shown). Primary Tg splenocytes became green only after activation, indicating normal

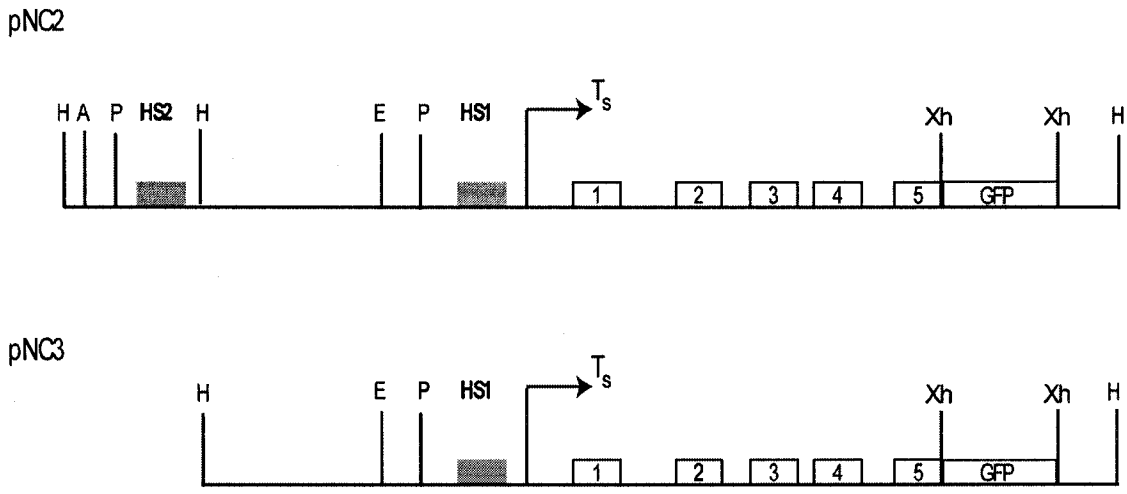


Figure 3-1. DNA map of the pNC2 and pNC3 reporter constructs

EGFP reporter constructs pNC2 and pNC3 containing 5.3 Kb and 3.6 Kb of upstream granzyme B sequence respectively. Granzyme B gene locus with DNase1 hypersensitive sites 1 and 2 (HS1, HS2) marked as grey boxes. The distances in kilobases of both HS's are relative to the transcription start site. Granzyme B exons are numbered 1 to 5 and major restriction sites (H, Hind III; A, Avr II; Ac, Acc I; E, Eco RI; P, Pst I) are as shown. The EGFP PCR product containing the last amino acids of the granzyme B gene excluding the stop codon was subconed into the Xho1 site in exon 5.

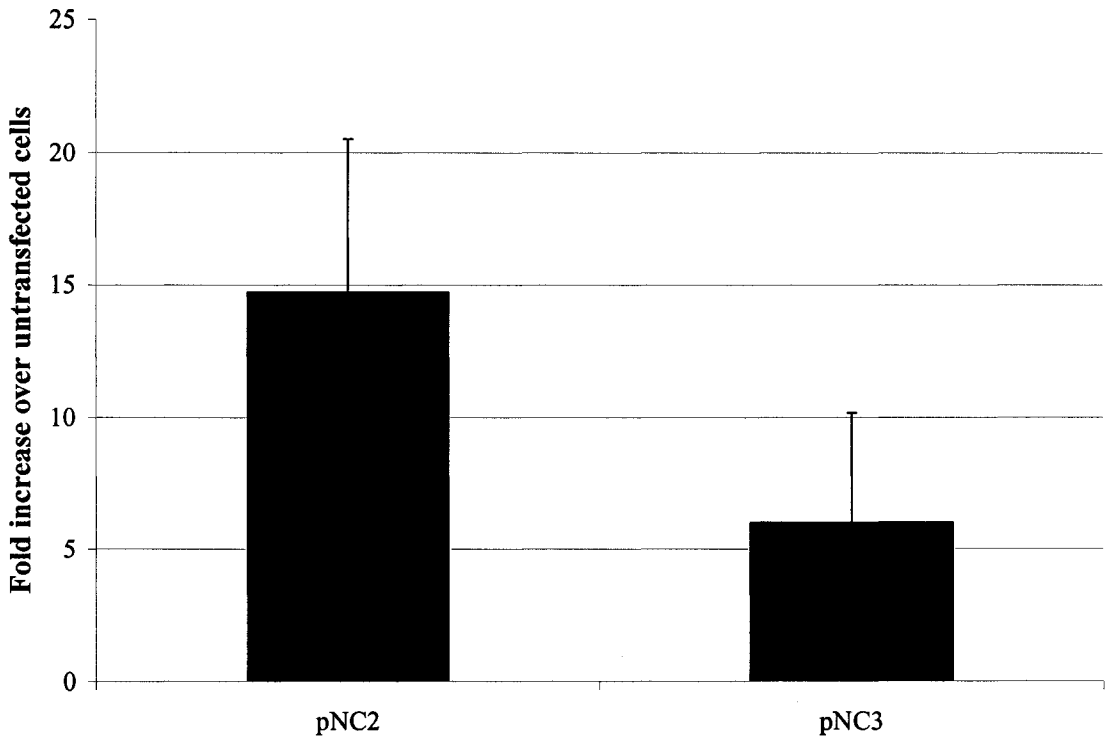


Figure 3-2. Average mean fluorescence of pNC2 and pNC3 CTLL-R8 clones

Average mean fluorescence intensities for all sorted clones in each stable transfection. The mean fluorescence intensities of all clones of each construct over the wild type fluorescence intensities of the untransfected CTLL-R8 cells plus standard deviations are represented.

induction of the transgene. This fluorescence was punctate when examined with confocal microscopy, suggesting the grB-EGFP fusion protein was correctly targeted to the cytolytic granules within these cells (Figure 3-3). This was further supported by the colocalisation of the green fluorescence with LysoTracker Red dye (Molecular Probes, Invitrogen Canada Inc., Burlington, ON) known to stain cytotoxic granules in CTLs (Ulrich H. von Andrian, Harvard Medical School, Boston MA, personal communication). However, not all of the activated Tg splenocytes contained green fluorescence.

3.2.2 Generation of a Tg CTL line expressing grB-EGFP under endogenous control

Since primary splenocytes can not be cultured indefinitely, we decided to derive a CTL line from isolated splenocytes of Tg mice. We derived this line by co-culturing the splenocytes from FVB/N Tg mice (H-2^q) with irradiated splenocytes from allogeneic Balb/c mice (H-2^d). The CTL line derived from Tg splenocytes contained around 10% green cells, although almost all the non-green cells stained positive using a αCD8a-PerCP antibody and the αgrB-PE antibody when analysed by FACS. All further analysis of the green CTLs was accomplished only after the Tg CTL line was first sorted for green fluorescence, unless stated otherwise. These results indicate that the

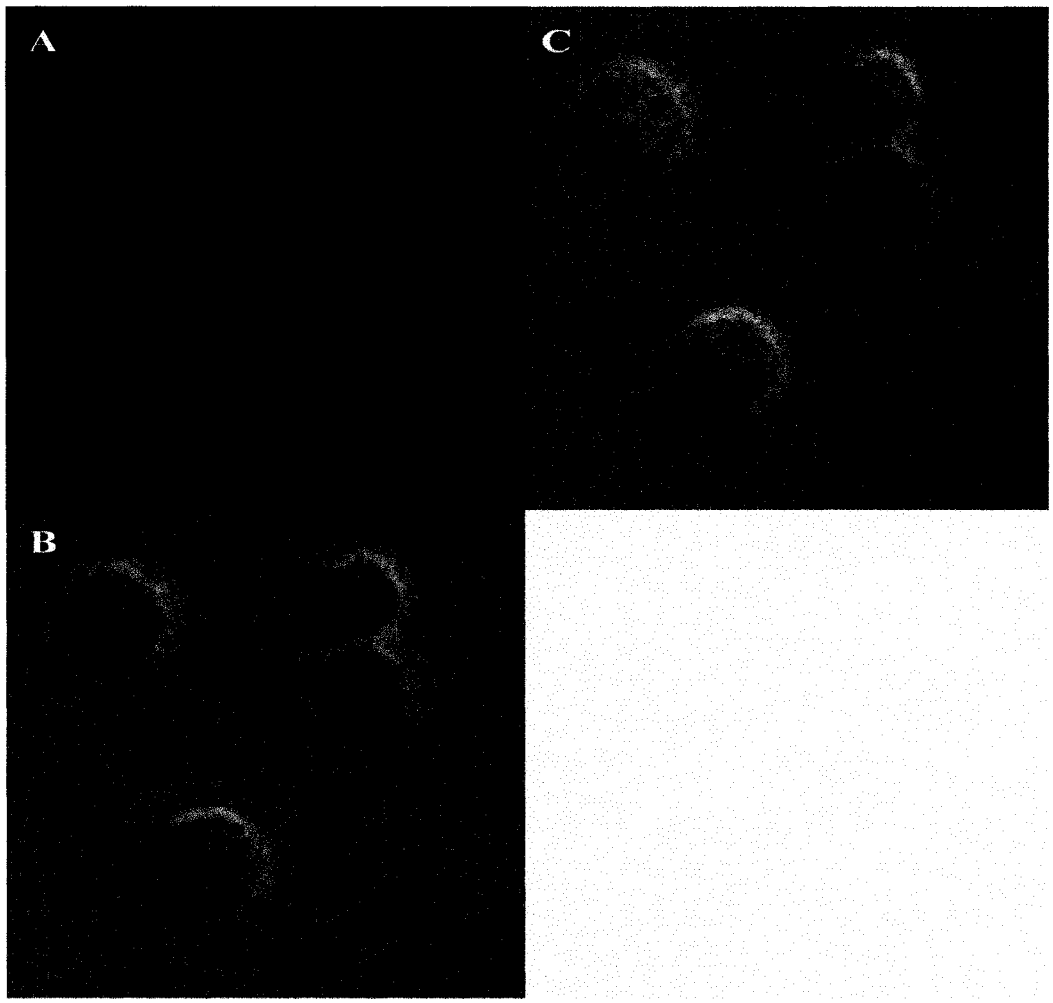


Figure 3-3. Confocal imagery of αCD3 stimulated Tg splenocytes

A. Confocal image of a representative sample of activated Tg splenocytes with grB-EGFP expression (shown in green) obtained by excitation at 488 nm, and emission filter of 505–530 nm. **B.** DIC image of the same cells showing field of view. **C.** Overlaid image of A and B.

grB-EGFP protein is properly targeted to cytotoxic granules, and the fusion protein is only expressed in activated CTLs.

