University of Alberta

Regulation of FasL Expression and Trafficking
in Cytotoxic T Lymphocytes

by

Jinshu He

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Immunology

Department of Medical Microbiology and Immunology

Fall 2009
Edmonton, Alberta

Examining Committee

Dr. Hanne Ostergaard, Medical Microbiology and Immunology

Dr. Michele Barry, Medical Microbiology and Immunology

Dr. Paige Lacy, Medicine

Dr. R. Chris Bleackley, Biochemistry

Dr. Tania Watts, Immunology, University of Toronto

ABSTRACT

Cytotoxic T lymphocytes (CTL) are differentiated $CD8^+$ T cells that eliminate virally infected cells and tumor cells. CTL lyse target cells by at least two distinct mechanisms: degranulation of cytolytic molecules and cell surface expression of Fas ligand (FasL), which induces apoptosis of Fas-expressing target cells. In addition to their defense function, these two cytolytic mechanisms also play crucial roles in homeostatic regulation and contribute to pathogenesis in many different model systems. To fully exploit killer cells in tumor and virus elimination, or dampen the immune response in, for example, autoimmune diseases, it is essential to understand the mechanisms that CTL employ to destroy target cells.

In contrast to the well-characterized degranulation mechanism, the regulation of FasL expression on the CTL cell surface remains elusive and even controversial. The prevailing model at the time I initiated my studies was that FasL is stored in cytolytic granules and that FasL cell surface expression would be subject to the same controls as degranulation. In this thesis, I revealed for the first time that there are two waves of FasL cell surface expression upon target cell engagement, which are differentially regulated by TCR signaling and perform distinct roles in CTL mediated responses. I demonstrated that CTL degranulation and FasL lytic mechanisms are fully independent with respect to stored component localization and regulation. Finally, based on cell fractionation and imaging studies, I suggested that FasL is stored in a recycling endosome

associated compartment, which is located in a special niche between the ER and mitochondria and uses a novel microtubule-independent secretory mechanism to translocate to the cell surface. Together, these findings provide important insight into the regulation and role of FasL in CTL mediated responses.

ACKNOWLEDGEMENTS

This thesis is the result of six years of work whereby I have been accompanied and supported by many people. It is a pleasure that I have the opportunity to express my gratitude for all of them.

I would like to thank my supervisor, Dr. Hanne Ostergaard, for her tremendous support and guidance over the years. Her enthusiasm and integral view on research inspired my growth as an independent researcher. I am really glad that I have come to get know her in my life. I also appreciate the loving support and encouragement I received from family and friends, especially my husband Zhenchang Xing, my mother Yanqiu Hu and my father Peilian He. I would like to thank my supervisory committee members and other members of my Ph.D. examining committee for their input into the project and providing me with invaluable comments on this thesis: Dr. Michele Barry, Dr. Paige Lacy, Dr. Tania Watts and Dr. R. Chris Bleackley. I would like to acknowledge Dr. Kevin Kane for constructive criticism during lab meetings and for helpful discussion and for critically reviewing manuscripts. Thanks to Dong-Er Gong for excellent technical assistance, Dr. Xuejun Sun and Gerry Barron for assistance with confocal microscopy, and Babara Simon for preparing many of the stock reagents and media used during this project. Many of the past and present members of Dr. Ostergaard's and Dr. Kane's laboratories have provided help and advice in this work. Financial support for the project was provided by the Canadian Institutes of Health Research and the Alberta Heritage Foundation for Medical Research.

Finally, I would like to thank everybody who was important to the successful realization of this thesis, as well as express my apology that I could not mention personally one by one.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AICD	Activation induced cell death
AP1	Activator protein 1
APC	Antigen presenting cell
APL	Altered peptide ligand
ARF	ADP-ribosylation factor
BAPTA-AM	1,2-Bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)
BCA	Bicinchoninic acid
Bcl-2	B-cell leukemia/ lymphoma 2
BCR	B cell receptor
BFA	Brefeldin A
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
BIG	BFA-inhibited GEF
Bim	Bcl‑2-interacting mediator of cell death
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
CHX	Cycloheximide
CKI	Casein kinase I
COPI	Coat protein I
CRAC	Calcium release-activated calcium channel
CRD	Cysteine-rich domain
CsA	Cyclosporin A
CTL	Cytotoxic T lymphocyte

DAG	Diacylglycerol
DC	Dendritic cell
dCS	Defined bovine calf serum
DD	Death domain
DIC	Differential interference contrast
DISC	Death-inducing signal complex
DMEM	Dulbecco’s modified essential medium
DMSO	Dimethyl sulfoxide
DN	Double negative
DOC	Deoxycholate
DP	Double positive
D-PBS	Dulbecco's phosphate- buffered saline
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early Endosome Antigen 1
Egr	Early growth response factor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ER-GIC	Endoplasmic reticulum-Golgi intermediate compartment
ERK	Extracellular regulated kinase
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain
FasL	Fas ligand
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Fyn	Fibroblast src/yes novel gene
GII	Glucosidase II

GBF1	Golgi BFA-resistance factor 1
GDP	Guanosine 5'-diphosphate
GEF	Guanine nucleotide exchange factor
gld	Generalized lymphoproliferative disease
GM-CSF	Granulocyte macrophage colony-stimulating factor
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine 5'-triphosphate
GVHD	Graft-versus-host disease
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule-1
ICER	Inducible cAMP early repressor
IFN-γ	Interferon-gamma
IL	Interleukin
IMM	Immobilized antibody
IP	Immunoprecipitation
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
LAMP1	Lysosomal-associated membrane protein 1
LAT	Linker for the activation of T cells
Lck	Lymphocyte-specific cytoplasmic protein tyrosine kinase
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen-1
lpr	Lymphoproliferation
LPS	Lipopolysaccharide

MACS	Magnetism-activated cell sorting
MAM	Mitochondria-associated ER membrane
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MMP	Matrix metalloprotease
MTOC	Microtubule organizing center
MVB	Multivesicular body
NFAT	Nuclear factor in activated T cells
NF-κB	Nuclear factor-kappa B
NK cell	Natural killer cell
NOD	Non-obese diabetic
NP	Nucleoprotein
NP-40	Nonidet P-40
ORAI1	Calcium release-activated calcium modulator 1
OVA	Ovalbumin
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PE	Phycoerythrin
PEL	Peritoneal exudate lymphocytes
PHA	Phytohemagglutinin
PI3K	Phosphoinositide-3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC-γ	Phospholipase C gamma
PMA	Phorbol 12-myristate 13-acetate
pMHC	Peptide-MHC
PMSF	Phenylmethylsulphonyl fluoride

PNS	Postnuclear supernatant
PRD	Proline-rich domain
PVDF	Polyvinylidene difluoride
RANTES	Regulated upon activation, normal T cell expressed and secreted
RasGRP	Ras guanyl nucleotide releasing protein
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute
SA	Self assembly
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH	Src homology
SLP-76	SH2-domain-containing leukocyte protein of 76 kDa
SNAREs	Soluble *N*-ethylmaleimide-sensitive attachment protein receptors
Sp-1	Specificity protein-1
SP	Single positive
STIM1	Stromal interaction molecule 1
T-bet	T-box expressed in T-cells
TCR	T cell receptor
TfR	Transferrin receptor
TGN	*Trans*-Golgi network
THD	TNF homology domain
TNFα	Tumor necrosis factor alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
TX-100	Triton X-100

VAMP-3	Vesicle-associated membrane protein 3
WB	Western blot
XL	Cross-linked antibody
ZAP-70	Zeta-associated protein-70

CHAPTER 1 : Introduction

The innate and adaptive immune systems

Detecting and eliminating invading microorganisms is required for the survival for all living creatures. Host defences against microorganisms that are recognized as foreign or "non-self" are mediated by the immune system, which consists of two tightly interregulated branches: innate and adaptive immunity. The innate immune system works rapidly to combat quickly replicating microbial invaders and gives rise to the acute inflammatory response, providing the first line of host defence. In contrast, the primary adaptive immune response takes longer to develop, typically 4–7 days after initial antigen exposure. This mechanism is highly specific for antigens and accounts for the generation of a rapid recall or memory response when re-exposed to the same antigenic challenge.

The cellular components of the innate immune system include macrophages, neutrophils, granulocytes, monocytes, mast cells, dendritic cells (DCs) and natural killer (NK) cells. These cells are usually present at the site of invasion or subsequently migrate to the site of inflammation and are readily available to contain pathogens by phagocytosis, producing antimicrobial peptides, or, in the case of NK cells, eliminating pathogen-infected cells. The targets of innate immune recognition are conserved pathogen-associated molecular patterns (PAMPs), which are invariant between microorganisms of a given class and are essential for microbial survival, thus 'escape mutants' are not generated. Unlike cells of the adaptive immune system, this system generally does not have memory

for specific pathogens. However, the innate immune response results in the expression of co-stimulatory molecules on the cell surface of antigen presenting cells (APC) – dendritic cells (DCs) and macrophages, and the production of pro-inflammatory cytokines, which control the initiation of the adaptive immune response, determine the differentiation of particular effector populations and influence further memory formation of the adaptive immune system.

The adaptive immune system is composed of two major types of lymphocytes: B cells and T cells. Lymphocytes provide both the specificity and memory which are characteristic of the adaptive immune response. Adaptive immune recognition relies on the generation of a random and highly diverse repertoire of antigen receptors — the T- and B-cell receptors (TCR and BCR) — followed by clonal selection and expansion of receptors with relevant specificities. B cells mature in the bone marrow and differentiate into antibody-secreting plasma cells upon activation to mediate humoral immunity. T cells develop in the thymus and give rise to cellular immunity. Two different kinds of T cells are involved in these functions. One type is $CD8^+$ T cells that differentiate into cytotoxic T lymphocytes (CTL) upon activation and directly eliminate cells infected with intracellular pathogens as well as transformed cells. It has been reported that transcription factor Eomesodermin (Eomes), a paralogue of T-bet (T-box expressed in T-cells), is involved in full effector differentiation of CTL (Pearce et al. 2003). The other type of T cell is the $CD4^+$ T cell, which are further categorized as T helper (T_H) cells, including T_H1, T_H2, T_H-17 cells, and regulatory T cells (Treg). T_H cells are mainly involved in intercellular co-operation between

haematopoietic cells in an immune response. T_H1 cells mediate protection against intracellular pathogens. T_H2 cells mediate humoral immunity and contribute to allergic responses. T_H-17 cells protect against extracellular bacteria and fungi, particularly at mucosal sites (Aujla et al. 2007). However, inappropriate T_H1 and T_H-17 responses give rise to autoimmune diseases, whereas allergic diseases result from inappropriate T_H2 responses (Murphy and Reiner 2002; Ansel et al. 2006; Ouyang et al. 2008). Treg cells are exported from the thymus as natural Treg cells or generated by the differentiation of naïve T cells in the periphery, suppressing immune responses and are essential for the maintenance of T cell homeostasis and peripheral tolerance under both physiological and immunopathological conditions including cancers (Sakaguchi 2005). In addition, so-called 'innate T cells' such as NKT and γδT cells also provide important host defense against pathogens, and exhibit characteristics of both innate and adaptive immunity.

T cell development in the thymus

T lymphocytes are produced in the thymus from circulating T cell progenitors. In the thymus, a diverse and polymorphic T-cell repertoire is generated by random recombination of discrete TCR α, β, γ and δ gene segments encoding multiple members of V, J and D gene regions, giving rise to T cells with a TCR containing either α and β chain, or γ and δ chain. The thymus provides an environment not only for the generation of T cell diversity, but also for the shaping of a peripheral T-cell pool that is self-MHC restricted and self-tolerant.

The initial T cell progenitors are double negative (DN) thymocytes, which do not express the T cell co-receptors CD4 and CD8. For cells that proceed along the αβ TCR pathway, upon entering into the thymus, DN thymocytes begin to rearrange their TCRβ locus and express the TCRβ chain. TCRβ chain pairs with pre-TCRα chains to form the pre-TCR complex, which initiates the signals for further development to $CD4^+$ $CD8^+$ double positive (DP) thymocytes. The TCRα locus then begins to rearrange and produces the second component chain of the mature αβ antigen receptor. The DP thymocytes then undergo positive and negative selection during which, thymocytes that do not bind self peptide-MHC complexes die by neglect, whereas those that bind self peptide-MHC complexes too strongly undergo apoptosis, and only those bearing TCR with low-affinity to self peptide-MHC receive signals for survival. This process enriches 'useful' T cells that are potentially reactive to foreign antigens, but not to self antigens, presented by self-MHC molecules. These selected DP thymocytes then differentiate into either CD4 or CD8 single positive (SP) thymocytes. Thymocytes that express TCRs that bind self peptide-MHC class I complex become $CD8^+$ T cells, whereas those that express TCRs that bind self peptide-MHC class II complex become $CD4^+$ T cells. These cells are then ready for export from the thymus as $CD62L^{hi}$ $CD69^{lo}$ mature but naïve T cells to peripheral lymphoid sites. Post-thymic T cells retain a low degree of self-reactivity; this reactivity is generally not sufficient to cause autoimmunity, but is essential for survival of naïve T cells and also enhances TCR sensitivity for foreign antigens (Davis et al. 2007).

T cell activation in the periphery

Once in the periphery, naïve T cells constantly circulate between the blood and secondary lymphoid organs, including spleen, peripheral and mesenteric lymph nodes and Peyer's patches, where they survey the antigens displayed by APCs, mainly DCs. As a consequence of infection, DCs process and present foreign antigens associated with both MHC class I and MHC class II molecules, and differentiate into mature APCs that express co-stimulatory molecules, produce cytokines and migrate to secondary lymphoid organs. Once a naïve T cell encounters and recognizes cognate antigen presented by mature DCs in the secondary lymphoid organs, they undergo dramatic proliferation (as much as 10^4-fold to 10^5-fold in as little as 8 days) through a process known as clonal expansion, and differentiate into effector T cells, either CTL or T helper cells. Effector T cells then leave the secondary lymphoid organs, migrate to the site of infection and eliminate the pathogen by killing infected cells, producing cytokines, and recruiting other leukocytes via chemokine production. Once the invading pathogen has been eliminated, activated T cells undergo a contraction phase through apoptosis, which is crucial for the maintenance of T cell homeostasis. After the contraction phase, only a fraction, typically 5%-10%, of pathogen-specific T cells survive. These cells eventually develop into memory T cells, typically with a frequency 10^2-10^3 fold larger than that of the initial naïve T cell population, which persist long-term and allow for a more rapid expansion of effector cells upon re-encounter with the same pathogen.

Signals from antigenic peptide-MHC (pMHC) (signal 1), co-stimulatory molecules (signal 2) and cytokines (signal 3) presented by mature DCs to naïve T cells all play key roles in their proliferative expansion and differentiation into effector, and eventually memory T cells. Among these factors, signal 3 is especially indispensible for the development of CTL cytolytic activity. It was reported that without signal 3 cytokines, including IL-12, CTL were defective in the cytolytic molecule granzyme B production (Curtsinger et al. 2005). Whether signal 3 is involved in Fas ligand (FasL) production, which induces apoptosis of Fas-expressing target cells, has not yet been described.