3.2.3 Functional analysis of the green Tg CTLs

Northern blot analysis of the sorted green Tg CTLs showed a larger transcript that stained positive for grB and EGFP cDNA probes (Figure 3-4), and western blot analysis showed a corresponding protein band that was reactive with both αgrB and αEGFP antibodies (Figure 3-5). Tritium and chromium release assays were performed as previously described (Garner et al., 1994) and indicated normal levels of DNA fragmentation and membrane damage in target cells killed by unsorted Tg CTLs as compared to WT CTLs (data not shown). This killing was reduced by the addition of EGTA in the presence of $MgCl_2$, which is known to inhibit the calcium-dependent degranulation of CTLs (Garner et al., 1994). Wildtype and sorted Tg CTLs were both incubated with immobilized αCD3 antibody to induce degranulation. Supernatants containing degranulated material of the sorted Tg CTL line exhibited higher levels of grB activity compared to a WT control when measured using an *in vitro* enzymatic assay. There was little activity detected in this assay when either of the CTL lines was incubated without αCD3 antibody, indicating that the green Tg CTLs degranulated normally in response to activation (Figure 3-6). The Tg CTL line was therefore able to degranulate normally and kill target cells efficiently.

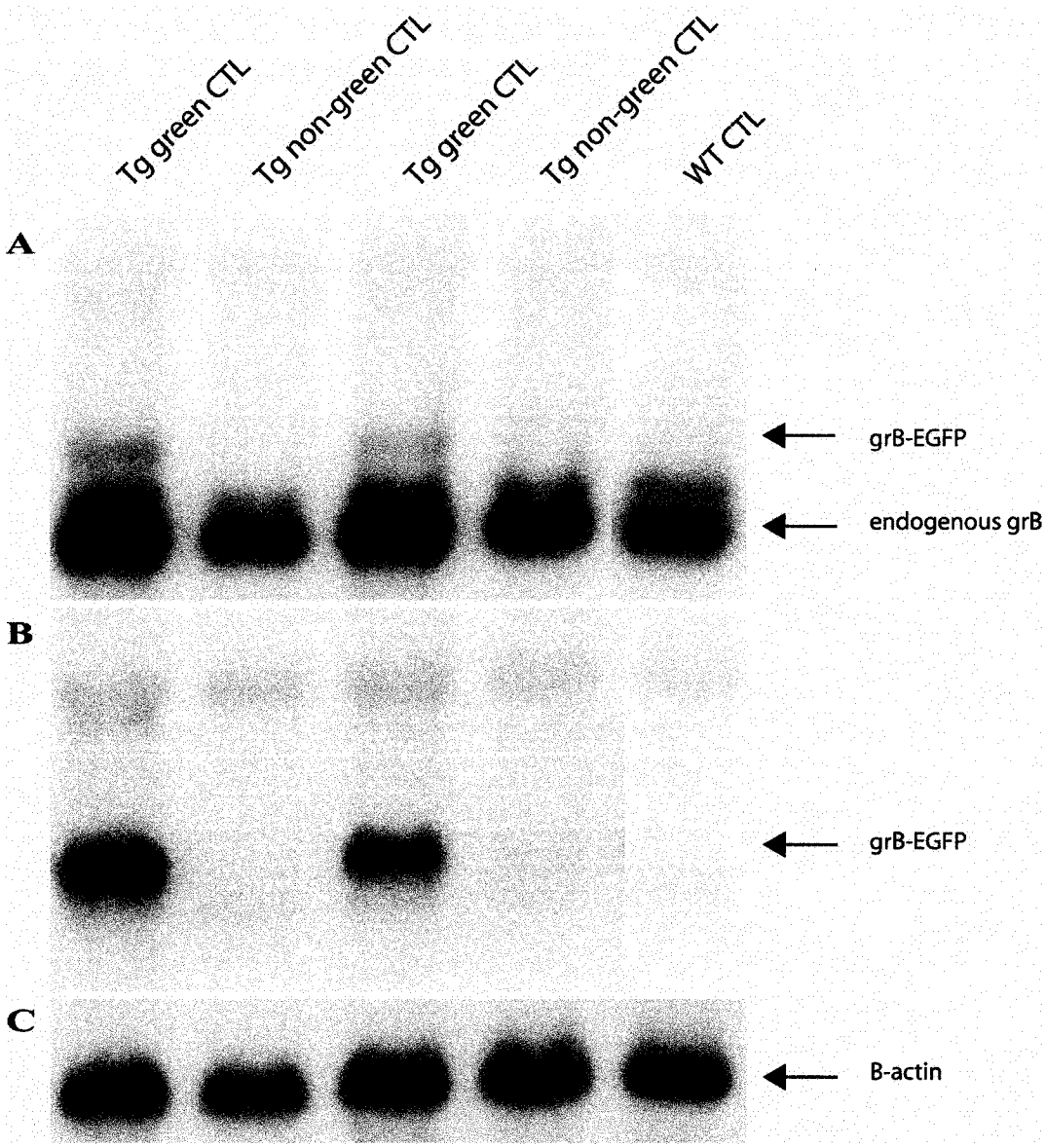


Figure 3-4. Northern Blot analysis of Tg and WT splenocytes

RNA of Tg CTLs separated based on green fluorescence (green and non-green, respectively) was collected from two separate Tg mice (first Tg mouse: lanes 2 and 3, second Tg mouse: 4 and 5), and from CTLs of a WT littermate control (lane 5). Samples were run and transferred to a nylon membrane. **A.** Membrane was probed with α^{32}P-labelled cDNA fragment of murine grB gene and visualised using autoradiography. **B.** Same membrane was stripped and re-probed using a α^{32}P-labelled cDNA fragment of EGFP transcript and visualised using autoradiography. The band that appeared overlayed with the upper grB positive band from the previous panel. **C.** Same membrane was stripped and re-probed with a α^{32}P-labelled fragment of the cDNA of the murine β-actin gene and visualised using autoradiography to demonstrate consistent loading of samples.

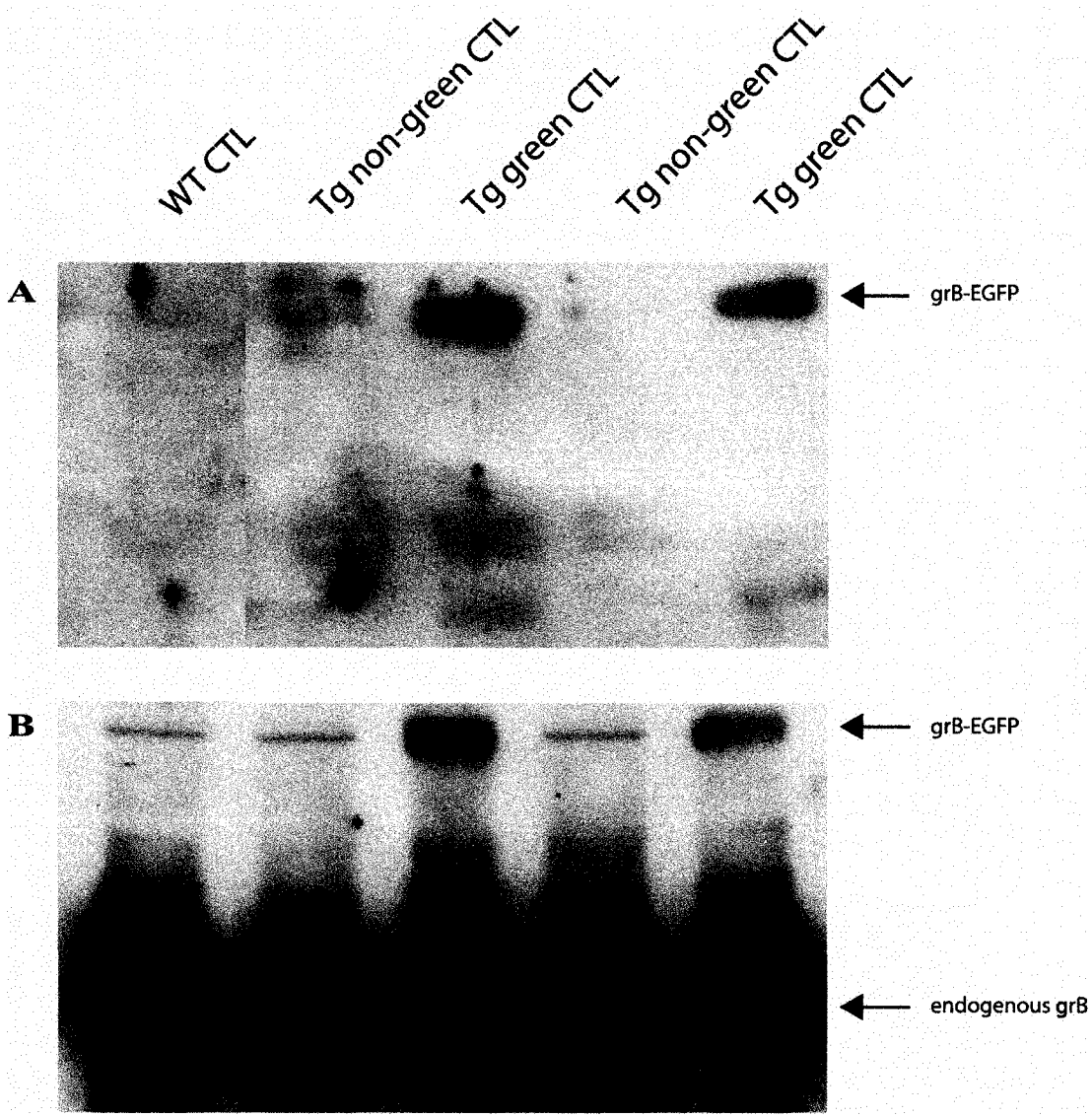


Figure 3-5. Western Blot analysis of Tg and WT splenocytes

Protein samples from $2x10^5$ CTLs/lane were collected from a WT littermate control (lane 1), and from two separate Tg mice. Tg CTLs were separated on basis of green fluorescence using a FACSAria machine into green and non-green populations (first Tg mouse: lanes 2 and 3, second Tg mouse: 4 and 5). The same mice that were used in the Northern blot analysis in the previous figure were used in the Western Blot analysis. Samples were resolved on a 12% SDS-polyacrylimide gel under reducing conditions, and transferred to a nitrocellulose membrane. **A.** The EGFP protein was visualised using an αEGFP antibody. **B.** An identical membrane was used to visualise the grB protein using an αgrB antibody. The upper bands from both membranes were of identical size.

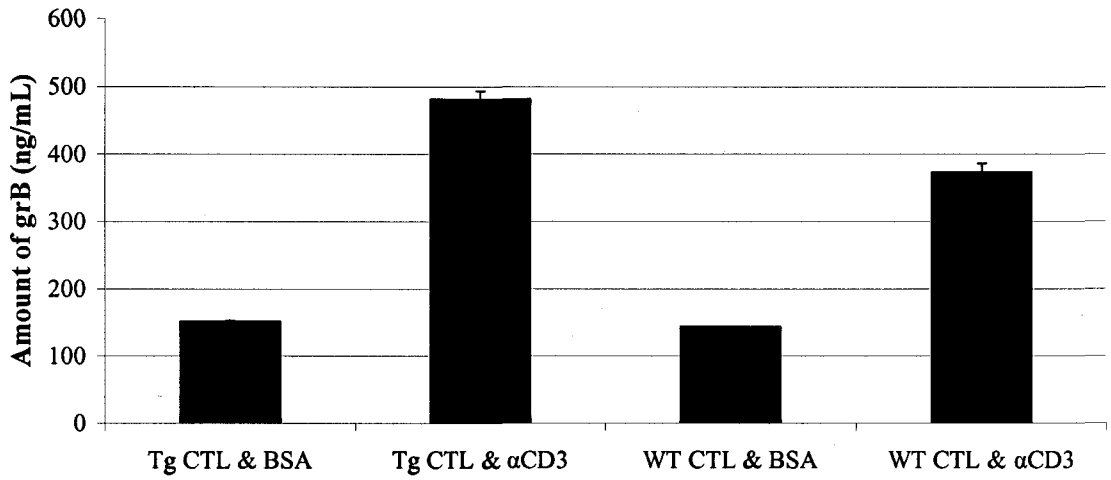


Figure 3-6. Degranulation assay of Tg and WT CTLs

Tg and WT CTLs were incubated with αCD3 coated beads, or beads coated with BSA. Supernatant was collected and measured for grB enzymatic activity as compared to standard curve produced with purified murine grB. Estimated concentrations of grB protein (ng/mL) are shown as mean and standard deviation of triplicate samples and is representative of at least two separate experiments.

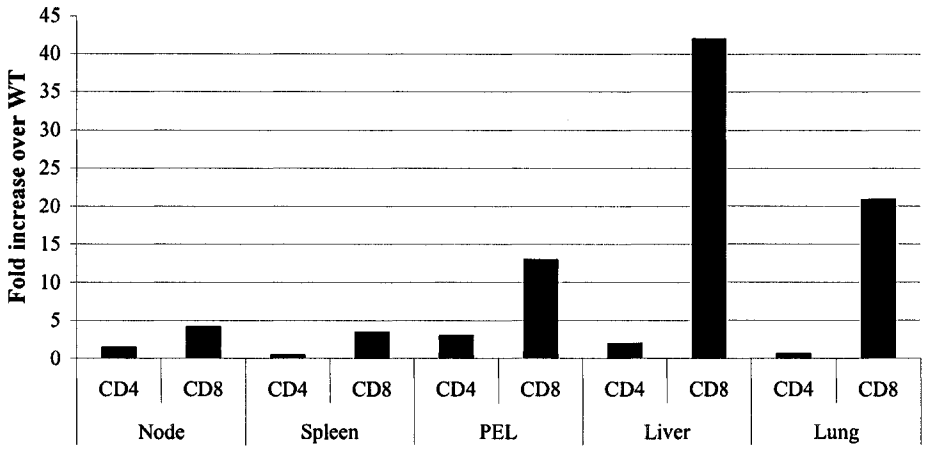
3.2.4 Green NK cells are not detected *in vivo* following LCMV infection

Transgenic and WT control mice were infected with LCMV to produce a systemic virus infection *in vivo*. $CD8^+$ and $CD4^+$ cells were examined from various tissues in the mouse at 3 and 7 days post-infection. There was no green fluorescence in any cells at day 3 (results not shown). This time point is when the population of natural killer (NK) cells peaks during acute LCMV infection (Biron et al., 2002; Welsh, 1978), however we were unable to observe any green fluorescent NK cells ($NK1.1^+/CD3^-$) in the infected Tg mice. We also generated lymphokine-activated killer (LAK) cells from the harvested splenocytes. These cells were also not green, although they stained positive with αgrB-PE antibody when analysed by FACS (data not shown). This evidence suggests that the grB-EGFP protein is not expressed in NK cells, despite the expression in these cells of the endogenous grB protein.

3.2.5 Green $CD8^+$ and $CD4^+$ T cells are generated *in vivo* following LCMV infection

At day 7 there was an increase in green fluorescent $CD8^+/CD3^+$ and $CD4^+/CD3^+$ cells in the infected Tg mouse when compared to the littermate control, most notably in the liver, lung and intra-peritoneal lavages when compared to the infected WT control (Figure 3-7A). The green $CD8^+/CD3^+$ cells ranged from 2 to 7% of the total population of $CD8^+/CD3^+$ cells. There was les than 0.5% of green $CD8^+/CD3^+$ cells in the WT mouse. This increase in green fluorescence corresponded to an increase in positive αgrB-PE

A



B

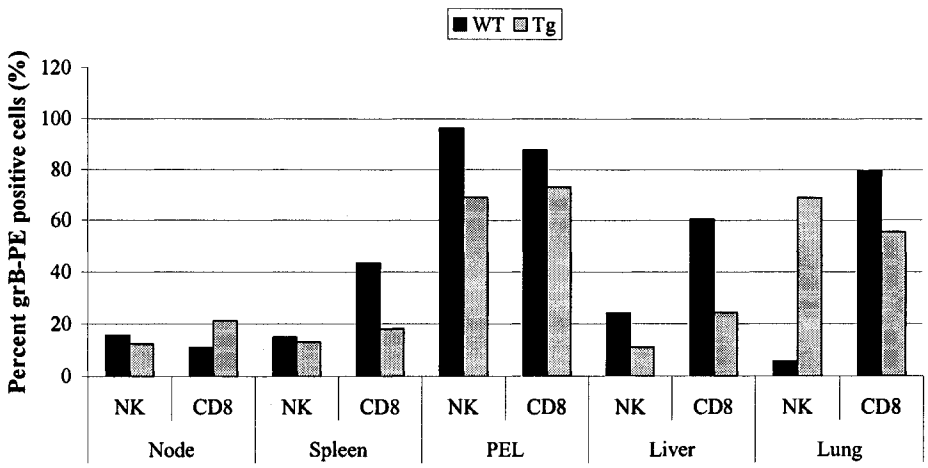


Figure 3-7. grB-EGFP positive CTLs are generated *in vivo* from LCMV infection

Transgenic and WT mice were infected i.p. with $2x10^5$ PFU of Armstrong strain LCMV. Various tissue samples were collected from 1 WT and 1 Tg mouse on day 7 post-infection. **A.** Green fluorescence was measured in $CD8^+/CD3^+$ (CD8) and $CD4^+/CD3^+$ (CD4) cells from various tissue samples. The values represent the percentage of CD8 or CD4 green cells found in the Tg mouse divided by the percentage of CD8 or CD4 green cells present in the WT animal, for each tissue sample. **B.** grB-PE staining was measured in $NK1.1^+/CD3^-$ (NK) and $CD8^+/CD3^+$ (CD8) cells from various tissue samples from 1 WT (black bars) and 1 Tg (grey bars) mouse. Values represent percentage of NK or CD8 cells that were also αgrB-PE positive.

antibody staining in these cells, which ranged from 20% to 90% αgrB-PE positive when analysed by FACS (Figure 3-7B).

3.3 Discussion

The green punctate patterning of the grB-EGFP protein in the CTLL-R8 clones suggested that the grB-EGFP was properly targeted to the cytotoxic granules of the CTLL-R8 cells, and that this fusion protein might be normally exocytosed during degranulation-induced target cell killing. Although CTLL-R8 cells express grB, they are not good models of primary CTLs, as they do not express the normal surface markers such as CD4 or CD8, and they do not readily kill target cells. However, because they are easily transfected, these cells are useful in transcriptional control studies of the grB promoter. The high expression of grB-EGFP in pNC2-transfected cells demonstrates the importance of the HS2 region for grB expression. Studies are now ongoing in the lab using truncated versions of this pNC2 construct in the 5' untranslated region of the grB locus to further define the HS2 region, and its importance in transcriptional control of the grB gene.

Since murine T cells cannot be easily transfected, the pNC2 construct was used to generate a transgenic mouse line. Splenocytes from Tg mice were used to generate a CTL line. These lines are generated routinely in the laboratory from primary murine splenocytes to create CTLs that kill allogeneic target cells of the same haplotype as the irradiated cells used to stimulate the CTL line. This Tg CTL line showed that the expression of the grB-EGFP protein did not affect the killing activity and normal function of these cells.