Intracellular signaling upon TCR engagement

Activation via the TCR triggers a cascade of intracellular biochemical events eventually leading to T cell proliferation and effector functions. TCR signals through its non-covalently associated CD3 chains. Recognition of pMHC by TCR results in phosphorylation of the ITAMs (immunoreceptor tyrosine-based activation motifs) of the CD3 complex by the src family kinases Lck (lymphocyte-specific cytoplasmic protein tyrosine kinase) and Fyn (fibroblast src/yes novel gene), which permits the recruitment and activation of the syk family protein tyrosine kinase ZAP-70 (zeta-associated protein-70). ZAP-70 subsequently phosphorylates the integral membrane adaptor protein LAT (linker for the activation of T cells). Phosphorylated LAT then recruits various cytoplasmic adapters either directly or through other adaptors, such as Grb2 (growth factor receptor-bound protein 2) and SLP-76 (SH2-domain-containing

leukocyte protein of 76 kDa) (Germain and Stefanová 1999). Grb2 is involved in the activation of Ras-Raf-ERK pathways (Zhang and Samelson 2000). SLP-76 in involved in the activation of PLC-γ (Phospholipase C gamma) (Germain and Stefanová 1999). Once activated, PLC-γ cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2), generating the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG binds to and activates novel protein kinase C (nPKC) and RasGRP (Ras guanyl nucleotide releasing protein). IP3 binds to its receptor on the endoplasmic reticulum (ER) membrane to release intracellular Ca^{2+} stores. Ca^{2+} flux in combination with DAG activates conventional PKC (cPKC). Depletion of the ER Ca^{2+} stores subsequently triggers STIM1 (Stromal interaction molecule 1) oligomerization, which then redistribute from the ER to the ER-plasma membrane junctions, and activates Orai pore-forming channel subunits in the plasma membrane to open the CRAC (Ca^{2+} release–activated Ca^{2+}) channel (Roos et al. 2005; Wu et al. 2006; Luik et al. 2006; Luik et al. 2008; Park et al. 2009) that allows sustained influx of extracellular Ca^{2+} into the cytoplasm. Calcium signaling is involved in regulating T cell proliferation, differentiation, apoptosis and effector functions. It also plays key roles in regulating intracellular vesicular trafficking.

Overview of cytotoxic T lymphocyte mediated cytotoxicity

Cytotoxic T lymphocytes (CTL) are differentiated $CD8^+$ T cells that eliminate tumor cells and cells infected with intracellular pathogens. CTL lyse target cells by at least two distinct well-characterized mechanisms: extracellular

Ca^{2+}-dependent degranulation of cytolytic molecules, including perforin and granzymes, and expression of Fas ligand (FasL), which induces apoptosis of Fas-expressing target cells (Barry and Bleackley 2002). CTL also secrete antiviral and inflammatory cytokines including IFN-γ (interferon-gamma) and TNFα (tumor necrosis factor alpha) (Kaech et al. 2002; Wherry and Ahmed 2004; Williams and Bevan 2007). In addition to the defense function, CTL cytolytic mechanisms also play crucial roles in homeostatic regulation and contribute to pathogenesis in many different model systems (Russell and Ley 2000), including autoimmune disorders (Vizler et al. 1999; Liblau et al. 2002; Walter and Santamaria 2005), graft-versus-host disease (GVHD) (Shlomchik 2007) and development of viral pathogenesis (Balkow et al. 2001; Ibuki et al. 2002; Ostler et al. 2002; Rutigliano and Graham 2004; Rehermann and Nascimbeni 2005).

During naïve $CD8^+$ T cell differentiation into CTL, they store cytolytic molecules in specialized secretory lysosomes. Upon engagement of a target cell bearing appropriate peptide/MHC complexes, degranulation of preformed cytolytic molecules, including perforin and granzymes, towards the target cell induces rapid apoptosis. Studies with perforin knockout mice have shown that this protein is essential for the elimination of infections with various viruses (Kägi et al. 1994a; Walsh et al. 1994a; Gupta et al. 2005). Recently, Migueles and colleagues showed that HIV-1-specific $CD8^+$ T cells from those rare HIV-1-infected individuals who are able to control viral replication without antiretroviral therapy, effectively suppress viral replication by granzyme B/ perforin-mediated

elimination of target cells (Migueles et al. 2008), further highlighting the crucial effect of degranulation in antiviral immunity.

FasL expression can be induced on CTL by antigen receptor engagement and confers the ability to kill Fas-expressing target cells. FasL participates in host defense against certain intracellular pathogens and transformed cells. Because functional Fas is constitutively expressed on a variety of tissues, as well as inducibly expressed in multiple lineages of immune cells, expression of FasL on CTL must be tightly regulated. Initial studies suggested that FasL mediated cytotoxicity relies on *de novo* FasL synthesis (Walsh et al. 1994b; Vignaux et al. 1995; Glass et al. 1996). Recently, however, it has been revealed that in addition to *de novo* synthesized FasL, there is a preformed pool of FasL stored in CTL (Li et al. 1998; Bossi and Griffiths 1999; Kojima et al. 2002), which is sufficient to mediate target cell killing without *de novo* protein synthesis (Li et al. 1998). However, the regulation and subcellular distribution of the preformed FasL in CTL remains elusive and even controversial. Li et al. suggested that preformed FasL is either constitutively expressed on the cell surface or stored in a Golgi-associated compartment (Li et al. 1998). Bossi and Griffiths demonstrated that preformed FasL is stored in cytolytic granules and degranulation controls FasL cell surface expression upon target cell engagement (Bossi and Griffiths 1999). Therefore, additional studies are required to resolve these conflicting observations.

Mechanism of degranulation

CTL and NK cells have lysosomally derived cytolytic granules, which have been designated "secretory lysosomes" because of their dual function in degradation and secretion (Burkhardt et al. 1990; Blott and Griffiths 2002). A mature secretory lysosome contains an electron-dense core normally found in secretory organelles and a multilamellar region typical of lysosomes (Burkhardt et al. 1990). Cytolytic granules contain cytolytic components such as perforin and granzymes, and lysosomal proteins such as LAMP1, LAMP2 and CD63. Perforin, a Ca^{2+}-dependent pore-forming protein that has homology to complement components, is an essential component of the killing mechanism of CTL and NK cells, and is the only molecule from the granules that can deliver granzymes into the target cell (Pipkin and Lieberman 2007). Granzymes are a group of serine proteases. The most abundant and best-characterized granzymes are granzyme A and granzyme B (Russell and Ley 2002; Lieberman and Fan 2003; Lord et al. 2003). While granzyme B cleaves procaspase 3 and the pro-apoptotic molecule Bid (Atkinson et al. 1998; Barry et al. 2000), thus activating programmed cell death, granzyme A induces nicks in single stranded DNA and prevents cellular repair (Shresta et al. 1999; Beresford et al. 1999), forcing cells into apoptosis (Bossi and Griffiths 2005). A recent model suggests that granzymes and perforin work together to induce target cell apoptosis, in which granzymes can be internalized by receptor-mediated endocytosis, and perforin then mediates the release of granzymes from intracellular vesicles, so that they can gain access to their substrates (Barry and Bleackley 2002). Recently, granzyme A has been

suggested to carry out alternative functions including activation of inflammatory pathways through pro-IL-1β processing in antigen presenting cells such as macrophages (Metkar et al. 2008).

In CTLs, after target cell conjugation, the microtubule organizing centre (MTOC) rapidly moves to the site of contact with the target cell (Kupfer and Dennert 1984; Kupfer et al. 1986; Stinchcombe et al. 2004). Cytolytic granules move along microtubules to the contact point with the target cell (Sancho et al. 2002), then a fraction of the granules fuse with the plasma membrane at a specific area within the immunological synapse, which is called "secretory synapse" (Stinchcombe et al. 2001a; Bossi et al. 2002), and the contents are directionally released to the target cell resulting in rapid target cell death (Trambas and Griffiths 2003).

Much has been learned over the past decade regarding the control of cytolytic granule movement, membrane docking and membrane fusion, mostly through the analysis of CTL from human patients with various secretion defects (Stinchcombe et al. 2004). Two essential effectors for degranulation have been identified: Rab27a (Ménasché et al. 2000; Haddad et al. 2001; Stinchcombe et al. 2001b) and Munc13-4 (Feldmann et al. 2003). Although the molecular or cellular mechanisms underlying their effects are not comprehensively defined, it has been shown that in the absence of Rab27a, a member of the large family of Ras-like GTPases involved in intracellular vesicle trafficking, the granules polarize towards the target cell, however they are unable to dock at the plasma membrane (Stinchcombe et al. 2001b), and remain bound to the MTOC suggesting that

Rab27a is required for microtubule detachment (Stinchcombe et al. 2004). In the deficiency of Munc13-4, a SNARE effector protein, cytolytic granules polarize and dock normally at the immunological synapse, but fail to fuse with the plasma membrane (Feldmann et al. 2003), indicating its involvement in granule membrane fusion. Recently, it was reported that upon target cell engagement, polarized cytotoxic granules in CTLs need further maturation steps, which involve the fusion of perforin/ granzyme-containing granules with an "exocytic vesicle" at the immunologic synapse, so allow for exocytosis of the cytolytic contents (Ménager et al. 2007). During this process, Munc13-4 was shown to regulate the assembly of the "exocytic vesicle", which is formed by the fusion of late lysosomes with the recycling endosomes carrying effectors of the exocytic machinery, including Munc13-4 and Rab27a (Ménager et al. 2007).

FasL structure

Fas ligand (FasL) was identified in 1993 as a 40-kD type II transmembrane glycoprotein belonging to the tumor necrosis factor (TNF) superfamily (Suda et al. 1993). It is predominantly expressed by activated T lymphocytes and NK cells (Suda et al. 1995; Montel et al. 1995; Nagata and Golstein 1995). FasL has been shown to mediate both apoptotic and inflammatory reactions. Furthermore, it can trigger signaling events that modulate T lymphocyte proliferation *in vitro* and *in vivo* (Suzuki and Fink 1998; Suzuki et al. 2000). FasL shares 25 to 30% sequence homology with other TNF superfamily members (Locksley et al. 2001). As illustrated in Figure 1-1, it has

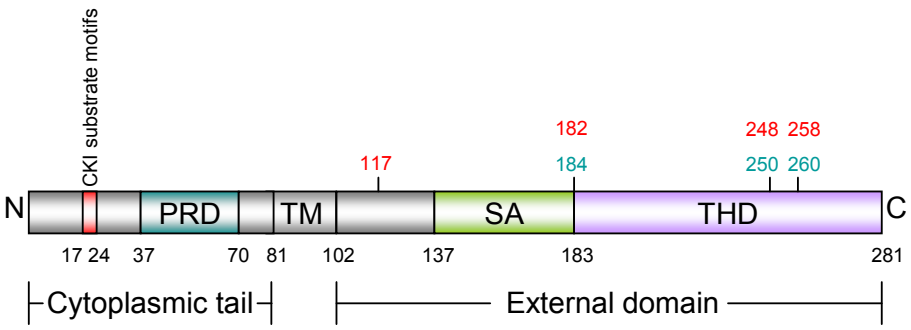


Figure 1-1. The structure of FasL. CKI substrate motifs, casein kinase I substrate motifs; PRD, proline-rich domain; TM, transmembrane domain; SA, self-assembly domain; THD, TNF homology domain. Murine FasL contains four potential N-glycosylation sites (N117, N182, N248, N258) (red), whereas human FasL contains three potential N-glycosylation sites (N184, N250, N260) (green).

an 80 amino acid cytoplasmic tail, which is the longest of the 19 known TNF family members (Bodmer et al. 2002). The cytoplasmic tail contains a striking proline-rich domain (PRD) spanning amino acids 37–70 (Janssen et al. 2003). The PRD consists of several potential SH3 (Src homology 3) domain- or WW-domain binding sites (Hane et al. 1995; Wenzel et al. 2001; Ghadimi et al. 2002; Baum et al. 2005), which are not found in any other TNF family members, suggesting a potentially unique role of the PRD in FasL regulation. The PRD has been suggested to be important for regulating FasL trafficking and FasL signaling (Blott et al. 2001; Sun et al. 2007). The FasL cytoplasmic tail also contains two conserved casein kinase I (CKI) substrate motifs, which serve as targets for casein kinase, resulting in serine/threonine phosphorylation (Sun et al. 2006; Sun et al. 2007), similar to what has been previously described for transmembrane TNFα (Watts et al. 1999). The CKI substrate motif has been implicated in regulating FasL signaling (Sun et al. 2006; Sun et al. 2007). The extracellular domain of FasL contains a self-assembly domain (SA) that is well conserved in all TNF family ligands (Orlinick et al. 1997a), which mediates the assembly of the bioactive FasL trimer. The conserved TNF homology domain (THD) close to the C-terminal end mediates highly specific binding to the cystein-rich domains (CRD) of the Fas receptor (Orlinick et al. 1997a; Orlinick et al. 1997b; Starling et al. 1997).

Although FasL is primarily a transmembrane protein, membrane bound FasL (mFasL) can be cleaved by matrix metalloprotease (MMP) from the cell surface to generate a soluble FasL (sFasL) of approximately 26 kDa (Kayagaki et

al. 1995). FasL "shedding" occurs from activated T cells, which was suggested to downregulate T cell mediated killing via FasL (Tanaka et al. 1998). sFasL has also been isolated from patients with large granular lymphocytic leukemia, NK cell lymphoma and a number of nonlymphoid tumor cells (Kavurma and Khachigian 2003). Emerging evidence demonstrates that natural sFasL is not capable of inducing significant apoptotic or inflammatory responses (Suda et al. 1997; Tanaka et al. 1998; Schneider et al. 1998; Hohlbaum et al. 2000), rather it opposes the activity of mFasL by binding Fas receptor as a decoy ligand (Suda et al. 1997; Schneider et al. 1998). However, a study showed that sFasL can induce apoptosis following association with extracellular matrix proteins (Aoki et al. 2001). Therefore, the function of sFasL might depend on the extracellular microenvironment.

Transcriptional regulation of FasL expression in T cells

FasL expression is tightly controlled in T cells and NK cells, which is induced only upon activation, conferring the ability to kill Fas^+ target populations. FasL can also be constitutively expressed by nonlymphoid cells such as the eye, where its potent proapoptotic activity is thought to contribute to the immune privilege of these organs (Griffith et al. 1995). FasL is also constitutively expressed by certain human and mouse malignant cells of both lymphoid and nonlymphoid origin, which was speculated to contribute to immune evasion (Hahne et al. 1995; O'Connell et al. 1996; Strand et al. 1996; O'Connell et al. 1999), although this remains a matter of controversy (Igney et al. 2005).

Inducible expression of FasL on the cell surface of T cells is tightly controlled at the transcriptional level through intricate interactions among various positive and negative transcriptional regulators, as well as through the recently recognized posttranslational regulation of FasL trafficking (Li et al. 1998; Bossi and Griffiths 1999; Kojima et al. 2002). Initial studies addressing FasL transcriptional regulation are based on mRNA analysis in a variety of primary and transformed lymphocytes (Suda et al. 1993; Hanabuchi et al. 1994). Such studies demonstrate FasL mRNA is absent in cells under resting conditions but is rapidly and transiently induced by a variety of extracellular stimuli. It was shown that Lck (Gonzalez-Garcia et al. 1997), ZAP-70 (Eischen et al. 1997), calcineurin (Anel et al. 1994), and Ras (Latinis et al. 1997) all influence FasL expression downstream of TCR signaling in T cells. In addition, cytokines such as IL-2 (Refaeli et al. 1998), or stress such as DNA damaging agents (Faris et al. 1998) all induce FasL transcription in T cells.

So far, putative binding sites for the transcription factors NFAT (Nuclear factor in activated T cells), NF-κB (Nuclear factor-kappa B), Sp-1 (specificity protein-1), Egr (early growth response factor), IRF (interferon regulatory factor), c-Myc/Max complex, are found along the FasL promoter (Kavurma and Khachigian 2003). Inducible FasL expression is regulated depending on the stimulus and in a cell specific manner. TCR induced FasL expression in T cells is mainly controlled by NFAT and the IRF family (Chow et al. 2000). DNA damage- induced FasL expression in T cells is regulated by the JNK pathway through the activation of the AP1 (activator protein 1) and NF-κB transcription

factors (Faris et al. 1998). IL-2 increased transcription of FasL was mediated via the Sp-1 and NFAT binding motifs on the FasL promoter (Xiao et al. 1999). In addition, the transcriptional repressor ICER (inducible cAMP early repressor) has been identified as a downregulator of FasL expression in T cells and NK cells (Bodor et al. 2002).