The Tg CTLs generated showed that though most of the Tg CTLs were grB-PE positive as expected, not all were grB-EGFP positive. This indicated that the endogenous protein was expressed in nearly all of the population, but the fusion protein was only expressed in a subpopulation of CTLs generated. Southern blot analysis of the non-green CTLs confirmed the presence of the transgene in the genome (data not shown). The subpopulation of green fluorescence cells had the same punctate pattern of green fluorescence, indicated proper trafficking of the fusion protein to the cytolytic granules of the CTLs. Northern blot analysis showed that the non-green CTLs did not transcribe the grB-EGFP transcript, indicating that these cells were not green because either the EGFP protein was being quenched in the acidic environment of the cytotoxic granules, or was being degraded. Western blotting indicated that the grB-EGFP protein was not cleaved or degraded in the green fluorescent cells. The grB-EGFP protein was therefore being transcribed, expressed and trafficked normally, but only in a smaller population (around 10%) of the activated Tg CTLs. This may be because the construct does not contain all the regulatory elements needed for the complete normal endogenous expression of the grB-EGFP protein. An interesting possibility is that the green fluorescent CTLs are a distinct subpopulation that expresses the grB-EGFP protein because these cells differ in some way to the non-green CTLs. This subset of green fluorescent CTLs were always found to be slightly larger than the non-green subset when examined by side scatter and forward scatter by FACS. However, any putative differences of the green subpopulation have yet to be defined, and require further study.

The Tg CTLs were analysed to determine if they were functionally normal, and therefore able to kill allogeneic target cells. The unsorted Tg CTLs were shown to induce normal levels of DNA fragmentation and membrane damage as measured by tritium and chromium release assays, indicating that the Tg CTLs contained intact killing machinery. Sorted green Tg CTLs were also able to cause mitochondrial depolarization indicated by the loss of TMRE staining (Invitrogen Canada Inc., Burlington, ON) to the same extent as WT CTLs (results not shown). The degranulation of the sorted green cells was induced normally by incubation with αCD3 antibody, and the amount of grB enzymatic activity in the degranulate was normal when compared to WT controls. Furthermore, the Tg CTLs did not degranulate when incubated with BSA control. These results together indicate the green sorted Tg CTLs were functionally normal when compared in killing assays, and in response to αCD3 stimulation to WT controls. The *in vitro* generated Tg CTLs were therefore unaffected by the expression of the grB-EFGP fusion protein, and degranulated and killed target cells normally.

The creation of this Tg mouse line could provide a powerful tool for the *in vivo* monitoring of activated CTLs using the grB-EGFP protein as a fluorescent activation "tag" to examine the kinetics of activation and movement of effector CTLs generated in the mouse during viral infection, tumourogenesis and graft rejection. Although the CTL line created *in vitro* from the Tg mouse splenocytes are functionally equivalent to other generated lines, we wanted to show that these green fluorescent CTLs can be produced in an *in vivo* model. We chose to infect our mice with the Armstrong strain of LCMV because the acute infection produced by this virus in mice produces approximately a 10

000-fold expansion in the number of virus-specific $CD8^+$ effector T cells (Khanolkar et al., 2002). This expansion of CTLs peaks around day 8 post-infection (Blattman et al., 2000; Homann et al., 2001) and can be found in both lymphoid and non-lymphoid tissues of the body (Kapasi et al., 2005; Zhou et al., 2004). This robust response would allow visualization of the green activated Tg CTLs. We also hoped to detect the presence of green NK cells in this experiment, as we did not yet know if the grB-EGFP construct would be expressed in these cells.

Day 3 post-infection is a time point expected to contain high numbers of expanded NK cells (Welsh, 1978) , and indeed tissue samples collected did contain $NK1.1^+/CD3^-$ cells. Although many of these cells were αgrB-PE positive when analysed with FACS, there was no green fluorescence in these cells. Lymphokine activated killer (LAK) cells were also generated to create what are essentially activated NK-like cells (Finkelstein and Miller, 1990; Grimm et al., 1982; Lindemann et al., 1989; Phillips and Lanier, 1986), and were again found to be αgrB-PE positive, but not green fluorescent. This data indicates that the grB-EGFP fusion protein is not expressed in NK cells of the Tg mouse. However, a subpopulation of $CD8^+/CD3^+$, and to a lesser extent $CD4^+/CD3^+$ T cells collected from tissues at day 7 post-infection did have green fluorescence. The number of green fluorescent cells was relatively small, but the highest percentages of green T cells occurred in the liver, lung and peritoneum. These are areas of high virus titre during infection, and are therefore areas where activated T cells are expected to congregate. These areas also contained the largest percentage of $CD8^+/CD3^+$ cells that stained positive for αgrB-PE antibody when analysed by FACS. This experiment showed that

green fluorescent CTLs could indeed be generated *in vivo* during a viral infection. The percentage of green CTLs compared to all αgrB-PE antibody positive CTLs was low, similar to the *in vitro* stimulated green CTLs generated from the Tg splenocytes. This again suggests that our construct is lacking all that is needed for complete normal endogenous expression of the grB-EGFP protein.

4. CTL-induced Granzyme B entry into target cells

4.1 Introduction

Although purified grB has been shown to be taken up by cells *in vitro* (Pinkoski et al., 2000; Pinkoski et al., 1998), this uptake has never been explicitly seen in more physiologically relevant conditions, as when CTLs kill a target cell. In this chapter, I will describe the uptake of grB from GrB WT CTLs into target cells as measured by αgrB-PE antibody staining. The αgrB-PE staining was found in target cells exposed to GrB WT CTLs, and not in target cells incubated with GrB KO CTLs, or in media alone. The transfer of endogenous grB into target cells was found to be time- and dose-dependent, since the target cells had increased αgrB-PE signal when exposed to more GrB WT CTLs, or when incubated with the CTLs for a longer period of time. When the microtubule-dependent degranulation of the CTLs was inhibited by colchecine incubation, the target cells did not accumulate αgrB-PE signal, indicating that this staining was also dependent on the degranulation of the CTLs.

Target cells were also observed to acquire green fluorescence when incubated with the grB-EGFP Tg CTLs, and not with WT CTLs. This transfer was target cell specific, since the Tg CTLs were activated with Balb/c (H-2^d) stimulators, and induced green fluorescent uptake in the allogeneic L1210 target cells (H-2^d). The EL4 cells (H-2^b) were not recognised by the Tg CTLs, and did not acquire the green fluorescence. This uptake

was also dependent on time of exposure of target to effector cells, and to degranulation of the CTLs, since the inhibition of degranulation by incubating the cells in the presence of EGTA stopped green fluorescent uptake in the L1210 target cells.

The presence of grB in target cells was also confirmed by the presence of grB enzymatic activity in target cell lysates that had been exposed to CTLs. This activity did not occur when syngeneic EL4 target cells were used, or when target cells were exposed to GrB KO CTLs. The enzymatic activity measured in these lysates was specific to grB protease activity, and not due to caspase activation in the apoptotic target cells, as caspase inhibitors did not significantly reduce the measured activity. These results definitively demonstrate *in vivo* grB entry into target cells after exposure to CTLs.

4.2 Results

4.2.1 GrB WT CTLs induce αgrB-PE transfer into target cells

Cytotoxic T lymphocytes (H-2^b) activated by Balb/c stimulators (H-2^d) were stained intracellularly with a conjugated human αgrB-PE antibody found to cross-react with the mouse protein. After incubation of target and effector CTL cells, the L1210 (H-2^d) target cells increased in mean fluorescence intensity of αgrB-PE staining (FL2 channel) when analysed with FACS. The staining was not present in target cells alone, and was absent when the CTL effector cells were fixed with paraformaldehyde prior to incubation with target cells, or when GrB KO CTLs are used in this assay (data not shown). The grB-PE staining was very high in target cells if CTLs were not removed prior to permeabilisation and antibody staining (Figure 4-1B). This staining occurred in any cell type that was permeabilised and stained in the presence of CTLs, whether or not these cells were recognized and killed by the effector cells (Figure 4-1A). After CTL depletion, there was 10% or less of total cells that are $CD8^+$ remaining (Figure 4-2A), and the αgrB-PE staining became time- and dose-dependent (Figure 4-2B, Figure 4-3 (A and B)). This staining was reduced by the inhibition of CTL degranulation (Figure 4-3 (A and B)). However, staining of EL4 (H-2^b) target cells was also found to be dose- and time-dependent (data not shown), in contrast to experiments described in Section 4.2.2, in which the EL4 target cells did not acquire significant levels of green fluorescence as compared to the L1210 target cells.

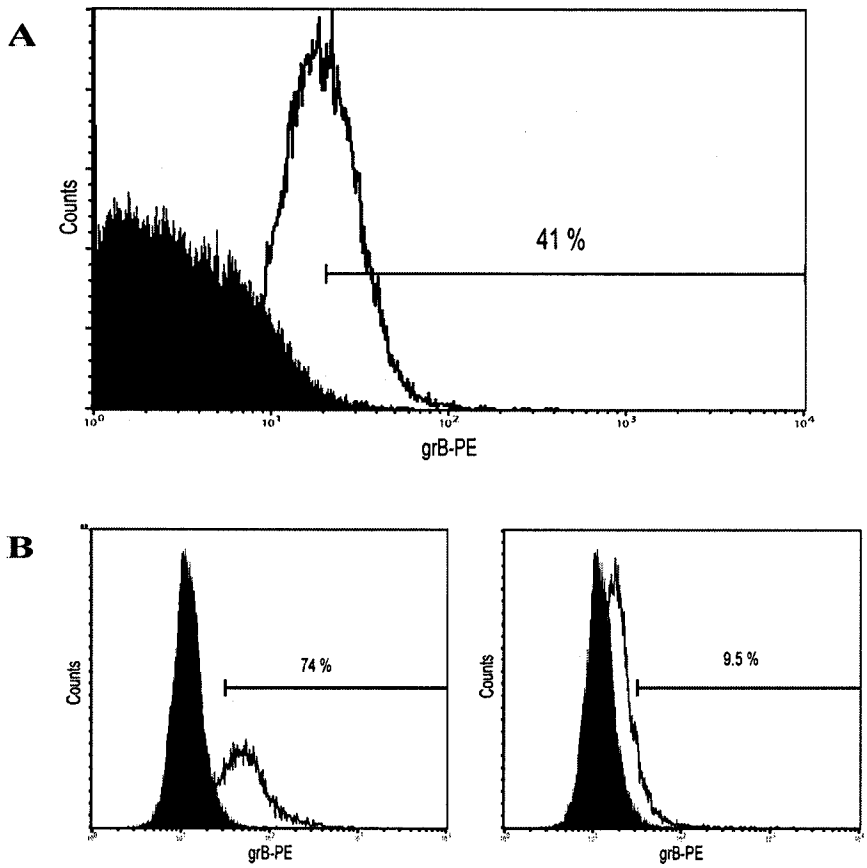
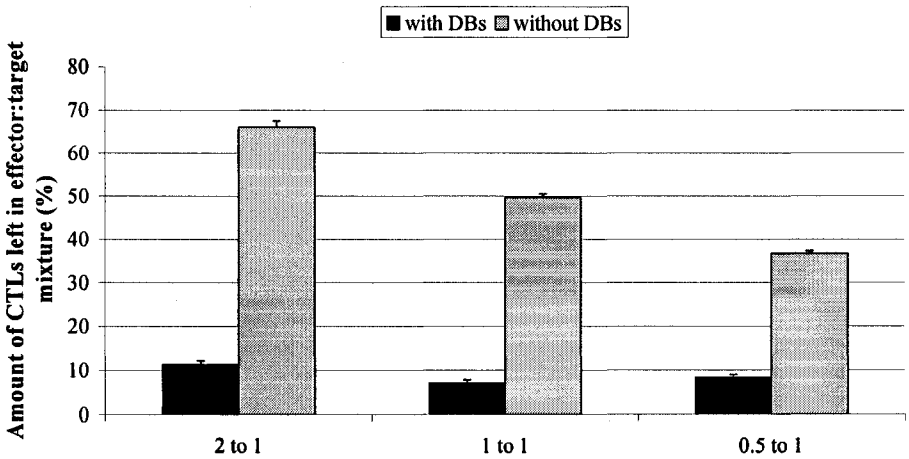


Figure 4-1. grB-PE antibody staining occurs non-specifically when grB WT CTLs are present during fixation and permeabilisation steps.

A. GrB KO CTLs previously stained with αCD8a-FITC antibody were either mixed with GrB WT CTLs or left alone, and samples were stained intracellularly for grB-PE. Cells were analysed using the FACSCalibre machine and GrB KO CTLs were identified based αCD8a-FITC staining. FACS histogram shows αgrB-PE antibody staining of GrB KO CTLs is elevated when GrB WT CTLs are present (unfilled peak) versus when they are absent (filled peak). Percentage of positive staining GrB KO CTLs is shown. Relative cell counts are represented on y-axis, versus αgrB-PE staining on a log scale. **B.** CTFR-stained allogeneic L1210 target cells (H-2^d) were incubated with H-2^d-stimulated GrB WT CTLs (2:1 E/T) for 30 minutes in RHFM/IL-2 media at 37°C, 5% CO_2. Cells were washed and incubated in RT EDTA/PBS, CTLs were then removed (right panel) with αCD8a antibody-coated magnetic beads, or were not removed (left panel) and cells were stained intracellularly with αgrB-PE antibody, and subsequently analysed using the FACSCalibur machine. Target cells were identified by CTFR staining, and were analysed for αgrB-PE antibody staining (unfilled peak) versus unstained controls (filled peak). Percentage of positive staining L1210 cells is shown. Relative cell counts are represented on y-axis, versus αgrB-PE staining on a log scale.

A



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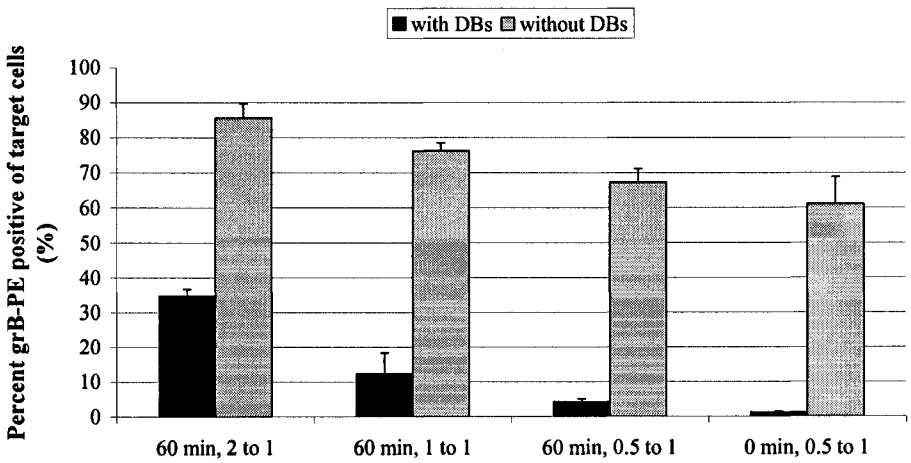
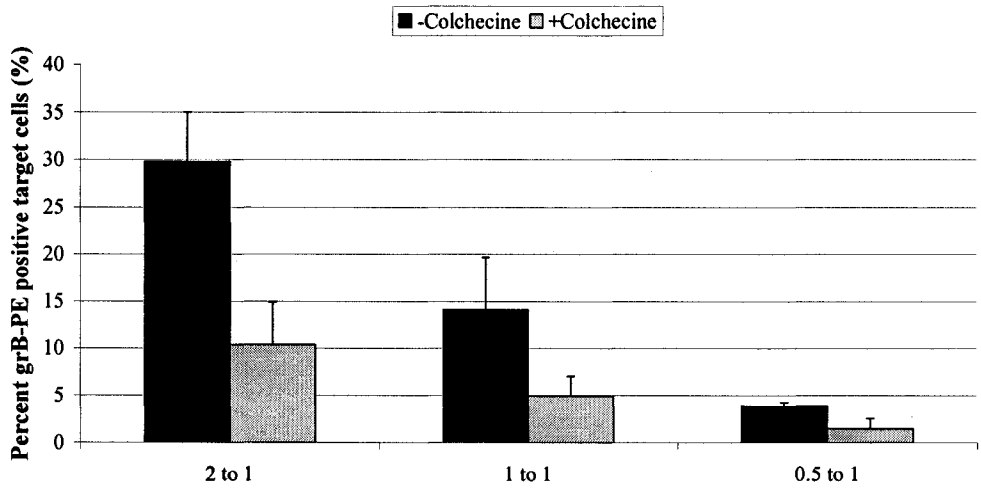


Figure 4-2. CTL depletion prior to αgrB-PE antibody staining of target L1210 cells renders the staining time- and dose- dependent

H-2^d-stimulated GrB WT CTLs were incubated with allogeneic CTFR-stained L1210 (H-2^d) target cells in RHFM/IL-2 media at 37°C, 5% CO_2 for 60 minutes (2:1, 1:1 or 0.5:1 E/T) or 0 minutes (0.5:1 E/T). CTLs were removed with αCD8a antibody-coated magnetic beads (with DBs, black bars) or were not removed (without DBs, grey bars) prior to αgrB-PE staining. Cells were washed and incubated in RT EDTA/PBS, then analysed using the FACSCalibur machine. Presented are the mean plus standard deviation of triplicate samples and is representative of at least two independent experiments. **A.** Percentage of $CD8^+$ cells remaining in total cell mixture was determined for all samples. **B.** Target cells were identified by CTFR staining, and assessed for percentage of target cells that acquired αgrB-PE staining.

A



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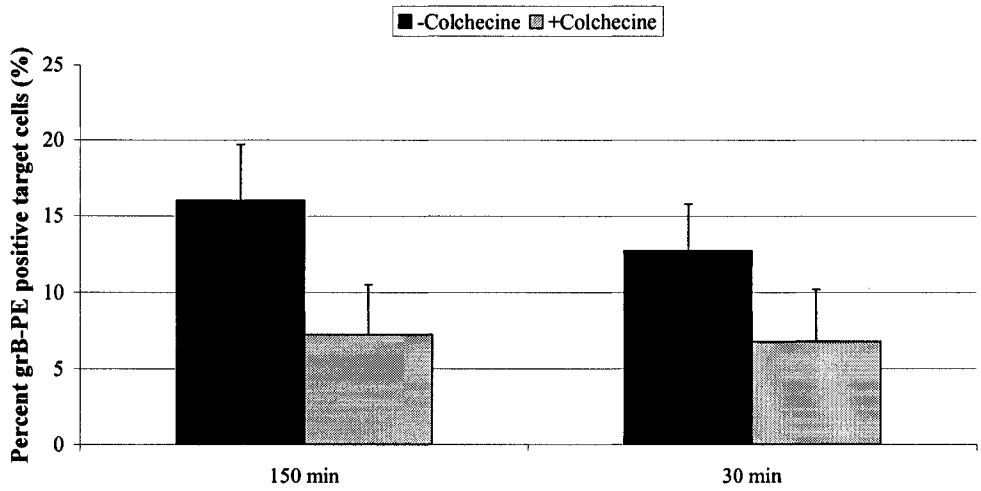

Figure 4-3. grB-PE antibody staining is dose- and time-dependent, and is reduced when CTL degranulation is inhibited

H-2^d-stimulated GrB WT CTLs were incubated with allogeneic CTFR-stained L1210 (H-2^d) target cells either with (grey bars) or without (black bars) previous incubation in media containing 20 mM colchecine. CTLs were removed with αCD8a antibody-coated magnetic beads prior to αgrB-PE staining. Cells were analysed by FACS. Target cells were identified by CTFR staining, and assessed for percentage of target cells that acquired αgrB-PE staining. Presented are the mean plus standard deviation of triplicate samples and is representative of at least three independent experiments. **A.** GrB WT CTLs were incubated with L1210 target cells at 2:1, 1:1 or 0.5:1 E/T for 90 minutes. **B.** GrB WT CTLs were incubated with L1210 target cells at 2:1 E/T for 150 or 30 minutes.