FasL induced apoptosis

The major function of FasL in T lymphocytes appears to be apoptosis induction. Apoptosis involves intrinsic and extrinsic pathways mediated respectively by the Bcl2 protein family and death receptor ligands including FasL (Strasser et al. 2000). FasL induces apoptosis by engaging Fas receptors on the target cell surface. Fas receptor activation leads to rapid recruitment of the adaptor protein FADD (Fas-associated death domain) via Fas intracellular death domain (DD) (Chinnaiyan et al. 1995), FADD in turn recruits procaspase 8 and/ or procaspase 10, forming a complex that is called the DISC (death-inducing signal complex) (Kischkel et al. 1995; Kischkel et al. 2001; Wang et al. 2001). Formation of the DISC results in the assembly of pro-caspase 8 and pro-caspase 10 in close proximity to each other, leading to their autoproteolytic activation, resulting in active caspase 8 and caspase 10 (Krammer et al. 2007). Fas mediated apoptotic pathways can diverge from this step in different cell types (Scaffidi et al. 1998). In type I cells (mitochondria independent) there is enough active caspase 8 that directly activates procaspase 3. In type II cells (mitochondria dependent) the small quantities of active caspase 8 is insufficient to process procaspase 3, but

sufficient to cleave the proapoptotic Bcl-2 family member Bid, resulting in the activation of the mitochondrial pathway with the release of cytochrome c, which eventually activates an enzymatic cascade of apoptosis.

Fas-FasL interactions in maintaining peripheral tolerance

Fas and FasL are known to play important regulatory roles in peripheral tolerance and T cell homeostasis, since Fas-FasL mutations in human and mice (*lpr/lpr* Fas deficient mice or *gld/gld* FasL deficient mice) lead to systemic autoimmunity and lymphoproliferative syndromes (Rieux-Laucat et al. 2003; Askenasy et al. 2005). The hallmark features of these syndromes are an accumulation of B cells and elevated levels of autoantibodies, as well as the development and expansion in peripheral lymphoid organs of the functionally impaired $TCR\alpha/\beta^{+}$ $CD3^{+}$ $B220^{+}$ $CD4^{-}$ $CD8^{-}$ abnormal double negative (DN) T cell population, which are normally found at very low levels (<1%) in the peripheral blood in human (Siegel et al. 2000). Fas is constitutively expressed on a variety of tissues (Watanabe-Fukunaga et al. 1992; Leithauser et al. 1993), and inducibly expressed in multiple lineages of immune cells, including T cells, B cells and DCs (Dhein et al. 1995; Daniel and Krammer 1994; Stranges et al. 2007). Also, although FasL is predominantly expressed on activated T cells and NK cells, B cells upregulate FasL expression in an autoimmune setting (Bonardelle et al. 2005). Therefore, it was unclear in which cells Fas or FasL functions to protect from autoimmunity in the intact immune system. Recent studies involving cell type-specific loss of Fas or FasL demonstrated that T cells, B cells and DCs are

all involved in the development of systemic autoimmunity and lymphoproliferative syndromes (Hao et al. 2004; Stranges et al. 2007; Mabrouk et al. 2008), the prevention of which requires both Fas-mediated elimination of activated APCs (both B cells and DCs) and chronically activated T cells. Briefly, these studies underlie the need for FasL expression by T cells to eliminate Fas expressing APCs presenting autoantigen in order to prevent the onset of autoimmune diseases (Stranges et al. 2007; Mabrouk et al. 2008). In this respect, B cells have dual roles, in that they function both as effector cells producing autoantibodies and as an APC subset. It was also shown that only mice with T cell Fas deficiency, but not mice with APC Fas deficiency, accumulated the abnormal DN T cell population (Stranges et al. 2007), demonstrating the critical role of Fas expression by T cells in eliminating the chronically activated autoreactive T cells.

FasL mediated cytotoxicity under pathological conditions

FasL-mediated cytotoxicity can also facilitate immunopathology. It was reported that although FasL is not essential for clearance of mouse hepatitis virus, it appears to contribute to viral pathogenesis by attacking target tissues (Ando et al. 1997; Parra et al. 2000; Balkow et al. 2001; Gremion et al. 2004). Furthermore, FasL on CTL may also contribute to the generation of autoimmune disease. For example, Fas/FasL is required for the induction of diabetes in the non-obese diabetic (NOD) mice (Itoh et al. 1997; Su et al. 2000). It was shown that CTL-mediated islet beta-cell destruction is mediated through FasL (Amrani et al. 1999).

Therefore, although signaling by Fas-FasL can down-modulate the systemic production of autoantibodies and the numbers of autoreactive T cells, FasL can increase organ-specific damage by mediating apoptosis of Fas-expressing target tissues. The balance between these two opposing effects might determine the outcome of specific autoimmune diseases.

FasL mediated cytotoxicity for pathogen and tumor clearance

Because of the complexity of the FasL/Fas system in regulating peripheral tolerance and other activities, it is not easy to use the Fas or FasL natural mutations or knockout mice to assess the significance of this pathway as an effector mechanism for cytotoxic lymphocytes *in vivo*. In contrast to the broadly accepted role of antiviral immunity mediated by degranulation, it was initially thought that FasL was largely dispensable for viral elimination or was redundant and was functional only in the context of perforin-deficiency in the relatively few systems that were examined (Russell and Ley 2002). However, more recent studies suggest that FasL may be required for certain types of immunity. For example, although degranulation is important for clearing acute infections, FasL may be important for clearing persistent infections (Rode et al. 2004; Zelinskyy et al. 2004; Shrestha and Diamond 2007). In addition, FasL may contribute, along with the perforin pathway, to the shaping of the diversity of escape variants of influenza (Price et al. 2005).

The mechanisms of CTL-mediated tumor regression *in vivo* remain to be fully understood. Although the perforin/ granzyme pathway has been considered

the dominant player over the FasL pathway (Henkart 1994; Van den Broek et al. 1996; Smyth et al. 2000), the relative importance of either of these lytic mechanisms *in vivo* remains unclear. More recent studies have shown that FasL- but not perforin-mediated lysis could be an important effector mechanism for the regression and/or elimination of some tumors *in vivo* (Seki et al. 2002; Caldwell et al. 2003; Dobrzanski et al. 2004). Epigenetic changes in tumor Fas levels may determine their sensitivity to FasL mediated killing (Maecker et al. 2002). Several studies in humans showed that the loss of Fas expression or function by different cancer types associates with a more malignant phenotype (Heane et al. 1996; Krammer et al. 1998; Owen-Schaub et al. 2000; Bergmann-Leitner et al. 2000). In this context, radiation, chemotherapeutic agents and cytokines such as IL-12 have been shown to enhance Fas expression by tumor cells (Maecker et al. 2002; Chakraborty et al. 2003; Lafleur et al. 2001), which may sensitize the primarily resistant tumor cells to Fas-mediated apoptosis, leading to enhanced CTL lytic activity in immunotherapy. In these situations, it is possible that in the *in vivo* milieu FasL-mediated anti-tumor effects may play an important role for optimal tumor regression. Taken together, these studies suggest that FasL expression and degranulation are not purely redundant mechanisms for killing but may have been selected to function under specific circumstances.

FasL in the maintenance of T cell homeostasis

Fas signaling is highly regulated during mature T cell activation and differentiation. Resting naïve T cells express little surface Fas. TCR stimulation

upregulates Fas expression and after interleukin 2 (IL-2) treatment for at least two days, cycling T cells become sensitive to Fas/FasL-mediated apoptosis upon repeated antigenic stimulation (Siegel et al. 2000). FasL mediated apoptosis of Fas^+ T cells has been implicated in the regulation of immune homeostasis (Lenardo et al. 1999), especially as a mediator of activation induced cell death (AICD). AICD, a process in which pre-activated T cells undergo cell death upon restimulation, affects mostly Fas-expressing T_H1 cells (Roberts et al. 2003), although controversy exists in some model systems that showed that loss of Fas by T_H1 cells did not provide them with a selective advantage over Fas-sufficient T cells during contraction phase *in vivo* (Stranges et al. 2007). The role of the Fas/FasL system in regulating CTL homeostasis after clonal expansion has been controversial. It was reported that the peripheral deletion of autoreactive CTL induced by cross-presentation of self-antigens involves Fas mediated killing (Kurts et al. 1998), whereas other *in vivo* studies suggested that the contraction phase of the LCMV-specific CTL response after infection is Fas-independent (Zimmermann et al. 1995; Nguyen et al. 2000; Reich et al. 2000). Consistent with these studies, a recent study in which analysis of mice with Fas loss restricted to T cells revealed that Fas controls autoimmune T cells, but not T cells responding to strong antigenic stimulation (Stranges et al. 2007). These observations suggest that different mechanisms of T cell deletion are invoked under different types of T cell responses. In this context, current models of CTL homeostasis control involve Bcl-2 family members, Bim (Bcl-2-interacting mediator of cell death) for instance, expressed by CTL and inflammatory cytokines including IL-12 and IFN-

γ (Harty and Badovinac 2008). A recent study in which a combined loss of Fas and Bim showed that shutdown of an acute CTL response to herpes simplex virus involved only Bim with no contribution by Fas, whereas both pathways synergized in eliminating antigen-stimulated CTL in chronic infection with murine γ-herpesvirus (Hughes et al. 2008). In another study, both Bim and Fas contribute to CTL contraction in acute LCMV infection, with Bim playing a larger role and the contribution of Fas varying with different T cell epitopes (Weant et al. 2008). Therefore, the contribution of Fas/FasL to T cell contraction appears to vary between different models. It was shown in both of the studies described above that the Fas pathway is important in lymph node contraction, whereas Bim functions by downsizing the T cell response in the spleen (Hughes et al. 2008; Weant et al. 2008). Taken together, multiple death pathways may function concurrently to maintain CTL homeostasis, and FasL might play a major role in regulating the homeostasis of chronically activated antigen-specific or autoreactive T cells.

FasL in the induction of inflammation

As mentioned earlier, in addition to the induced expression on activated T lymphocytes and NK cells, FasL can also be constitutively expressed by nonlymphoid cells in the eye and certain human and mouse malignant cells. This pattern of FasL expression led to the "counterattack" hypothesis, which suggests that the potent proapoptotic activity of FasL to attack Fas^+ inflammatory cells mediates immune privilege by protecting tissues and tumors from immune attack

(Griffith and Ferguson 1997; O'Connell et al. 1999). Based on this hypothesis, it was speculated that graft rejection could be prevented if cells or organs were transfected with FasL before transplantation. However, more and more evidence revealed that ectopic FasL expression caused rapid allograft rejection and profound inflammation (Kang et al. 1997; Restifo et al. 2000), highlighting the potential nonapoptotic functions mediated by FasL.

Recent evidence suggests that in addition to mediating apoptosis, ligation of Fas also contributes to a variety of nonapoptotic signaling, including inflammation (O'Connell et al. 2001; Ma et al. 2004), early T cell development (Kabra et al. 2001) and proliferation (Zhang et al. 1998), depending on the cellular microenvironment (Peter et al. 2007). Fas-FasL can induce inflammatory responses either directly or indirectly. In a direct way, Fas ligation can trigger caspase-mediated processing and secretion of pro-inflammatory cytokines, including IL-1β and IL-18, or directly upregulate expression of various cytokines and chemokines in some cell types (O'Connell et al. 2001). For example, it was shown that Fas ligation induces DCs to rapidly produce IL-23 and CXC and CC chemokines, resulting in enhanced chemoattraction of neutrophils and T cells *in vitro*, indicating that Fas-FasL system might provide a link between innate responses and adaptive immunity by promoting DC cytokine and chemokine production (Kidoya et al. 2005; Guo et al. 2005), although it remains to be determined if this occurs under physiological conditions. It was demonstrated that activation of ERK1/2 and caspases is necessary for Fas-induced chemokine production in DCs (Guo et al. 2005). So far, the molecular and cellular

mechanisms underlying the switch between apoptosis and activation by Fas signaling are largely unknown. It was reported that apoptosis induction requires Fas internalization in type I cells (Algeciras-Schimnich et al. 2002; Lee et al. 2006) and when internalization was blocked, Fas signaling activated the proliferative ERK and NF-κB signaling pathways (Lee et al. 2006). Hence, the subcellular localization of Fas might play important roles in controlling activation of distinct signaling cascades to determine divergent cellular fates. Recently, a signaling threshold model was also proposed by the group led by Krammer (Bentele et al. 2004; Lavrik et al. 2007). The authors found that Fas stimulation with a low concentration of soluble agonist anti-Fas did not result in apoptosis but in MAP kinase activation in type I cells (Lavrik et al. 2007). It would be of interest to examine whether Fas ligation by different levels of membrane bound FasL could result in the same outcomes. It would also be interesting to examine whether there are potential connections between the localization model and the signaling threshold model.

FasL may also regulate inflammation through a more indirect route. It has been suggested that FasL induced inflammation might be secondary to apoptosis (O'Connell et al. 2001), in which local excessive Fas mediated apoptosis results in cytokine release from apoptotic cells, which could generate a local inflammatory milieu, triggering recruitment of inflammatory cells. This scenario is conceivable for the situation of FasL-overexpressing allograft transplantation in which, the sudden introduction of a $FasL^+$ allograft into the host results in a local excessive Fas mediated apoptosis. This scenario is also possible for FasL induced

chronic hepatitis pathogenesis, in which massive FasL induced apoptosis of Fas-expressing hepatocytes will further precipitate inflammatory tissue damage. Therefore, locally excessive FasL-mediated apoptosis may not be 'silent' with respect to the immune system, but may instead trigger inflammatory responses.

FasL reverse signaling

In addition to triggering Fas signaling as a ligand, it has been suggested that FasL also has the capacity to transduce "reverse signaling" to modulate TCR signals. Reverse signaling has also been associated with other members of the TNF superfamily (Sun and Fink 2007). Its common outcomes are changes in proliferation and cytokine production profiles of the affected cells (Janssen et al. 2003). FasL reverse signaling seems to provide a positive costimulatory signal required for optimal thymocyte maturation (Boursalian and Fink 2003), maximal $CD8^+$ T cell proliferation *in vitro* and *in vivo* (Suzuki and Fink 1998; Suzuki et al. 2000; Suzuki and Fink 2000) in mice. However, FasL reverse signals also lead to cell-cycle arrest and cell death in murine $CD4^+$ T cells *in vitro* and *in vivo* (Desbarats et al. 1998). The molecular basis of the apparent dual role of FasL reverse signaling has not been elucidated. Sun and colleagues showed that the reverse signals through FasL as costimulation requires TCR coengagement in CTL, driving FasL association with select SH3 domain containing proteins via amino acids 45-54 in the PRD, activating PI3K and MAPK pathways, mediating nuclear translocation of the transcription factors NFAT and AP-1, and enhancing IFN-γ production (Sun et al. 2006; Sun et al. 2007). The authors further showed

that serine phosphorylation within the CKI binding sites within FasL cytoplasmic tail are involved in NFAT activation (Sun et al. 2007), although the biological consequences of this NFAT activation upon FasL costimulation has not been described. Therefore, distinct FasL reverse signaling pathways might provide an additional level of regulation for T cell responses. Further study is required to examine the different biological roles of FasL reverse signaling in the apperant opposing effects in $CD8^+$ and $CD4^+$ T cells and to decipher the molecular basis of these effects.

Relationship between degranulation and FasL expression

As mentioned earlier, FasL expression and degranulation are not purely redundant mechanisms for killing but may have been selected to function under specific circumstances. Furthermore, there appears to be considerable heterogeneity of CTL with respect to their usage of the perforin-dependent or FasL-dependent pathways for mediating target cell lysis. For example, it was shown that different CTL clones vary considerably in their ability to mediate degranulation-independent cell lysis (Ostergaard and Clark 1989). Furthermore, immunization of mice with tumor cells results in peritoneal exudate lymphocytes (PEL), which include extremely efficient CTLs that appear to mediate primarily FasL-mediated, but not perforin/granzyme B-mediated killing (Helgason et al. 1992; Berke et al. 1993; Li et al. 1998). Therefore, not all CTL are equivalent in their employment of the two major pathways for target cell killing.

Early studies revealed that there were two distinct cytolytic pathways mediated by CTL according to their Ca^{2+} dependence; a wholly Ca^{2+}-dependent pathway of degranulation and a Ca^{2+}-independent pathway of unknown mechanism (Ostergaard et al. 1987; Trenn et al. 1987). Additional studies revealed that this alternate pathway was FasL mediated killing (Kojima et al. 1994; Rouvier et al. 1993; Kägi et al. 1994b; Lowin et al. 1994). Further evidence suggested that these are indeed distinct killing pathways since CTL signaling leading to degranulation and FasL expression are distinct (Esser et al. 1996; Esser et al. 1998) and CTL from Ashen mice are defective in degranulation, but are still able to kill via the FasL pathway (Haddad et al. 2001). More recently, however, it was shown in human CTL that FasL localizes to cytolytic granules, and degranulation leads to FasL cell surface expression (Bossi and Griffiths 1999) suggesting that FasL and granule release would be under similar controls, and would both, therefore be Ca^{2+}-dependent (Figure 1-2). Thus the relationship between degranulation and FasL expression remains ambiguous.

Regulated vesicle exocytosis

Vesicular transport is the predominant mechanism for the transport of proteins and lipids between membrane-bound organelles, and between organelles and the plasma membrane in eukaryotic cells. Usually, carrier vesicles that are loaded with soluble and membrane-bound cargo bud off a donor compartment, then transport to and dock and fuse with a target membrane to deliver their cargo (Bonifacino and Glick 2004). A variety of proteins, including the ARF family of

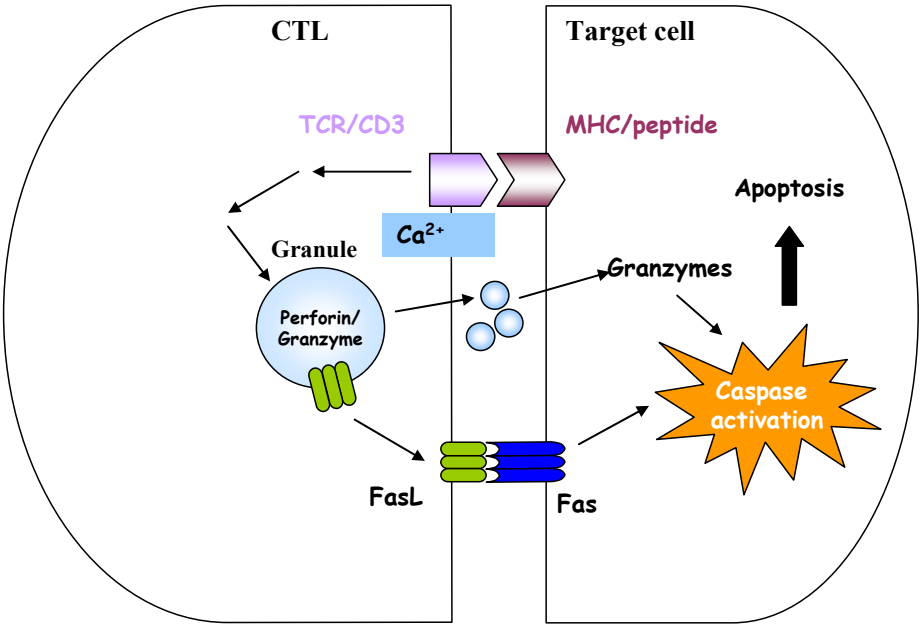


Figure 1-2. Prevailing model of the two mechanisms used by CTL to destroy target cell at the time I initiated my studies. In this model, FasL is stored in cytolytic granules and FasL cell surface expression would be subject to the same controls as degranulation.

small GTPases (D'Souza-Schorey and Chavrier 2006), the Rab family of small GTPases (Pfeffer and Aivazian 2004), SNAREs (Stow et al. 2006) and their relevant effector proteins, play a key role in orchestrating the distinct steps of vesicular trafficking (Bock et al. 2001). Typically, ARF proteins regulate vesicle budding, Rab proteins are involved in vesicle transport and docking and SNAREs mediate membrane fusion. Different ARF, Rab and SNARE proteins all have distinct subcellular localizations as well as cell-type specific effects, and are believed to regulate specific steps in intracellular trafficking (Advani et al. 1998; Pfeffer and Aivazian 2004; D'Souza-Schorey and Chavrier 2006; Stow et al. 2006).

As shown in Figure 1-3, newly synthesized molecules destined for secretion or expression on the cell surface are moved towards *trans*-Golgi network (TGN), from which they are packaged into vesicles transported either along the constitutive pathway or regulated pathway for exocytosis (Burgess and Kelly 1987). Constitutive protein secretion occurs either through small carrier vesicles going directly to the cell surface, or via the late recycling endosomes trafficking to the cell surface. During regulated exocytosis, molecules are stored in secretory granules in the cell until a signal is received for their release, this pathway is critical for the correct biological functioning of many different cells derived from the hemopoietic lineage (Stinchcombe and Griffiths 1999). In CTL, the regulated secretory pathway has been closely associated with cytotoxic functions, such as the well established degranulation pathway (Stinchcombe and Griffiths 1999; Blott and Griffiths 2002). As described earlier, cytotoxic molecules

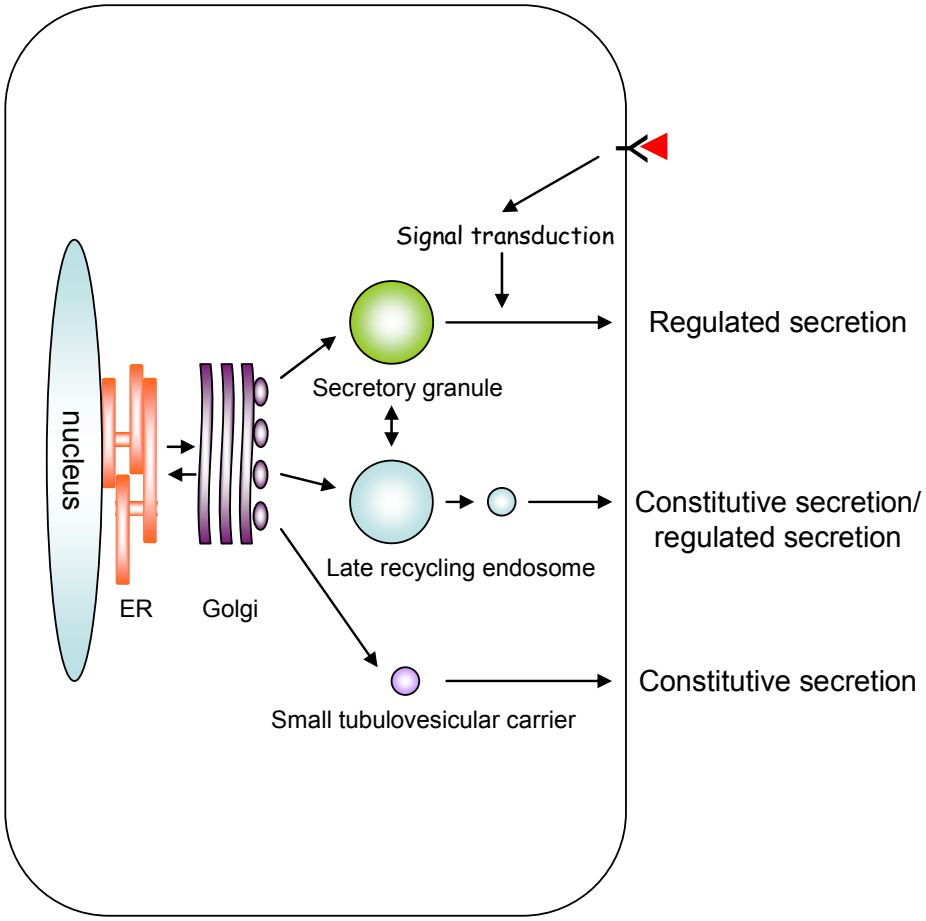


Figure 1-3. Constitutive secretion versus regulated vesicle exocytosis. Newly synthesized molecules destined for secretion or expression on the cell surface are moved towards *trans*-Golgi network (TGN), from which, they are packaged into vesicles transported either along the constitutive pathway or regulated pathway for exocytosis. Constitutive protein secretion occurs either through small carrier vesicles going directly to the cell surface, or via the late recycling endosomes trafficking to the cell surface. In addition, the late recycling endosomes may be involved in regulated vesicle exocytosis. During regulated exocytosis, molecules are stored in secretory granules in the cell until a signal is received for their release.

are stored in cytolytic granules in resting CTL, then upon target cell engagement, the granules are released in a Rab27a/ Munc13-4 dependent manner. Recently, the involvement of the late recycling endosomes in degranulation has been described in T cells, indicating it may also play key roles in regulated vesicle exocytosis (Ménager et al. 2007). In addition, the regulated secretion of other molecules by $CD8^+$ T cells has been described including stored RANTES (Catalfamo et al. 2004) and CXCR1 (Gasser et al. 2005). So far, the knowledge regarding the subcellular localization and trafficking of FasL remains elusive and even controversial. Further studies will be required to identify the FasL storage compartment in CTL and the regulatory molecules involved in its trafficking.

Hypothesis

The hypothesis for the present study is that the degranulation of cytolytic granules and FasL cell surface expression pathways of CTL-mediated lysis are independently controlled cytolytic mechanisms that serve non-redundant functions in CTL.

Study objectives

The specific questions that were addressed include:

1. Are degranulation of cytolytic granules and FasL cell surface expression pathways of CTL-mediated lysis independently controlled cytolytic mechanisms? If so, how are they differentially regulated?
2. How is induced FasL cell surface expression regulated in CTL upon target cell engagement?
3. What are the CTL-mediated effector functions of stored and *de novo* synthesized FasL?
4. What is the nature of the compartment in which preformed FasL is stored?

In this thesis I will present data to demonstrate that FasL is stored in a compartment distinct from the cytolytic granules. The remainder of my thesis is focused primarily on examining the regulation and function of the stored, preformed FasL in CTL. Through these studies I will gain insight to the contribution of FasL to CTL-mediated killing, which could have future implications on vaccine design for appropriate pathogen clearance by CTL.

CHAPTER 2 : Materials and Methods

Cells

The murine alloreactive $CD8^+$ CTL clones AB.1 (H-2^d anti-H-2^b) and clone11 (H-2^k anti-H-2^b) were described previously (Blakely et al. 1987; Kane et al. 1989). CTL clone 3/4, which is specific for H-$2D^b$-restricted $NP_{366-374}$ peptide (ASNENMETM) derived from nucleoprotein of A/PR/8/34 (H1N1) influenza virus, has been described previously (Kane and Mescher 1993). OT-I CTL which is specific for H-$2K^b$-restricted OVA peptide (SIINFEKL) was generated in the lab as described later. CTL clones were maintained by weekly stimulation with irradiated (2,500 rad) C57BL/6 splenocytes alone (for AB.1 and clone 11), or pulsed with 200 μg/ml $NP_{366-374}$ peptide (for clone 3/4) or 10 μM OVAp (for OT-I) and 10 U/ ml of murine recombinant IL-2 and were used 5–6 days later. CTL clones were cultured in CTL clone medium: RPMI supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml penicillin/streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 53 nM β-mercaptoethanol. The human CTL line (hCD8) was a generous gift from Dr. R. C. Bleackley (University of Alberta) and was described previously (Atkinson et al. 1998). hCD8 was maintained by weekly restimulation with irradiated RPMI-8666 cells in RPMI containing 10% fetal bovine serum and 10 U/ml recombinant IL-2.

The L1210 lymphoma cell lines expressing chimeric class I MHC (L1210/K^b) (Durairaj et al. 2003) or Fas (L.Fas) was a generous gift from Dr. K. P. Kane (University of Alberta). EL4 cells were obtained from American Type

Culture Collection (ATCC, Manassas, VA). NIH-3T3 cells were a gift from Dr. J. C. Stone (University of Alberta) and were cultured in DMEM supplemented with 10% FCS. COS-1 cells were a gift from Dr. J. Elliott (University of Alberta). Cell lines L1210, L1210/K^b, L.Fas, EL4 and COS-1 were grown in DMEM supplemented with 8% defined bovine calf serum (dCS).

In vitro generation of bone marrow–derived DCs (BMDC)

Bone marrow–derived DCs (BMDC) were generated by cultures of C57BL/6-derived bone marrow in RPMI supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml penicillin/streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 53 nM β-mercaptoethanol and 5 ng/ml of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF). After 9 days, DC maturation was induced by overnight incubation with 0.5 μg/ml of LPS. Mature DCs were pulsed at 10^8 cells/ ml in FCS for 60 min at 37 °C with 10 μM SIINFEKL peptide, then washed 3 times with 2% FCS/ RPMI and used for primary OT-I $CD8^+$ T cells co-culture.

In vitro generation of OT-I CTL

For OT-I CTL generation, OT-I T cells were purified from spleens of female OT-I (RAG1-/-) Tg mice (C57BL/6 background) (a generous gift from Dr. K. P. Kane) with EasySep® mouse $CD8^+$ T cells enrichment kit (STEMCELL Technologies, Vancouver, Canada). 10^5 purified OT-I T cells and 2×10^5 10 μM SIINFEKL-pulsed mature BMDCs were co-cultured in 200 μl of CTL clone

media in 96-well tissue culture treated plates. Alternatively, 5×10^5 purified OT-I T cells and 5×10^6 10 μM SIINFEKL-pulsed splenocytes from female B57CL/6 mice were co-cultured in 2 ml of CTL clone media in 24-well tissue culture treated plates. On day 4, cultures were supplemented with 10 U/ml of IL-2. On day 7, the cells were split and supplemented with fresh media containing 10 U/ml IL-2. On day14, the effector cells were restimulated with SIINFEKL-pulsed irradiated splenocytes from C57BL/6 mice in 2 ml of CTL clone medium containing 10 U/ml IL-2, followed by a period of rest prior to maintenance in culture as described above.

In vivo allo-Ag priming and preparation of PEL and spleen-derived CD8$^+$ T cells

Eight to ten week old female BALB/c mice were primed by intraperitoneal injection of 2×10^7 EL4 cells. Ten to twelve days after priming, the mice were sacrificed and peritoneal exudate lymphocytes (PEL) from 5 mice were harvested by rinsing peritoneal cavities with PBS and pooled. No tumor cells were detectable in the crude PEL. Crude PEL were applied to tissue culture treated plate for 30 min at 37°C to get rid of the adherent cells. Splenocytes were also collected 12 days after priming. CD8$^+$ T cells were then purified with MACS® CD8$^+$ T cells isolation kit (Milteny Biotech, Gladbach, Germany) and used immediately for assay. All animal studies have been approved by the University Animal Policy and Welfare Committee at the University of Alberta.

Antibodies

The hybridomas producing 145-2C11 (anti-CD3ε), M17/5.2 (anti-LFA-1), 2.4G2 (anti-mouse FcR) and Y3 (anti-H-2K^b) were obtained from the American Type Culture Collection (ATCC). Antibodies were purified from these hybridomas as described previously (Berg NN and Ostergaard HL, 1995). PE-conjugated anti-mouse FasL (MFL3), biotin-conjugated anti-mouse FasL (MFL3), anti-human FasL (NOK-1), anti-CD107a (LAMP-) (1D4B), FITC-conjugated anti-CD107a (1D4B), PE-Cy5-conjugated anti-CD8α (Ly-2) (53-6.7), anti-active caspase-3 (C92-605), anti-Fas (Jo-2) and anti-cytochrome C (7H8.2C12) were purchased from BD Pharmingen (San Jose, California). Anti-mouse FasL antibody (clone101626) was purchased from R&D Systems (Minneapolis, MN). Anti-GM130 (35/GM130), anti-STIM1 (44/GOK), and anti-Rab4 (7/Rab4) were purchased from BD Transduction Laboratories (San Jose, California). Anti-cytochrome C (6H2.B4) was purchased from BioLegend (San Diego, CA). Goat anti-cathepsin D polyclonal antibody (G-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor® 647 anti-mouse Granzyme B (16G6) and anti-transferrin receptor (CD71) were purchased from eBioscience Inc (San Diego, CA). Anti-EEA1, anti-Golgi 58K and anti-β-COP were purchased from Sigma-Aldrich (St. Louis, MO). Anti-Rab11 was purchased from Cell Signaling Technologies (Danvers, MA). Rabbit anti-calnexin polyclonal antibody was purchased from Stressgen (Victoria, Canada). Rabbit antiserum H2, specific for GIIβ, was developed in our laboratory as described previously (Arendt CW and Ostergaard HL, 1997). Rabbit anti-hamster IgG, FITC-conjugated donkey

anti-rabbit IgG, anti-rat IgG-HRP, anti-mouse IgG-HRP, anti-rabbit IgG-HRP and anti-goat IgG-HRP were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor 546, Alexa Fluor 488, Alexa Fluor 555 and Alexa Fluor 647 conjugated streptavidin and secondary antibodies were purchased from Molecular Probes (Eugene, OR).