4.2.2 Tg CTLs induce grB-EGFP transfer into target cells

To determine if the grB-EGFP fusion protein is physically transferred to target cells, CTFR-stained L1210 (H-2^d) and EL4 (H-2^b) target cells were analysed by FACS for green fluorescence after incubation with Tg or WT CTLs (H-2^q) that were activated with Balb/c stimulators (H-2^d). Target cells were identified by CTFR staining and the percentage of target cells that had acquired green fluorescence was measured (Figure 4-4). The L1210 target cells became green after exposure to the CTLs, suggesting the fusion protein was taken up by these cells. In contrast, the EL4 cells that were not recognized by the CTL effectors did not accumulate green fluorescence (Figure 4-5). The amount of green fluorescence also increased with time of exposure, as L1210 target cells exposed to the Tg CTLs for longer periods accumulated more green fluorescence (Figure 4-6). When degranulation of Tg CTLs was inhibited by the addition of EGTA, the green fluorescence was no longer transferred to the L1210 target cells (Figure 4-7).

4.2.3 GrB WT CTLs induce transfer of grB enzymatic activity into target cells

Target cells were lysed and examined for grB enzymatic activity using an *in vitro* enzymatic assay. Whole cells lysates of allogeneic L1210 target cells incubated with GrB WT CTLs showed an increase of grB enzymatic activity. This activity was not present in lysates of L1210 cells alone, or on L1210 cells incubated with GrB KO CTLs. The syngeneic EL4 target cells did not exhibit high levels of grB enzymatic activity, even

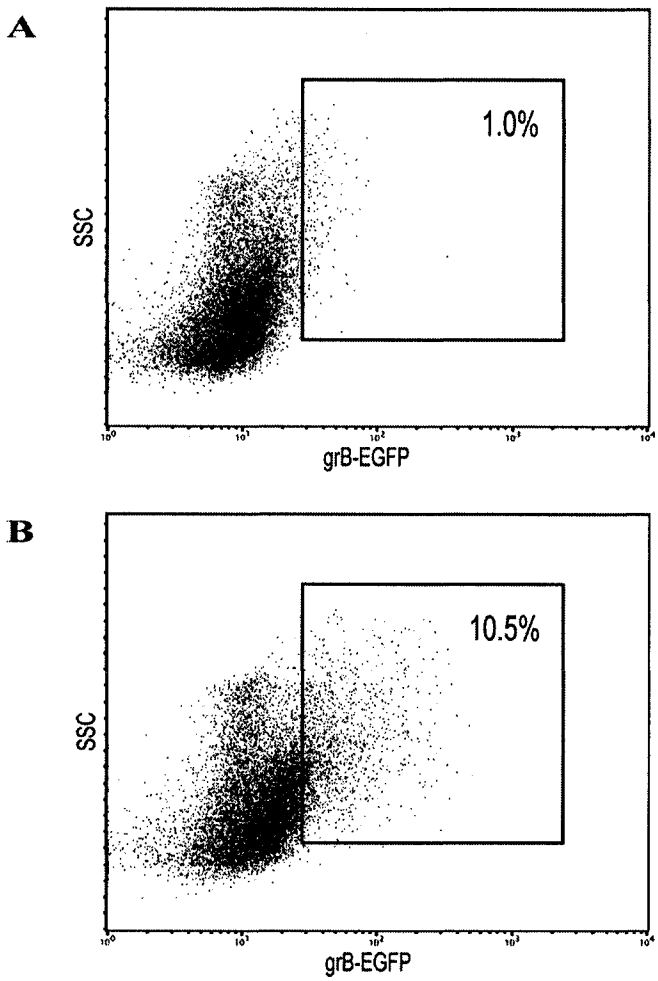

Figure 4-4. L1210 target cells acquire green fluorescence after exposure to Tg CTLs

Allogeneic L1210 target cells (H-2^d) were stained with CTFR and incubated with H-2^d-stimulated **A.** WT CTLs, or **B.** Tg CTLs at a ratio of 2:1 E/T. Cells were washed and incubated with EDTA/PBS and then analysed using the FACSCalibur machine. Target cells were identified by CTFR staining, and were analysed for grB-EGFP fluorescence as shown. FACS profiles of L1210 target cells show side scatter (SSC) versus grB-EGFP on a log scale. Numbers in profiles represent the percentage of target cells that are found in the rectangular gate and are therefore grB-EGFP positive. Panels are representatives of many such experiments.

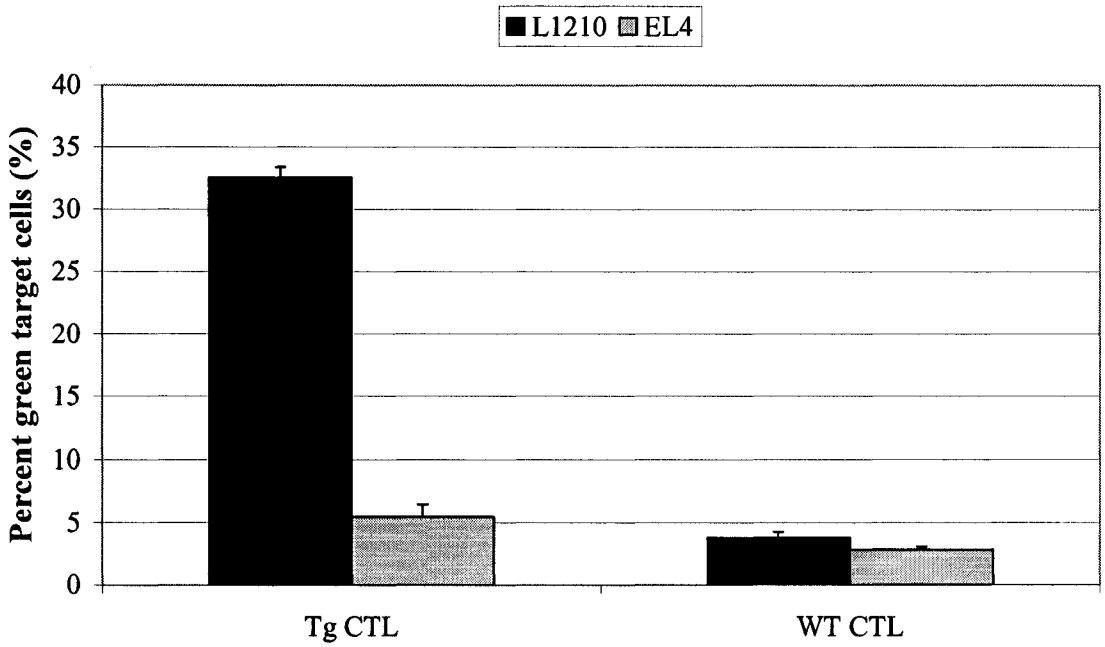

Figure 4-5. Accumulation of green grB-EGFP fluorescence is target specific

H-2^d-stimulated Tg and WT CTLs were incubated with allogeneic CTFR-stained L1210 (H-2^d) and EL4 (H-2^b) target cells (2:1 E/T) for 150 minutes in RHFM/IL-2 media at 37°C, 5% CO_2. Cells were washed and incubated in RT EDTA/PBS, then analysed using the FACSCalibur machine. Target cells were identified by CTFR staining, and assessed for grB-EGFP fluorescence. Percentage of target L1210 cells (black bars) and EL4 cells (grey bars) that are green after incubation with Tg CTLs (Tg) or WT CTLs (WT) are shown as mean plus standard deviation of triplicate samples, and is representative of at least 3 independent experiments.

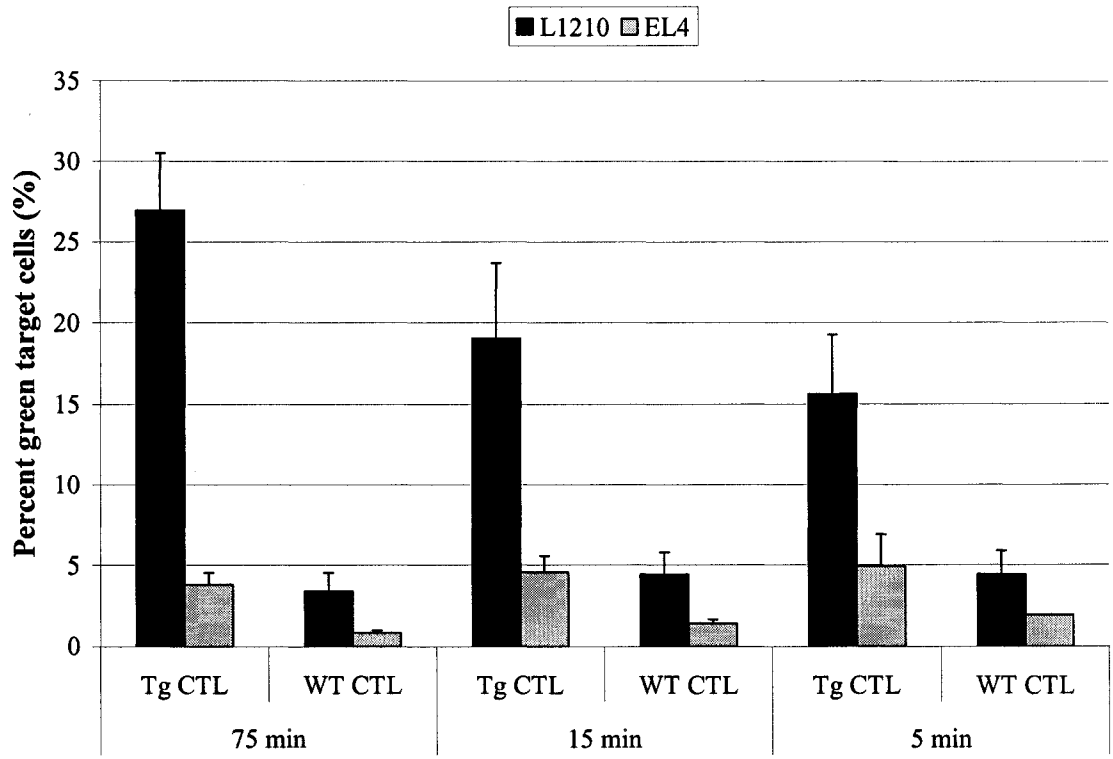

Figure 4-6. Accumulation of grB-EGFP fluorescence is time-dependent

H-2^d-stimulated Tg and WT CTLs were incubated with allogeneic CTFR-stained L1210 (H-2^d) and EL4 (H-2^b) target cells (2:1 E/T) for 75, 15 and 5 minutes in RHFM/IL-2 media at 37°C, 5% CO_2. Cells were washed and incubated in RT EDTA/PBS, then analysed using the FACSCalibur machine. Target cells were identified by CTFR staining, and assessed for grB-EGFP fluorescence. Percentage of target L1210 cells (black bars) and EL4 cells (grey bars) that were green fluorescent after incubation with Tg CTLs (Tg) or WT CTLs (WT) are shown as mean plus standard deviation of triplicate samples, and is representative of at least 3 independent experiments.