Reagents

Streptavidin Agarose and NeutrAvidin were purchased from Thermo Fisher Scientific Inc (Waltham, MA). Surfactant-free, 5.2 μm white sulfate latex beads were purchased from Interfacial Dynamics Corp (Eugene, OR). CHX, EGTA, PMA, colchicine, PKH67 green or PKH26 red fluorescent dye were purchased from Sigma-Aldrich (St. Louis, MO). Ionomycin, Brefeldin A, cyclosporin A, BAPTA-AM and saponin were purchased from Calbiochem (San Diego, CA). Cell Tracker Orange CMRA and Cell Tracker Blue CMAC were purchased from Molecular Probes (Eugene, OR). Effectene transfection kit was purchased from Qiagen (Mississauga, ON). 60% OptiPrep solution was purchased from Sigma-Aldrich (St. Louis, MO). $Na^{51}CrO_4$ (^{51}Cr) was purchased from PerkinElmer. $NP_{366\text{-}374}$ peptide (ASNENMETM) was synthesized by Sigma-Genosys (Canada). OVA peptide (SIINFEKL) and its low affinity variant G4 (SIIGFEKL) were synthesized by GeneScript Corp (Piscataway, NJ).

COS-1 cell transient transfection

COS-1 cells were seeded on a glass coverslip in 12-well plate 24 h before transfection. Transient transfection of COS-1 cells with mouse FasL cDNA in eukaryotic expression vector PSRαSD7 (gift from Dr. John Elliott, University of Alberta) was carried out using the Effectene Transfection reagent (Qiagen) according to the manufacturer's instructions, with a 1:50 DNA to Effectene reagent ratio. FasL expression was examined by confocal microscopy 24 h post transfection.

Target cell pulsing with peptides

EL4 cells were incubated in FCS at 1 x 10^7 cells/ml with the indicated concentration of $NP_{366-374}$ or OVA peptides at 37°C for 1 h. Cells were then washed with 2% FCS/ RPMI, resuspended in ice cold 4% CS in RPMI, and used as target cells for stimulation of CTL clones.

Target cell stimulation of CTL

To discriminate between effector and target cells, target cells were stained with PKH67 green or PKH26 red fluorescent dye (Sigma-Aldrich) according to the manufacturer's instructions, with modification. Briefly, 10^7 cells were washed in PBS and resuspended in 400 µl of the diluent C provided with the dye. The dye was diluted to 10 µM in 400 µl of diluent C. Equal volumes of cells and dye were combined and incubated for 3 min at room temperature, followed by addition of 2 ml calf serum (CS) and further incubation for 1 min to adsorb the

excess dye and stop further uptake. Cells were then extensively washed, incubated for 1 h in 4% CS in PBS at 37°C then washed once and resuspended at 1×10^6 cells/ml in ice cold 4% CS in RPMI for assay. This target cell prestaining was used in the majority of figures presented in chapter 3 unless mentioned by staining the cells with anti-CD8 and gating on the $CD8^+$ population to examine CTL cells. All the experiments in other chapters use anti-CD8 staining to discriminate between CTL and target cells.

CTL clones or purified $CD8^+$ T cells were conjugated with stained target cells in ice cold 4% CS in RPMI at a ratio of 1:1 and centrifuged at 200 x *g* for 3 min at 4°C then incubated at 37°C for the indicated time. Where indicated, cells were preincubated with 10 μg/ml cycloheximide (CHX), colchicine, cyclosporin A (CsA), or BAPTA-AM at the indicated concentration, or carrier control for 30-45 min and included in the assay, except for BAPTA-AM, which was washed out before CTL and target cell conjugate formation. When used, 4 mM EGTA/ 3 mM $MgCl_2$ were added before mixing CTL and target cells. After stimulation, effector and target cell conjugates were separated with 5 mM EDTA in PBS then stained for CD8α, FasL, and CD107a. After gating on the $CD8^+$ cells to examine only the CTLs, the expression of FasL and CD107a was assessed.

Antibody stimulation of CTL

For immobilized anti-CD3 mAb stimulation, wells of a 24-well flat bottom Falcon 3912 microtitre plate (Becton Dickinson, Oxnard, CA) were coated with 10 μg/ml 145-2C11 overnight at 4 °C. Wells were washed and blocked with 2%

BSA in PBS then 10^6 CTL were added to each well and incubated at 37 °C for the indicated time. After stimulation, cells were detached from the plate with ice cold 5 mM EDTA in PBS then stained for FasL and CD107a. For soluble, cross-linked anti-CD3 mAb stimulation, 10^7 cells/ml in PBS containing 2% CS was incubated with 20 μg/ml 145-2C11 on ice for 15 min, then washed and resuspended at 2×10^6 cells/ml. Rabbit anti-hamster IgG was added to 2 μg/ml and cells were incubated at 37 °C for the indicated time, washed and then stained for FasL and CD107a.

Isolation of H-2K^b

The H-2K^b was purified from EL4 cells by immunoaffinity chromatography as described (Kane et al. 1989) with modification. Briefly, TX-100 lysate from 1 x 10^{10} EL4 cell membrane was passed over a Y3 affinity column preceded by a Sepharose 4B precolumn. Elution was performed using elution buffer: 0.5% DOC, 0.05 M diethylamine, 650 mM NaCl, pH 11.5. The H-2K^b column fractions were characterized by BCA protein assay, ELISA, and by allo-reactive CTL degranulation response as previously described (Kane et al. 1989; Kane and Mescher 1993).

Coating latex beads with ICAM-1/ H-2K^b and stimulation of allo-reactive CTL

Latex beads were incubated at 1 x 10^7/ml with 0.06 μg/ml of purified ICAM-1 (gift from Dr. Andy Kokaji, University of Alberta, Canada) and various concentration of H-2K^b at 4°C overnight with rotation. The concentration of

ICAM-1 was selected by incubating latex beads with various concentrations of ICAM-1, then its level on the coated beads was compared by flow cytometry to EL4 cells. A concentration of 0.06 μg/ml ICAM-1 resulted in a similar level to staining as EL4. After incubation, equal volumes of 2% BSA/ PBS was added to the latex beads, incubating at room temperature for 30 min with rotation. The latex beads were then washed with 0.1% BSA/ PBS, and resuspended in ice cold 4% CS in RPMI for assay. CTL clones were conjugated with coated latex beads in ice cold 4% CS in RPMI at a ratio of 1:2 for 5 min, and centrifuged at 200 x *g* for 3 min at 4°C and then incubated at 37°C for the indicated times. After stimulation, beads and target cell conjugates were separated with 5 mM EDTA in PBS and then stained as described above.

Cell staining and flow cytometry

After stimulation, degranulation of CTL was measured by cell surface expression of CD107a (Wolint et al. 2004) by staining with FITC-conjugated anti-CD107a monoclonal antibody or FITC-conjugated rat IgG isotype control (BD Pharmingen). In contrast to the previously described method (Wolint et al. 2004), we did not add Brefeldin A as a Golgi stop as there was no difference in CD107a expression with or without the Golgi stop (data not shown). Cell surface FasL was revealed with PE-conjugated anti-FasL monoclonal antibody or PE-conjugated hamster IgG isotype control (BD Pharmingen) diluted in 5 mM EDTA/ 4% CS/ PBS. All cell surface staining was performed on ice for 30 min. For intracellular FasL detection, cells were fixed with 2% paraformaldehyde for

20 min at room temperature, then stained in permeabilization buffer (0.2% saponin/ 4% CS in PBS) and analyzed. Data was acquired on a BD Biosciences FACscan flow cytometer and analyzed with CellQuest software (BD BioSciences, San Jose, CA), or Biosciences FACSCanto flow cytometer and analyzed with FACSDiva software (BD BioSciences, San Jose, CA) or FlowJo software (Tree Star Inc., Ashland, OR).

Target cell active caspase-3 assay for CTL-mediated cytotoxicity

Target cell apoptosis was assessed by the activation of caspase-3 as described (Jerome et al. 2003), with modification. Briefly, CTL was labelled with PKH26 red dye as described earlier. 8×10^5 CTLs were combined with 2×10^5 target cells at a ratio of 4:1, pelleted at 200 x *g* for 3 min at 4°C, followed by incubation for 1.5 h at 37°C in the presence or absence of 4 mM EGTA/3 mM $MgCl_2$. After incubation, conjugates were disrupted with 5 mM EDTA in D-PBS. Cells were stained in permeabilization buffer (0.2% saponin, 5 mM EDTA, 4% dCS in D-PBS) with anti-active caspase-3 mAb followed by FITC-conjugated donkey anti-rabbit IgG and analyzed by flow cytometry.

Conjugate assay

CTL were labelled with PKH26 red dye or Cell Tracker Orange CMRA and equal numbers of CTL clone AB.1 and target cells were mixed, pelleted at 200 x *g* for 3 min at 4°C, and incubated for 15 min at 37°C. After incubation for the indicated time, the cells were vortexed to separate non-specific binding and

fixed with 2% paraformaldehyde and analyzed by FACS. The percentage of conjugates was evaluated on red-positive cells by analyzing forward scatter versus red fluorescence (Gismondi et al. 2004).

Confocal microscopy

CTL were adhered to poly-L-lysine (Sigma) coverslips for 10 min at room temperature, and fixed with methanol for 10 min at -20°C. Cells were washed and blocked with 2% normal donkey serum plus 1% BSA in PBS. Cells were stained with the indicated primary antibody followed by the appropriate labeled secondary antibody. When biotinylated antibodies were employed, coverslips were blocked with 0.1 mg/ml NeutrAvidin® (Pierce Biotechnology, USA or Molecular Probes, USA) and 1% BSA diluted in PBS. As secondary antibody background and crossreactivity control, each cell image was stained with one first antibody and appropriate two secondary antibodies. In all cases, secondary antibody background was negligible and showed species specific staining. The coverslips were mounted with glycerol/PBS (9:1). Samples were examined with Zeiss LSM510 confocal microscope. Plan-Apochromat 63 x /1.4 Oil DIC objective lens was employed. Z-stack images (interval=0.2μm) were acquired and subjected to deconvolution and 3-D reconstruction (for some experiments) using Huygens Essential software (Scientific Volume Imaging BV, Netherlands). Visualization and colocalization analysis were performed with Imaris software (Bitplane AG, Switzerland). For colocalization analysis, the detection threshold was empirically determined as 20% of the maximum intensity of each channel to

minimize background, and a new channel that contains only voxels that represent the colocalization result was generated. For each sample, five microscope fields containing stained cells were randomly selected to undergo Z-stack scanning.

^{51}Cr release assay for antigen-specific killing and bystander killing

Target cells were labeled with 100–150 μCi of $Na^{51}CrO_4$ (^{51}Cr) at 37°C for 1 h in FCS. After washing targets four times with 5% FCS in RPMI 1640, they were plated at 10^4 ^{51}Cr-labeled cells/well and mixed with AB.1 in V-bottom microtiter plates at indicated E:T ratios in triplicate. For bystander killing assay, 10^4 ^{51}Cr labeled non-Ag-bearing target and 10^4 unlabeled cognate target were mixed with AB.1 at indicated E:T ratios. After 4 h incubation at 37°C, plates were centrifuged for 5 min at 500 x *g*, and 25 μl of supernatant was collected and counted in a MicroBeta TriLux liquid scintillation counter (PerkinElmer). Percent specific lysis was determined as (experimental release – spontaneous release)/(maximal release – spontaneous release) x 100.

De novo FasL induction with immobilized anti-CD3 and bystander killing assay

For immobilized anti-CD3 mAb stimulation, wells of a 96-well flat bottom Falcon 353912 flexible plate (Becton Dickinson, Oxnard, CA) were coated with 50 μl of 10 μg/ml 145-2C11 overnight at 4°C. Wells were washed and blocked with 2% BSA in PBS then 10^5 CTL were added to each well and incubated at 37°C for 2 hr for FasL synthesis induction. 10^4 ^{51}Cr labeled non-Ag-bearing

target were then added to each well. After 4 h incubation at 37°C, bystander killing was detected and analyzed as above.

Preparation of OptiPrep continuous density gradient

50% OptiPrep stock was prepared by mixing 5 part of 60% OptiPrep™ (Sigma, Aldrich) with 1 part of Optiprep diluent (0.25 M Sucrose, 6 mM EDTA, 60 mM HEPES, 6 mM PMSF, 6× protease inhibitor cocktail, pH 7.4). 2 ml of 30%, 25%, 20%, 15%, 10%, 5% OptiPrep were then made by diluting 50% OptiPrep stock with homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES, 1 mM PMSF, 1× protease inhibitor cocktail, pH 7.4). A discontinuous gradient was formed by top-layering with solutions of different prercentage of OptiPrep (from 30% to 0) in a 14 ml Polyallomer tube (Beckman), then stored at 4°C overnight to allow for the diffusion across the interfaces to form a continuous gradient.

Subcellular fractionation of CTL

1×10^8 CTL cells were washed three times with D-PBS, followed by treatment with 1 mM serine cysteine protease inhibitor PMSF for 5 min at room temperature. Cells were then washed once with ice-cold homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES, 1 mM PMSF, 1× protease inhibitor cocktail, pH 7.4), pelleted and resuspended in 2 ml ice-cold homogenization buffer. The cell suspension was subjected to homogenization through a ball-bearing cell homogenizer (14 micron clearance) (Isobiotec,

Heidelberg, Germany). The homogenate was centrifuged at 1000 × *g* for 10 min to produce a postnuclear supernatant (PNS). PNS was layered on top of the 12 ml OptiPrep continuous density gradient (30%-0%), and centrifuged at 100,000 × g_{ave} for 2 hr using Beckman SW41Ti swinging bucket rotor with Beckman Coulter Optima L-100 XP ultracentrifuge. Fractions of 1ml were collected from bottom of the tube by tube puncture, and 40 μl of each fraction was subjected to 10% SDS-PAGE gel separation and examined by western blotting.

FasL immunoprecipitation

1 x 10^7 CTL cells were lysed by incubation with 1ml 1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris pH 7.6 and 1 mM sodium vanadate) at 4°C for 20 min with rotation. Lysates were then centrifuged at 16,000 × *g* for 10 min at 4°C. Post-nuclear lysates were incubated with 1 μg biotin-conjugated anti-FasL monoclonal antibody (MFL3) or biotin-conjugated Armenian hamster isotype control for 1hr on ice, followed by incubation with 25 μl of 50% Streptavidin Agarose slurry for 2 hr at 4°C with rotation. Immunoprecipitates were pelleted and washed three times with 1% NP-40 lysis buffer, then resuspended in 80 μl of 1x Laemmli reducing sample buffer and boiled for 3 min, then separated by 10% SDS-PAGE. For N-glycosidase F (PNGase F) and Endoglycosidase H (endo H) digestion, immunoprecipitates were incubated in 30 μl of PBS containing 50 mM EDTA, 1% NP-40, 0.15% SDS, 1%

β-mercaptoethanol, 1 mM PMSF, 5 μg/ml MMP inhibitor, and protease inhibitor cocktail for 16 h at 37 °C.

Western blotting

Samples were loaded onto SDS-PAGE gels that were run at 9 mA overnight. The gels were then transferred to polyvinylidene difluoride (PVDF) membranes at 200 mA for 5-6 hr. Immunoblotting was performed using the appropriate primary and HRP-coupled secondary Abs and was visualized by ECL (enhanced chemiluminescence) (PerkinElmer Life Science, USA). When multiple blots were performed on the same membrane, the membrane was stripped in buffer containing β-mercaptoethanol, SDS and Tris-HCl, pH 6.7 at 56°C for 30 min followed by 15 min with rocking.

Reproducibility of results

Unless otherwise stated, experiments were repeated at least three times and representative data are shown.

Statistical analysis

For most experiments, results from three independent experiments were averaged and the Standard Error of the Mean (SEM) was shown. For some assays, results from a single experiment were averaged and the Standard Deviation (SD) was indicated for triplicated samples.

CHAPTER 3 : Cytotoxic T Lymphocytes Contain and Utilize Intracellular Stores of FasL Distinct From Cytolytic Granules

Most of the data presented in this chapter is reproduced, with permission, from:

Jin-Shu He and Hanne L. Ostergaard

The Journal of Immunology, 2007, 179:2339-2348

A. Introduction

Cytotoxic T lymphocytes (CTL) are differentiated $CD8^+$ T cells that eliminate tumor cells and cells infected with intracellular pathogens. CTL lyse target cells by at least two distinct well-characterized general mechanisms: extracellular Ca^{2+}-dependent degranulation of cytolytic molecules, including perforin and Granzyme B, and expression of Fas ligand (FasL), which induces apoptosis of Fas-expressing target cells (Barry and Bleackley 2002). In addition to their defense function, these two cytolytic mechanisms also play crucial roles in homeostatic regulation and contribute to pathogenesis in many different systems (Russell and Ley 2002).

CTL have specialized secretory lysosomes (Trambas and Griffiths 2003). Upon engagement of a target cell bearing appropriate peptide/MHC complexes, cytolytic granules move along microtubules to the contact point with the target cell (Sancho et al. 2002), then a fraction of the granules fuse with the plasma membrane (Lyubchenko et al. 2001; Stinchcombe et al. 2001a) and the contents

are directionally released to the target cell resulting in rapid target cell death (Trambas and Griffiths 2003). In contrast, the regulation of FasL expression on the CTL cell surface is not fully established and there are several conflicting studies in the literature. FasL is not constitutively expressed on CTL and must be induced (Vignaux et al. 1995; Glass et al. 1996). FasL upregulation was shown to require extracellular Ca^{2+} (Vignaux et al. 1995; Glass et al. 1996), however the lytic phase of FasL-mediated lysis could occur in the absence of extracellular Ca^{2+}. In contrast, our data indicate that both the induction phase and lytic phases of degranulation-independent lysis can occur in the absence of extracellular Ca^{2+} (Ostergaard and Clark 1987; Ostergaard et al. 1987). FasL upregulation was also shown to require protein synthesis (Vignaux et al. 1995; Glass et al. 1996), however Li et al. suggested that upregulation of FasL expression on *ex vivo* CTL did not require protein synthesis and that there were pre-existing pools of FasL (Li et al. 1998). Consistent with the existence of pre-synthesized FasL pools, FasL was shown to localize in lysosomal lytic granules and was subject to degranulation controlled cell surface delivery (Bossi and Griffiths 1999; Kojima et al. 2002). These conflicting observations regarding FasL expression remain to be resolved.

In this chapter, I investigated TCR-regulated FasL cell surface expression upon target cell engagement in alloreactive CTL clones and *in vivo* primed $CD8^+$ T cells. I revealed that there are two waves of FasL cell surface expression: a rapid, extracellular Ca^{2+}-independent, preformed FasL expression and a delayed, extracellular Ca^{2+}-dependent, *de novo* FasL expression. Also, my data indicate

that FasL is stored in a regulated secretion compartment distinct from cytolytic granules, by confocal microscopy. Furthermore, I show that preformed functional FasL undergoes TCR-regulated cell surface expression that can be distinguished from the exocytosis of cytolytic granules. Finally, further investigation of FasL expression in different CTL clones reveals that not all CTL are equal in their ability to mobilize preformed FasL to the cell surface upon target cell engagement, suggesting an additional level of regulation leading to cell surface transport of preformed FasL.

B. Results

Both extracellular Ca^{2+} -independent and -dependent FasL cell surface expression occur on CTL upon target cell recognition

When alloreactive mouse CTL clone AB.1 was stimulated with its cognate target cells L1210/K^b, I found that there was rapid expression of FasL on the cell surface (Figure 3-1A). Substantial expression was detected at 15 min and increased until 2 hours. Surprisingly, Ca^{2+} chelation with EGTA revealed that there are two phases of FasL expression on the cell surface: a rapid extracellular Ca^{2+}-independent phase followed by a later extracellular Ca^{2+}-dependent phase. In contrast, no FasL cell surface expression was observed upon negative control target cell L1210 conjugation (Figure 3-1A). Although the early phase of FasL expression is extracellular Ca^{2+}-independent, its expression exhibited some delay under Ca^{2+} chelation when L1210/K^b was used as a target (Figure 3-1A). However, I found that this delay depends on effector/ target cell combination,

since it did not occur when AB.1 was stimulated with EL4 target cells (Figure 3-2B). I can conclude that at the population level, there are two waves of FasL surface expression on the CTL clone in response to target cell engagement. It is unclear whether it is the same cells that undergo two waves of FasL expression, but of the two waves of FasL that are expressed on the CTL surface, the early wave is likely translocated from internal Ca^{2+}-independent stores and the latter wave is Ca^{2+}-dependent.

Conventional assays to examine CTL exocytosis of cytolytic granules, which generally measure granzyme A activity from CTL supernatants, evaluate degranulation at a population level, while the FasL experiments determine the expression on individual cells by FACS. To more directly compare FasL cell surface expression and degranulation, I used a modification of the sensitive FACS-based degranulation assay employed by Wolint et al. whereby degranulation is measured by the appearance of cell surface CD107a (LAMP1), which is found in CTL granules (Wolint et al. 2004), to re-examine the extracellular Ca^{2+} requirement for degranulation. Consistent with conventional enzymatic assays, CTL degranulation, as measured by CD107a cell surface expression, stimulated by target cells, absolutely required extracellular Ca^{2+} (Figure 3-1B). Taken together, these observations indicate that the early FasL cell surface expression and degranulation occur downstream of distinct signaling pathways with respect to their extracellular Ca^{2+} requirements.

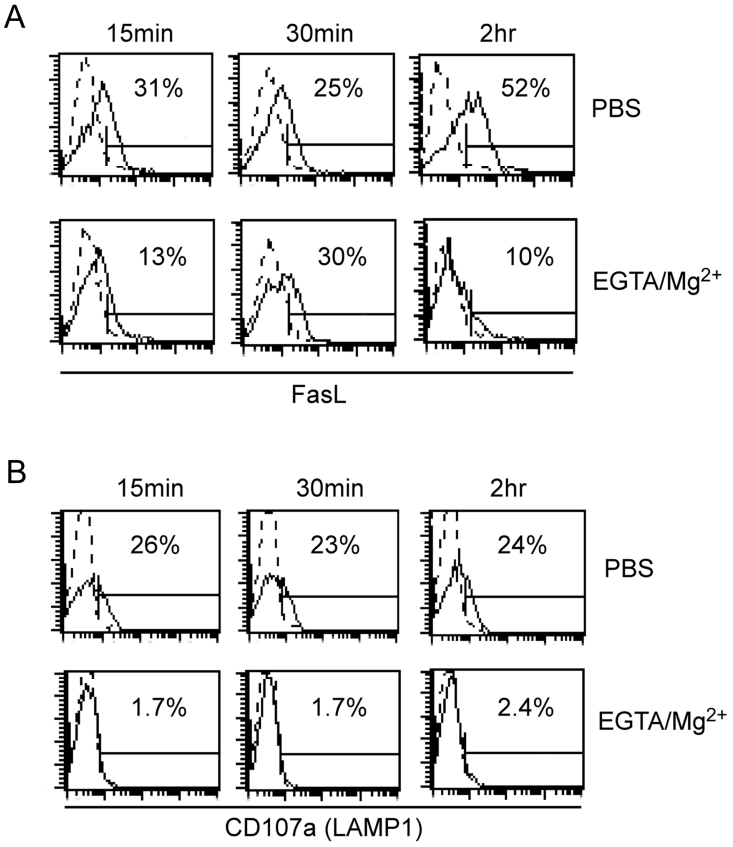
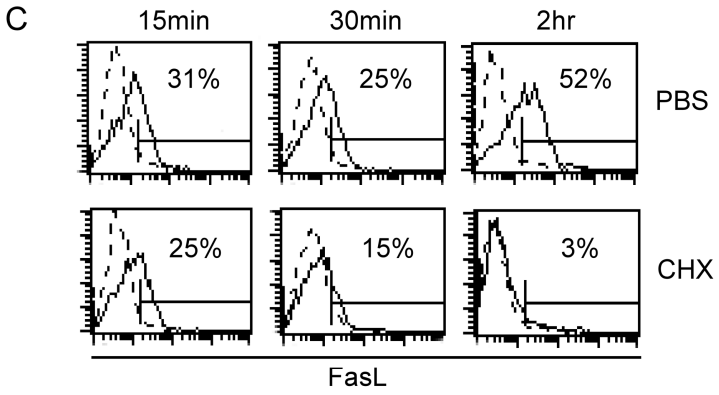
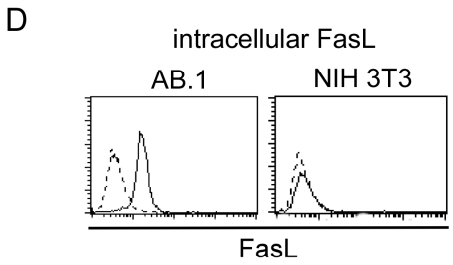
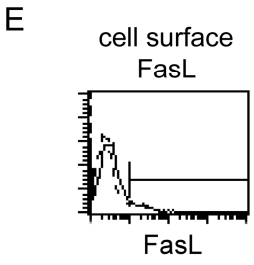
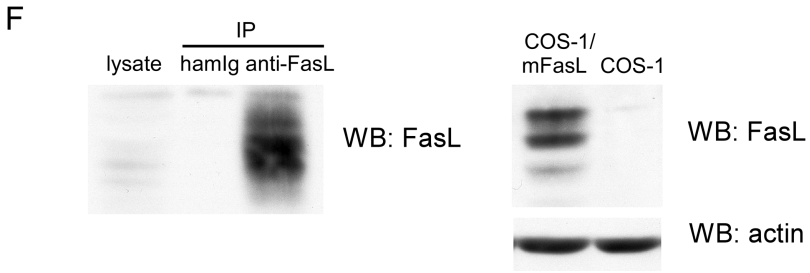


Figure 3-1. Target recognition by CTLs stimulates both extracellular Ca^{2+}-independent, stored pools of FasL and extracellular Ca^{2+}-dependent, *de novo* synthesized FasL cell surface expression. CTL clone AB.1 was incubated in the presence or absence of 4 mM EGTA/ 3mM $MgCl_2$ with cognate target cell L1210/K^b (——) or untransfected L1210-negative control (– – –). After the indicated time, the specific conjugates were separated with 5 mM EDTA, and surface FasL (*A*) or CD107a (*B*) on gated AB.1 was determined by flow cytometry. Indicated values are the percentage of FasL (*A*)- or CD107a (*B*)-positive AB.1. *C*, AB.1 was preincubated with 10 μg/ml CHX for 45 min, then stimulated with cognate target cells L1210/K^b (——) or negative control L1210 (– – –), and assessed for surface FasL expression at the indicated time. (Data presented in *A* and *C* are from the same experiment.) *D*, Unstimulated AB.1 or NIH3T3 cells as a FasL-negative control were subjected to intracellular FasL staining (——) or isotype Ab control staining (– – –) and analyzed by flow cytometry. *E*, Basal cell surface FasL level on unstimulated AB.1 was measured (——). Background (– – –) represents the isotype Ab staining. *F*, Immunoblot of FasL IP of unstimulated AB.1 is shown. A sample of postnuclear lysate is also shown. Additionally, COS-1 cells were either transiently transfected with mouse FasL cDNA (COS-1/mFasL), or mock transfected (COS-1), immunoblots of the postnuclear cell lysates probed with antibodies against FasL and actin are shown. In all cases, data are representative of at least three independent experiments.


C
15min
30min
2hr
31%
25%
52%
PBS
25%
15%
3%
CHX
FasL


D
intracellular FasL
AB.1
NIH 3T3
FasL


E
cell surface
FasL
FasL


F
IP
lysate
hamIg anti-FasL
WB: FasL
COS-1/
mFasL
COS-1
WB: FasL
WB: actin

Pre-synthesized FasL undergoes rapid TCR-regulated mobilization to the cell surface upon target cell engagement

Given the rapid expression of extracellular Ca^{2+}-independent FasL at the cell surface, I next examined whether *de novo* protein synthesis is required for this to occur. Pretreatment with the protein synthesis inhibitor cycloheximide (CHX) did not have a significant impact on early FasL cell surface expression (15 min) by AB.1 CTL upon target cell engagement (Figure 3-1C), indicating that new protein synthesis was not required to observe cell surface FasL at the early time point. In contrast, *de no*vo protein synthesis was absolutely required for the delayed (2 hr), extracellular Ca^{2+}-dependent FasL cell surface expression (Figure 3-1C). Flow cytometry revealed a significant basal level of intracellular FasL (Figure 3-1D), but there was little cell surface FasL expressed in resting CTL clone AB.1 (Figure 3-1E). Constitutive cellular FasL expression was confirmed by immunoblotting (Figure 3-1F). To confirm the specificity of the FasL antibody used for immunoblotting in this study, I transfected COS-1 cells with mouse FasL and stained with the antibody. The anti-FasL (clone 101626) detected FasL of the lysates of the transfected COS-1 but not the untransfected cells (Figure 3-1F). Because of the high basal level of cellular FasL protein, but only inducible expression on the cell surface, I conclude that there is a pre-existing pool of FasL, which is stored in a TCR-regulated secretory compartment in CTL, that undergoes immediate, extracellular Ca^{2+}-independent cell surface transport upon target cell engagement.

The rapidly expressed pool of FasL is sufficient to lead to CTL mediated killing of Fas-expressing target cells

I next sought to determine if the early Ca^{2+}-independent FasL expression on CTL was sufficient to induce target cell death. I also preferred to use an assay that could measure induction of cell death more rapidly than a standard 4-hour killing assay to minimize contributions of non-detectable degranulation or possible low levels of *de novo* FasL expression. The triggering of apoptosis via Fas involves a caspase activation cascade, within which, caspase-3 is activated downstream of caspase-8 activation via both mitochondria-dependent and -independent pathways (Barry and Bleackley 2002). Additionally, caspase-3 can be a direct substrate for granzyme B in intact cells (Atkinson et al. 1998). Therefore, I selected caspase-3 activation as an early indicator of target cell apoptosis via both the FasL and degranulation pathways. CTL clone AB.1 induced caspase-3 activation of target cell apoptosis was investigated by flow cytometry and the level of cleaved, active caspase-3 in Fas^{hi} EL-4 (H-2^b) cells and Fas^{lo} L1210/K^b cells (Figure 3-2A) was compared in the presence or absence of EGTA/Mg^{2+}. I reasoned that, in the absence of extracellular Ca^{2+}, the Ca^{2+}-dependent FasL and degranulation mechanisms would be excluded and the cytolytic effect of the extracellular Ca^{2+}-independent, preformed FasL pool alone could be selectively examined. I first confirmed that AB.1 shows both rapid Ca^{2+}-independent and delayed Ca^{2+}-dependent FasL cell surface expression upon EL4 engagement (Figure 3-2B). I observed that, after a 1.5-hour incubation with CTL clone AB.1 in the presence of 4 mM EGTA, EL4 still undergoes a significant level