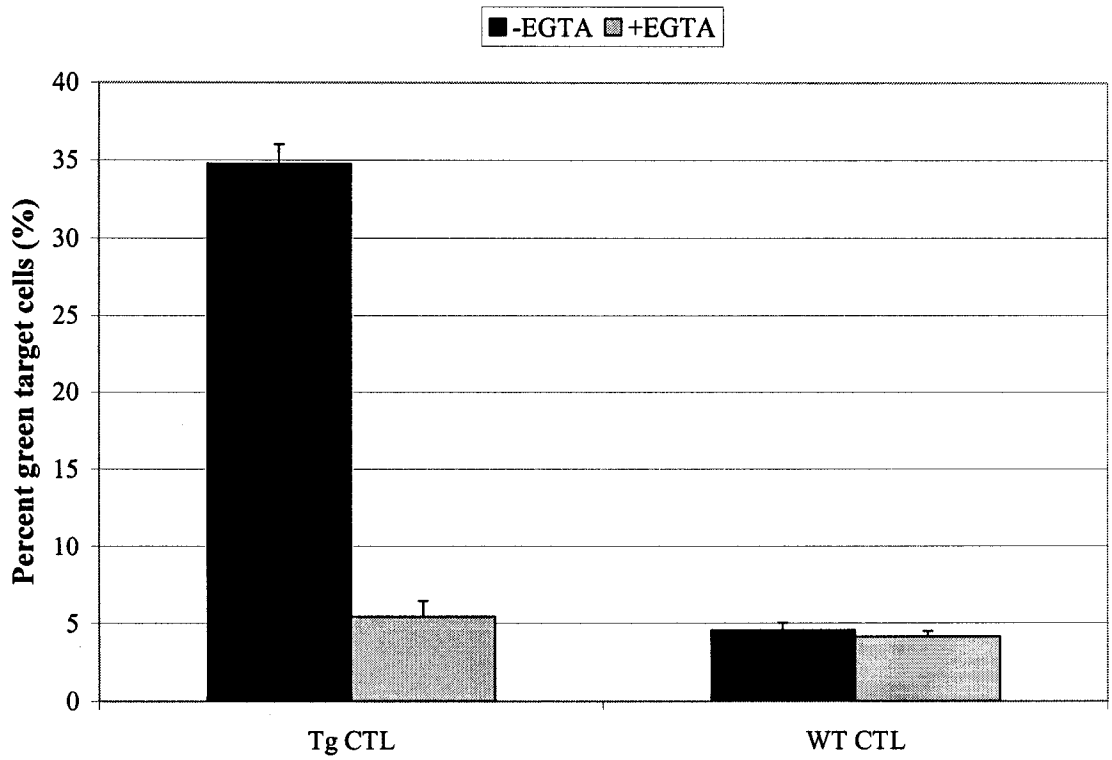

Figure 4-7. Accumulation of grB-EGFP fluorescence is dependent on CTL degranulation

H-2^d-stimulated Tg and WT CTLs were incubated with allogeneic CTFR-stained L1210 (H-2^d) target cells (2:1 E/T) for 150 minutes in RHFM/IL-2 media at 37°C, 5% CO_2 either with (grey bars) or without (black bars) the addition of 5 mM EGTA (pH 8) and 10 mM $MgCl_2$ to inhibit calcium-dependent degranulation of CTLs. Cells were washed and incubated in RT EDTA/PBS, then analysed using the FACSCalibur machine. Target cells were identified by CTFR staining, and assessed for grB-EGFP fluorescence. Percentage of target L1210 cells that are green after incubation with Tg CTLs (Tg) or WT CTLs (WT) are shown as mean plus standard deviation of triplicate samples, and is representative of at least 3 independent experiments.

when incubated with GrB WT CTLs (Figure 4-8). The colourmetric substrate used in this assay has been shown to be cleaved at a low level by caspases (C. Ewen, University of Alberta, personal communication). This assay was therefore repeated in the presence of the general caspase inhibitor zVAD-fmk (50 μM final concentration, Kamiya Biomedical Co., Seattle, WA) to determine if the colourmetric change was due to the activation of endogenous caspases present in the target cells during CTL-induced apoptosis. However, the activity was only slightly reduced when the inhibitor was first added to the lysates, as compared to the addition of the carrier DMSO alone (results not shown). This indicates that the activity measured in these lysates was indeed the activity of the grB from the CTLs, and not of endogenous activated caspases in the target cells.

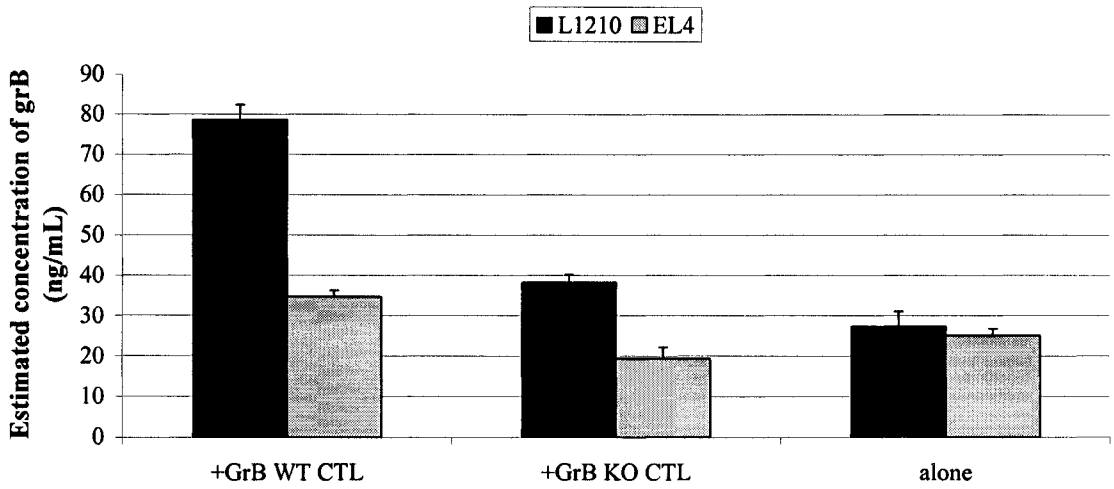

Figure 4-8. Cell lysates of allogeneic L1210 target cells, but not syngeneic EL4 target cells, acquire grB enzymatic activity after incubation with GrB WT CTLs

H-2^d-stimulated GrB WT or GrB KO CTLs were incubated with allogeneic L1210 (H-2^d) target cells or syngeneic EL4 (H-2^b) target cells (2:1 E/T) in RHFM/IL-2 media at 37°C, 5% CO_2 for 45 minutes, or target cells alone were also incubated. CTLs were removed with αCD8a antibody-coated magnetic beads and target cells were lysed. Whole cell lysates of L1210 cells (black bars) and EL4 cells (grey bars) were analysed for grB enzymatic activity. Estimated concentrations of grB (ng/mL) are shown as mean and standard deviation of triplicate samples and is representative of at least three independent experiments.

4.3 Discussion

Although the presence of grB in target cells is implied by the cleavage of target protein substrates, it has never been physically documented in target cells killed by CTLs. Experiments using αgrB-PE antibody staining showed endogenous grB transfer into allogeneic L1210 target cells in a time- and dose-dependent manner, since the signal accumulated when the target cells were exposed to a greater number of CTLs or for a longer time. The αgrB-PE staining of the L1210 target cells was also dependent on functional degranulation machinery of the CTLs, since the microtubule inhibitor colchecine decreased αgrB-PE staining in these cells. Colchecine was used to inhibit degranulation because EGTA did not inhibit αgrB-PE staining. It has been found in the laboratory EGTA does not inhibit degranulation in some other experimental conditions, as when degranulate is measured for grB enzymatic activity (C. Ewen, University of Alberta, personal communication). Degranulation is dependent on microtubule function, so the inhibition of αgrB-PE antibody staining by the microtubule-inhibiting drug colchecine demonstrates conclusively that the αgrB-PE staining is indeed degranulation-dependent.

The αgrB-PE antibody staining experiments also demonstrated αgrB-PE transfer from GrB WT CTLs into syngeneic EL4 target cells (results not shown). Since the EL4 cells are syngeneic targets, it is assumed that the CTLs will not attack these cells. Indeed, this staining does not reflect the level of apoptosis in the EL4 cells, since tritium and chromium release assays indicated that GrB WT CTLs do not kill syngeneic EL4 target

cells. It is possible that the αgrB-PE staining was due to surface-bound grB on the EL4 cells, but both EL4 and L1210 cells that have been exposed to GrB WT CTLs showed very little αgrB-PE positive surface staining (results not shown). The EL4 cells may have acquired grB protein nonspecifically because the CTLs spontaneously degranulate at a low level into the supernatant, due to continual activation of the CTLs by stimulator cells each week. The released granule contents, including grB and perforin, would therefore be present in the media during the killing assay. When the perforin protein is released in media of relatively high calcium concentration (1.5 mM) and without being in close proximity to a membrane, it is quickly inactivated (Henkart et al., 1984). This suggests that although grB may be present in the media, uptake of the protease into cells would be independent of perforin. This perforin-independent uptake of grB would result in grB being harmlessly sequestered in endocytic vesicles in the EL4 cells (Froelich et al., 1996). In contrast, the L1210 target cells were specifically targeted by the CTLs. This targeting facilitated a concentrated uptake of all granule contents released into the effector-target synapse, allowing both grB and perforin to enter the target L1210 cells to initiate apoptosis.