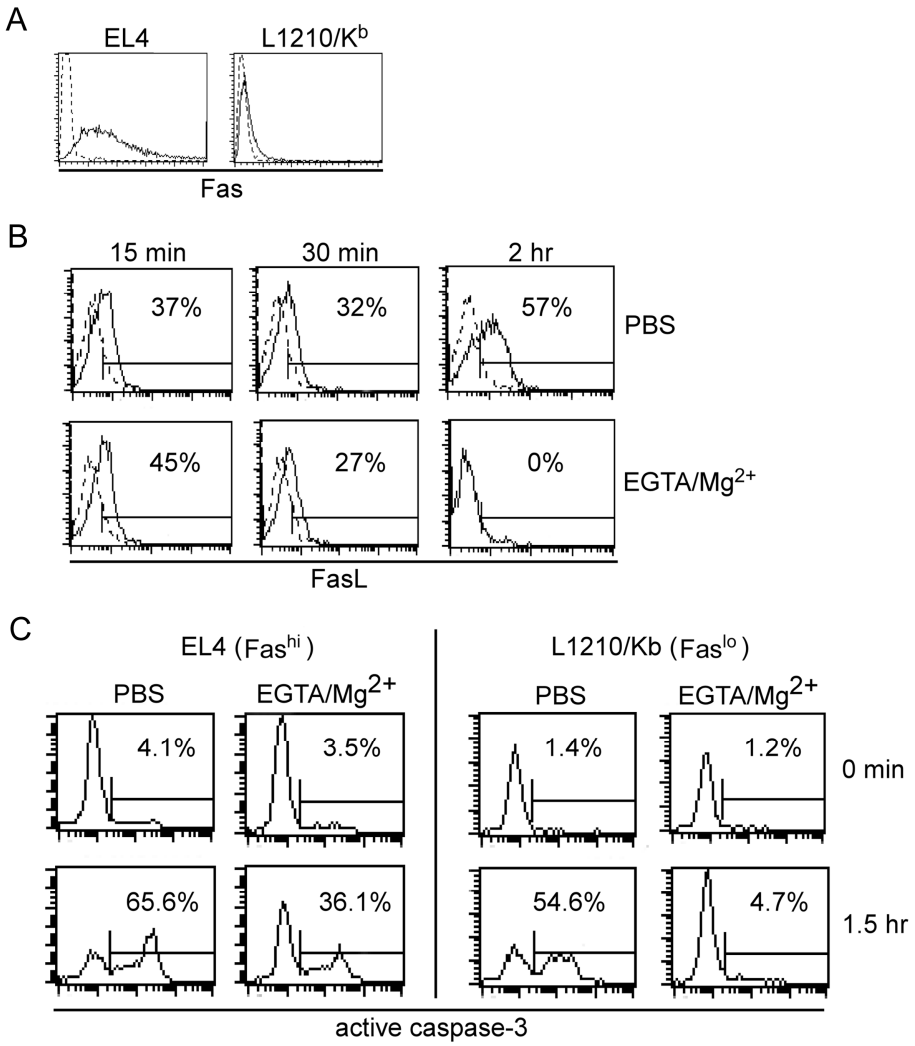


Figure 3-2. The early extracellular Ca^{2+}-independent FasL expression is sufficient to induce apoptosis of Fas^{+}, but not Fas^{-}, target cells. *A*, Fas surface expression on EL4 and L1210/K^b target cells were examined by flow cytometry. *B*, CTL clone AB.1 was incubated in the presence or absence of 4 mM EGTA/3 mM Mg^{2+} with cognate target cell EL4. After the indicated time, the specific conjugates were separated with 5 mM EDTA, and surface FasL on gated AB.1 was determined by flow cytometry (——). Background (– – –) indicates a zero time point control in which AB.1 and EL4 were mixed and placed on ice for 15 min. Indicated values are the percentage of cell surface FasL-positive AB.1 upon EL4 engagement subtracted by that of zero time point control. *C*, AB.1 was incubated for the indicated time with EL4 (Fas^{hi}) or L1210/K^b (Fas^{lo}) target cells in the presence or absence of 4 mM EGTA/ 3 mM $MgCl_2$. Cells were stained with an Ab specific for activated caspase-3 as an early marker of target cell apoptosis. Data signify expression of intracellular active caspase-3 on the gated target cell population. In *A*, data are representative of two independent experiments. In *B* and *C*, data are representative of at least three independent experiments.

of caspase-3 activation, which, in contrast, is completely blocked in the L1210/K^b cells that express limited amount of Fas (Figure 3-2C). This observation indicates that the rapidly expressed pool of FasL is indeed sufficient to induce target cell apoptosis. These data are consistent with our previous study showing that AB.1 is a potent mediator of extracellular Ca^{2+}-independent target cell killing assessed by ^{51}Cr-release assay (Ostergaard et al. 1987).

Anti-TCR stimulates two distinct patterns of FasL surface expression

I next examined whether TCR stimulation alone can induce the same pattern of FasL cell surface expression induced by target cells. Our laboratory has previously shown that solid-phase or plate-bound anti-TCR/CD3 is required to trigger degranulation; crosslinked anti-TCR/CD3 is unable to stimulate any detectable degranulation by granzyme A enzymatic assay (Berg et al. 1998). However, both stimulation methods can induce tyrosine phosphorylation and activation of other downstream signaling pathways (Berg et al. 1998). Similarly, I wanted to determine which TCR stimulation method can trigger FasL cell surface expression, as it had previously been shown that plate-bound anti-TCR triggers Ca^{2+}-dependent cell-surface expression of *de novo* synthesized FasL (Vignaux et al. 1995; Glass et al. 1996). Consistent with our earlier study (Berg et al. 1998), I found that only plate-bound, but not crosslinked anti-CD3 induces Ca^{2+}-dependent degranulation as measured by CD107a cell surface expression (Figure 3-3A). In contrast, I demonstrated that both crosslinked and immobilized anti-CD3 can induce FasL cell surface expression on the CTL (Figure 3-3B). Moreover, FasL

cell surface expression patterns induced by these two different methods are characteristically distinct. TCR stimulation by anti-TCR antibody crosslinking induces extracellular Ca^{2+}-independent, rapid and transient, preformed FasL cell surface expression, which mimics the rapidly expressed pool of FasL upon target cell stimulation. In contrast, immobilized anti-CD3 stimulation results in an extracellular Ca^{2+}-dependent, delayed, *de novo* FasL cell surface expression pattern as was previously published (Vignaux et al. 1995; Glass et al. 1996), which is similar to the late FasL cell surface expression upon target cell conjugation. An intriguing observation is that, although immobilized anti-CD3 provides a more sustained stimulation than crosslinked anti-CD3 (Berg et al. 1998), it did not elicit the early (15 min) FasL cell surface expression (Figure 3-3B), indicating that perhaps there are distinct signaling requirements for the two different FasL expression patterns. I also examined surface FasL expression 5 and 10 min post immobilized anti-CD3 stimulation and detected no increased expression (Figure 3-3C), excluding the possibility that there is rapid FasL cell surface expression followed by rapid turnover. It should be noted that CTL undergo relatively rapid attachment and TCR signaling within 10 min of addition to the immobilized anti-CD3 (Berg et al. 1998), suggesting that CTL are adhered and signaling at the early time point. Taken together, these data indicate there are two TCR-dependent signaling pathways that lead to different patterns of FasL CTL cell surface expression upon TCR engagement.

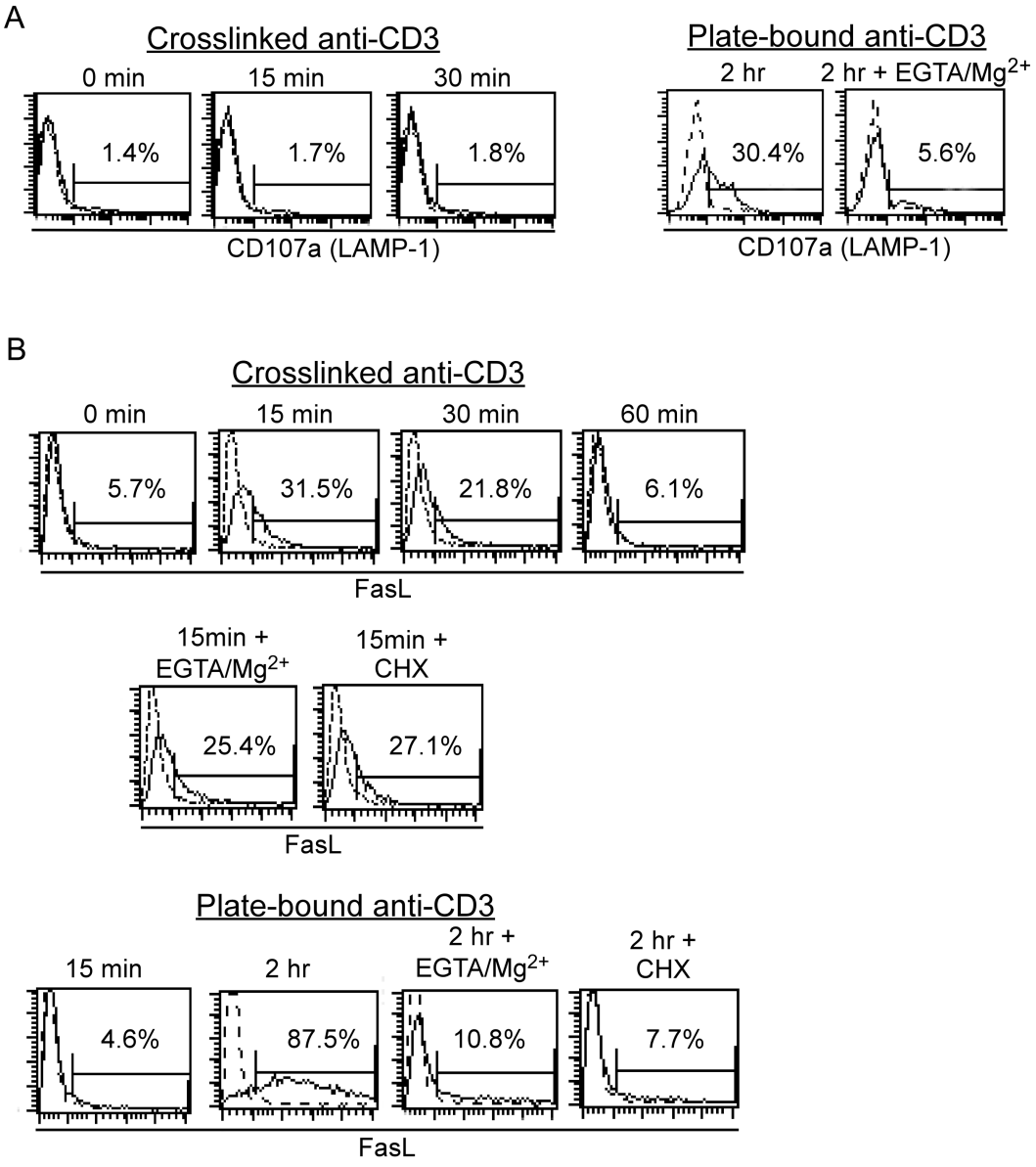
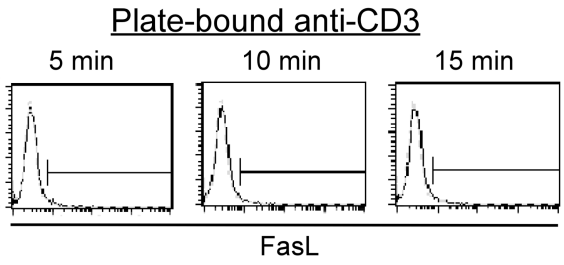


Figure 3-3. Soluble cross-linked and plate-bound anti-CD3ε induce distinct patterns of FasL cell surface expression; however, only plate-bound anti-CD3 triggers CTL degranulation. CTL clone AB.1 was treated for the indicated time with either soluble cross-linked or plate-bound anti-CD3 (145-2C11). Cells were stimulated in the presence or absence of 4 mM EGTA, 3 mM $MgCl_2$, or 10 μg/ml CHX. AB.1 was detached from the anti-CD3-bound plate with 5 mM EDTA treatment at 4°C for 10 min prior to staining. Cell surface CD107a (*A*) or FasL (*B* & *C*) expression was measured on stimulated cells (——) or control cells (– – –). Data are representative of at least three independent experiments.

C



Stored FasL cell surface delivery is microtubule-independent

CTL cytolytic granules have been shown to move along microtubules to accumulate at the target cell contact point (Sancho et al. 2002) and MHC class I-stimulated degranulation is disrupted with colchicine (O'Rourke et al. 1991). I wanted to determine if microtubules are required for stored FasL cell surface expression. To disrupt microtubule-based granule movement, AB.1 was pre-treated with colchicine (0.1 μM-5 mM), which binds tubulin and blocks microtubule polymerization, then FasL and CD107a cell surface expression was assessed at 15 min after target cell stimulation. For these experiments, I performed simultaneous staining of FasL and CD107a and the FACS profiles of cells treated with and without 100 μM colchicine is shown (Figure 3-4A), along with a colchicine titration (Figure 3-4B). At all concentrations examined, colchicine did not inhibit rapid FasL cell surface delivery (Figure 3-4B). In contrast, degranulation as measured by CD107a cell surface expression was significantly impaired by colchicine in a concentration-dependent manner (Figure 3-4B). The same concentrations that blocked CD107a cell surface expression also inhibited degranulation as measured by release of Granzyme A enzymatic activity from cells and reorientation of the microtubule organizing center towards the contact point (Lysechko and Ostergaard, unpublished data). I further confirmed that colchicine treatment did not impair effector-target conjugate formation at 15 min after target cell incubation ensuring that lack of degranulation is not due to insufficient conjugate formation (Figure 3-4C). These data indicate that preformed FasL expression is not dependent on microtubules, unlike CTL

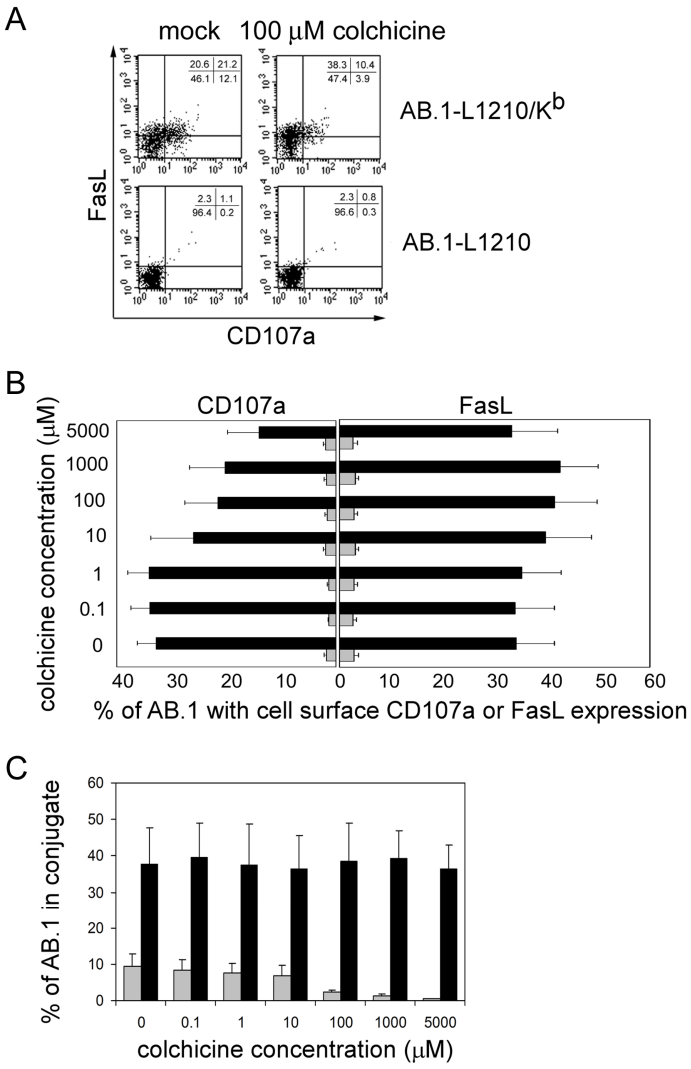


Figure 3-4. Cell surface expression of preformed pool of FasL is microtubule independent. AB.1 cells were pretreated with ethanol carrier control or the indicated concentration (0.1 μM–5 mM) of colchicine, then stimulated with cognate target cell L1210/K^b (black bar) or negative control L1210 (grey bar) for 15 min. Cells were stained with CD8α (PE.Cy5), FasL (PE), and CD107a (FITC). After gating on the $CD8^+$ cells to examine only the CTLs, the expression of FasL and CD107a was assessed. *A*, Typical FACS plot. *B*, CD107a and FasL surface expression was determined at different colchicine concentrations. Indicated values are percentage of AB.1 positive for FasL or CD107a expression. *C*, PKH26 red dye-stained AB.1 cells were treated with colchicine and stimulated with unstained L1210 or L1210/K^b as in *A*. After 15 min of stimulation, cells were vortexed to eliminate nonspecific binding and fixed. The percentage of AB.1 in conjugates was assessed by focusing on red fluorescence-positive cells and analyzing forward scatter. In *B* and *C*, data are the average of three independent experiments; error bars indicate SEM.

degranulation, and further illustrate the differential control of exocytosis of cytolytic granules and rapid FasL transport to the cell surface.