Although grB would not be active in the endocytic vesicles of the EL4 cells, the protease would have measurable enzymatic activity when released from these compartments after making target cell lysates. Instead, the αgrB-PE staining present in EL4 cells when visualised by FACS did not correlate with the low levels of grB enzymatic activity found within the EL4 cell lysates when measured in the enzymatic assay (Section 4.2.3). These paradoxical results were not the result of a difference in experimental methods of the

killing assays performed in both of these procedures. In both experiments the EL4 cells were exposed to the same E/T ratio of CTLs, and for the same length of time. The CTLs were also removed in a similar manner in both protocols. Nonetheless, αgrB-PE staining indicated the presence of grB protein in the EL4 target cells, yet the enzymatic activity of the lysates showed no grB enzymatic activity. In contrast, the activity of the lysates from the L1210 cells incubated with CTLs was much higher, and correlated with high αgrB-PE staining of these target cells. It is therefore unlikely that the αgrB-PE staining in the EL4 cells was due to the sequestration of grB into endocytic compartments. It is possible the difference in enzymatic activity between the lysates of these two different cell lines was due to caspase activity, because grB has similar substrate specificity to caspases. The observed difference in enzymatic activity would then be the result of caspase activation in the dying L1210 cells. Since the syngeneic EL4 cells do not undergo apoptosis and therefore caspase activation by the CTLs, the EL4 cells would not exhibit increased enzymatic activity. This was however not the case, as caspase inhibitors did not significantly reduce the level of enzymatic activity of the cell lysates.

The contradictory results of the uptake of grB into syngeneic EL4 target cells reported in this thesis are intriguing. The syngeneic EL4 cells accumulate αgrB-PE staining when exposed to CTLs, but the EL4 cells do not undergo apoptosis, and the lysates of these cells do not show any significant grB enzymatic activity. The EL4 cells may remain viable after grB uptake due to an intrinsic resistance to grB-mediated apoptosis. If EL4 cells expressed an inhibitor to grB, then the cells could accumulate αgrB-PE staining without undergoing apoptosis. The presence of an inhibitor would also reduce the

measurable enzymatic activity of grB in the CTL-treated EL4 cell lysates. Members of a family of protease inhibitors, called serpins, have been found to inhibit grB activity by binding covalently to the protease. Human CTLs express the serpin PI-9, a potent inhibitor of grB thought to protect the CTL from nonspecific grB release during target cell killing (Sun et al., 1996). Murine sertoli cells express serpina3n, a serpin that is important in the immunoprotective properties of these cells (Sipione et al., 2006). The expression of a potentially novel grB inhibitor in the EL4 cells would be an exciting discovery. Further studies to determine the cause of the resistance of EL4 cells to grB-mediated killing are therefore warranted.

The experiments using Tg CTLs have shown that exposure to allogeneic targets (L1210 cells) caused transfer of grB-EGFP into these target cells. This transfer was target specific, since EL4 cells that did not share the same haplotype as the irradiated cells used to stimulate the CTLs did not accumulate grB-EGFP signal. This result differed from the results seen with αgrB-PE staining in the EL4 cells. The sensitivity of these two methods of grB detection may contribute to this difference. The grB-EGFP protein is dimmer than the αgrB-PE antibody, and is therefore less detectable at low levels of protein concentration. The grB-EGFP accumulation in L1210 cells occurred in a similar manner as the increase of αgrB-PE antibody staining in these cells. The grB-EGFP accumulated in the L1210 target cells in a time-dependent manner. This transfer depended on the degranulation machinery of the CTL, and was inhibited by the presence of EGTA. These experiments conclusively show that CTLs transfer grB-EGFP into L1210 target cells.

In total, the experiments of grB uptake into target cells have shown that grB did indeed transfer into target cells. This transfer was degranulation-, time- and dose-dependent. Most of these experiments show this transfer was also target-specific, although syngeneic EL4 target cells also accumulated αgrB-PE antibody signal that did not result in apoptosis of the EL4 cells. The grB present in these syngeneic targets was not enzymatically active, and may reflect an intrinsic inhibition of grB activity by the EL4 target cells. The presence of grB inhibitory molecule in the EL4 cells must be confirmed with further research of this phenomenon.

5. Future Directions

The results outlined in this thesis have introduced some areas requiring future research. The generation of a CTL line from grB-EGFP Tg mice has shown that not all CTLs that expressed endogenous grB also expressed the fusion protein. This difference in the expression patterns of grB and grB-EGFP suggested that the transgene does not contain all the regulatory elements needed for absolute endogenous expression. Determination of these unknown elements would allow the creation of a transgene that would be normally expressed in CTLs, thus eliminating the necessity of sorting the green fluorescent CTLs, as has been described in this thesis. The creation of this fusion transgene will facilitate the relatively simple assembly of different grB-EGFP genomic constructs, and future studies of grB gene transcriptional regulation will be easily measured by FACS.

Another possible reason for the differential expression of the transgenic and the endogenous protein is that the grB-EGFP fusion construct may be preferentially expressed in a subset of CTLs. Different subsets of $CD8^+$ T cells have been discovered, including the so-called "effector" and "memory" groups of CTLs, however these broad grouping have also been further subdivided. This transgene may differentiate between two types of "effector" CTLs. In support of this concept, it has been found that a cytolytic population of human effector CTLs that highly express both perforin and grB can be distinguished from other $CD8^+$ T cells by expression of various surface markers (Rey et al., 2006). Intriguingly, the green fluorescent Tg CTLs described in this thesis

also seem to form a distinct population from the other Tg CTLs. The green cells are consistently larger when viewed by FACS than the non-green Tg CTLs (results not shown), and a similar group of large CTLs are also observed in the WT CTL lines, suggesting that this population of CTLs may exist in each CTL line generated. Further research to determine if these green Tg CTLs differ in surface marker staining or in functional assays from the non-green Tg CTLs is therefore necessary.

An important area of future research that is needed to broaden the findings of this thesis is the paradoxical results of grB uptake into EL4 cells. The EL4 cells are syngeneic targets of the WT GrB CTLs, and do not undergo apoptosis as measured by chromium and tritium release assays. However, these cells appear to accumulate αgrB-PE antibody signal after exposure to GrB WT CTLs, in a time- and dose-dependent manner. Furthermore, when lysates are made from the EL4 cells, there is no significant grB enzymatic activity measured. One explanation of these results is that the EL4 cells express a serpin, or other similar grB protein inhibitor. These serpins have been found to be expressed by viruses to avoid CTL-induced apoptosis of infected cells. This finding would greatly increase the understanding of tumour cell immune evasion. EL4 cells are tumour cells of lymphoid origin, and it is possible that an inhibitor to grB may be expressed in these cells to allow the cancer cells to avoid elimination by CTLs. An important first step in identifying a potential grB inhibitor expressed in these cells would be to examine the grB protein found in these cells by Western blotting. Since the serpins inhibit grB activity through covalently binding, a grB-serpin complex would appear as a higher molecular weight band than grB protein alone. Other approaches could also be

used to determine the cause of αgrB-PE accumulation in EL4 cells. A similar killing assay could be performed, but with CTLs derived from another mouse background, and activated with stimulators of the same haplotype as the EL4 cells. These newly derived CTLs would therefore kill the EL4 cells, and not the L1210 cells. The target cells could then be stained with αgrB-PE antibody to determine the pattern of grB accumulation under these experimental conditions.

One of the most important results reported in this thesis has been the creation of the Tg grB-EGFP mouse line, as there are many exciting possibilities for using these *in vivo* labelled fluorescent CTLs in other experimental methods to determine many important aspects of CTL effector function. In the scope of this thesis, these fluorescent CTLs were primarily generated to demonstrate grB uptake into target cells. However, these cells could be very useful to define other aspects CTL function, such as uptake of grB into target cells. The Tg CTLs would be valuable in determining the exact mechanisms involved in grB uptake into target cells, as the molecular machinery has not yet been completely elucidated. For example, the process of granule exocytosis can be visualised at the immunological synapse using fluorescent imaging of CTLs that have bound to artificial planar bilayers (Dustin et al., 2006). The grB-EGFP CTLs would allow live imaging of granule exocytosis using these methods, and the addition of stimulatory or inhibitory proteins into this artificial bilayer would enable the molecular dissection of this pathway.

Although the *in vitro* generated Tg CTLs are useful for some protocols, including the grB uptake studies outlined in this thesis, the Tg mice are also a powerful tool to elucidate activation and effector function of CTLs *in vivo*. These mice can be used to analyse the mechanisms of action of CTLs generated in the body, as during an infection. As described in this thesis, the *in vivo* generation of green fluorescent CTLs was observed when Tg mice were infected with LCMV. This mouse line could also be used in a variety of other *in vivo* models of immune function. For example, the kinetics and spatial distribution of CTLs during tumourigenesis could be studied by the injection of tumour cells into these mice. If a solid tumour is formed, it could be excised to gather information on the activation level of the CTL infiltrate. The activated CTLs could also be collected and cultured *in vitro*, and killing assay performed on the tumour cell line to observe whether grB accumulates in the target tumour cells. Mouse transplantation models could be applied to these Tg mice as well. The graft versus host disease (GvHD) phenomenon, as well as transplant rejection, could be observed in these mice, to determine the temporal and spatial control of these green effector CTLs in these models.

In this thesis, the *in vivo* generated green CTLs were extracted from different tissue samples of the LCMV-infected mice. This method allowed an examination of effector CTLs present in each gross tissue sample, but did not allow live observation of the CTLs as the infection progressed in the animals. Since the grB-EGFP molecule is a live marker of CTLs, this Tg mouse line is uniquely equipped for the live analysis of *in vivo* CTLs. For example, the use of intravital multiphoton microscopy has allowed researchers to examine CTL action in a whole mouse (Mempel et al., 2006). The Tg CTLs would

provide an activation marker for effector CTLs, and would also allow the live visualisation of grB-EGFP transfer into target cells *in vivo*. The murine immunological models described in the previous paragraph could all be performed to collect live data with the use of this intravital microscopy technique. The generation of the Tg mice has therefore created an enormous array of possibilities of live imaging of grB-EGFP expressing CTLs, to examine the transcriptional regulation of the grB gene, the exocytosis of grB-EGFP from the CTL, and the *in vivo* actions of effector CTLs in mouse models of infection, tumourigenesis, and graft rejection. In conclusion, the generation of a grB-EGFP Tg mouse line has provided many new opportunities and exciting avenues of future studies.

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