Stored FasL cell surface delivery is calcineurin-independent

It has been shown that the calcineurin inhibitor cyclosporin A (CsA) inhibits CTL degranulation (Dutz et al. 1993) and partially inhibits FasL mediated killing (Anel et al. 1994). However, it was not clear if the calcineurin-dependent cytotoxicity was mediated by stored or *de novo* synthesized FasL. In contrast, I found that early FasL cell surface translocation (15 min) is calcineurin-independent (Figure 3-5A), which I confirmed is indispensable for degranulation (Figure 3-5C). Cell surface FasL was also examined at 2hr post stimulation, the time at which only *de novo*, but not stored FasL, is expressed on the cell surface. I found that *de novo* FasL cell surface expression is calcineurin-dependent (Figure 3-5B). These data further indicate that stored FasL cell surface translocation is differentially regulated from degranulation and *de novo* FasL cell surface expression.

Either phorbol ester PMA alone or calcium ionophore ionomycin alone is sufficient to induce FasL cell surface expression

I observed that PMA alone or ionomycin alone was sufficient to stimulate rapid (15 min) stored FasL cell surface translocation by CTLs in the presence of CHX (Figure 3-6A & 6B). It is important to note that there is no significant difference between PMA and ionomycin regarding their ability to elicit FasL

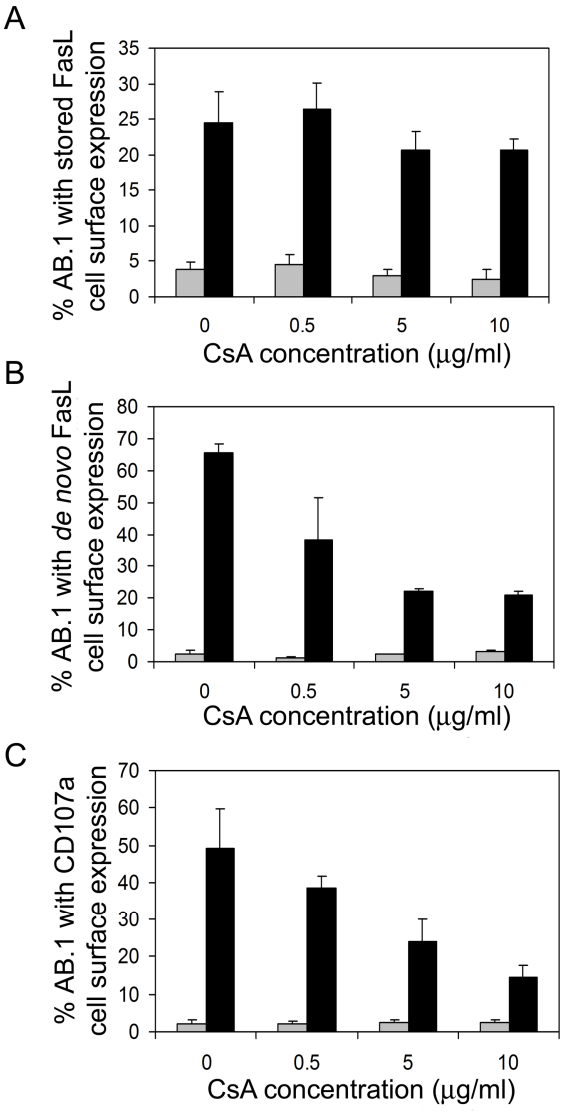


Figure 3-5. Cell surface expression of the stored FasL is calcineurin independent. AB.1 cells were pretreated with ethanol carrier control or the indicated concentration (0.5–10 μg/ml) of cyclosporin A (CsA), then stimulated with cognate target cell L1210/K^b (black bar) or negative control L1210 (grey bar) for either 15 min (*A* and *C*) or 2 hr (*B*). Cells were stained with CD8α (PE.Cy5), FasL (PE) and CD107a (FITC). After gating on the $CD8^+$ cells to examine only the CTLs, the expression of early FasL (*A*), delayed FasL (*B*), and CD107a (*C*) was assessed. Indicated values are percentage of AB.1 positive for FasL (*A* and *B*) or CD107a (*C*) expression. Data are the average of three independent experiments; error bars indicate SEM.

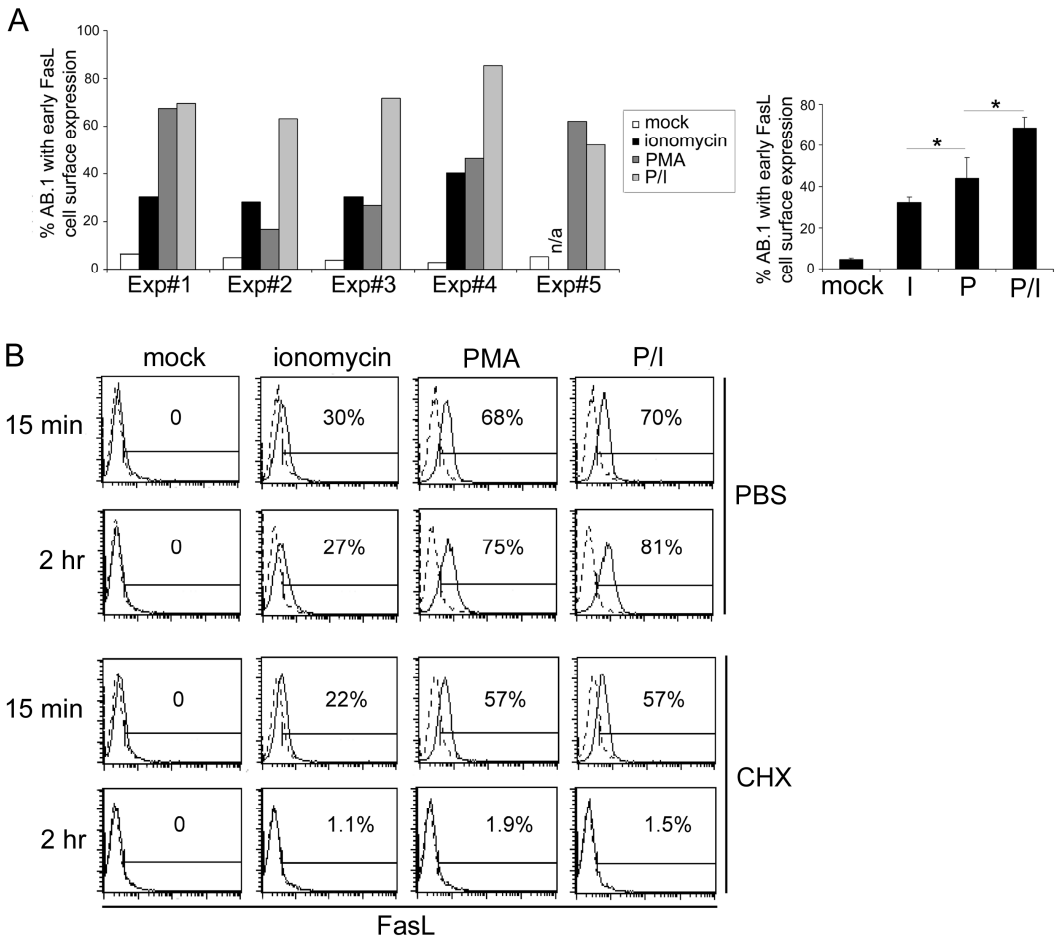


Figure 3-6. Either Ca^{2+} ionophore alone or phorbol ester alone is sufficient to elicit FasL cell surface expression from both the stored and the *de novo* synthesized pool. *A*, AB.1 was pre-incubated with cycloheximide (CHX), then stimulated with either 2.5 μM ionomycin alone (I), or 10 ng/ml PMA alone (P), or 10 ng/ml PMA plus 0.5 μM ionomycin (P/I) or carrier control DMSO for 15 min. The percentage of AB.1 with cell surface FasL expression was detected by flow cytometry to examine the stored FasL translocation. Results from five independent experiments (left panel) and the average of these five independent experiments (right panel) are shown. Error bars indicate SEM. Statistical significance was assessed by one-way ANOVA (* $p > 0.05$, not significant). (n/a: not available). *B*, AB.1 was pre-incubated with CHX or left untreated, then stimulated with either ionomycin alone, PMA alone, or PMA plus ionomycin (P/I) or carrier control DMSO for either 15 min or 2 hr. The percentage of AB.1 with cell surface FasL expression was detected by flow cytometry to examine the stored FasL translocation and *de novo* FasL cell surface expression upon stimulation (——). Negative control (– – –) was AB.1 treated with DMSO carrier control. Indicated values are the percentage of cell surface FasL-positive AB.1 upon stimulation subtracted by that of DMSO treatment. Data are representative of at least three independent experiments.

translocation from the stored pool ($p > 0.05$) (Figure 3-6A). In contrast, it was established that both PMA and ionomycin are required to stimulate degranulation (Berrebi et al. 1987). These data provide further evidence supporting that stored FasL is differentially regulated from cytolytic granules. I also found that either ionomycin alone or PMA alone can stimulate *de novo* FasL cell surface expression 2h after stimulation, which was blocked by CHX (Figure 3-6B), suggesting a differential signaling requirement between degranulation pathway and *de novo* FasL cell surface expression, the only I have identified thus far.

FasL is found in vesicles that are distinct from cytolytic granules

The distinct signaling and stimulation requirements and microtubule dependence for rapid FasL cell surface expression and degranulation suggests that these two processes are independently controlled. It has been suggested that FasL is stored in cytolytic granules (Bossi and Griffiths. 1999; Kojima et al. 2002), implying that signaling requirements for degranulation and FasL expression would be identical. However, my results above, indicating distinct signaling requirements for these two mechanisms, strongly suggest colocalization would be unlikely. I therefore compared the intracellular localization of preformed FasL and cytolytic granules in AB.1 by confocal microscopy. Using cathepsin D, LAMP1, perforin and granzyme B as markers for cytolytic granules, I demonstrated that the FasL intracellular storage compartment completely segregates from cytolytic granules (Figure 3-7A). Cytolytic granules stained with granule cargo marker cathepsin D and granule membrane marker LAMP1 as a

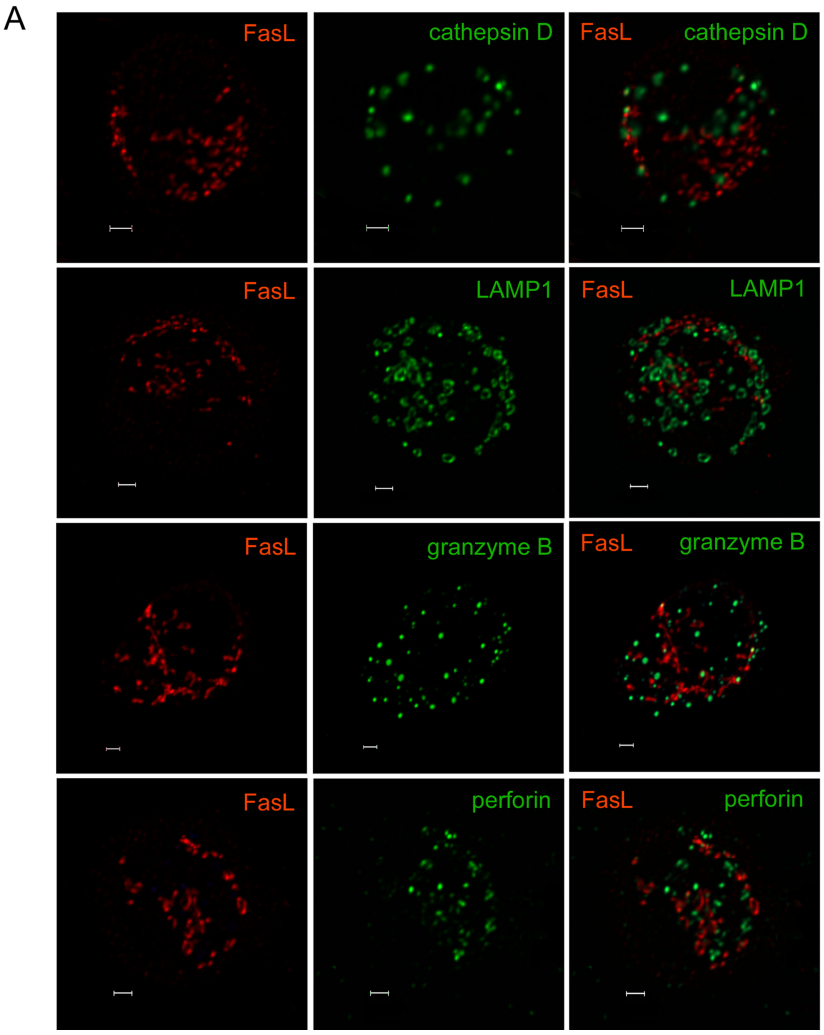
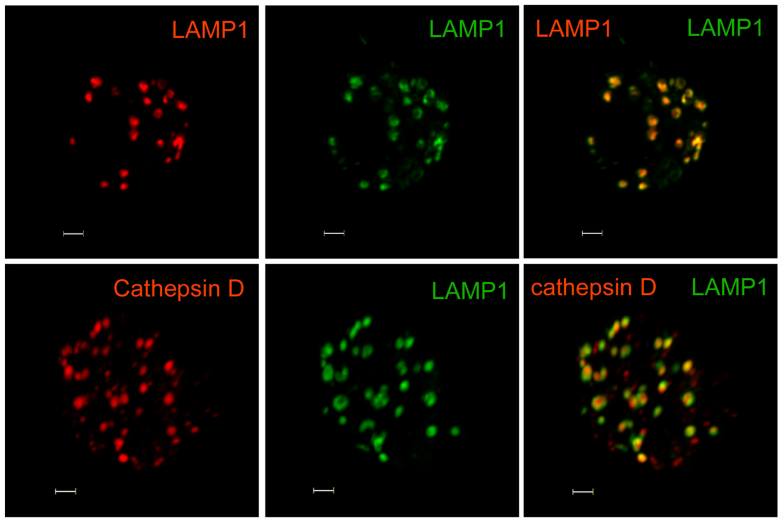
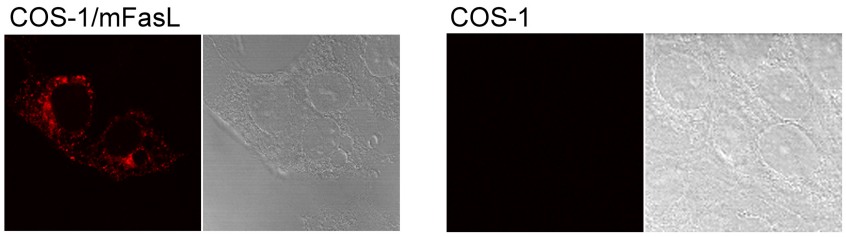


Figure 3-7. Stored FasL segregates from cytolytic granules in murine CTL. *A*, AB.1 cells were stained with Abs specific for FasL (red) and the cytolytic granule markers cathepsin D, LAMP1, granzyme B, or perforin (green) and the appropriate secondary Abs and analyzed by confocal microscopy. *B*, As a positive control, AB.1 cells were stained with rat anti-LAMP1 primary Ab followed by both Alexa Fluor 488 donkey anti-rat IgG and Alexa Fluor 594 donkey anti-rat IgG. Another positive control was cathepsin D (red) and LAMP1 (green) costaining. *C*, COS-1/mFasL and COS-1 cells were stained with biotinylated anti-FasL Ab (MFL3) followed by Alexa Fluor 546 streptavidin and detected by confocal microscopy. *D*, AB.1 were stained with biotin-conjugated anti-FasL (MFL3), or biotin-conjugated hamster IgG isotype control, followed by streptavidin Alexa 546, or streptavidin Alexa 546 staining alone, and subjected to confocal microscopy. In *A*, *B* and *D*, Z-stack images were acquired (interval, 0.2 μm) and subjected to deconvolution and three-dimensional reconstruction. Representative projections of the reconstructed three-dimensional images are shown. Scale bars represent 1 μm. Data are representative of at least three independent experiments.

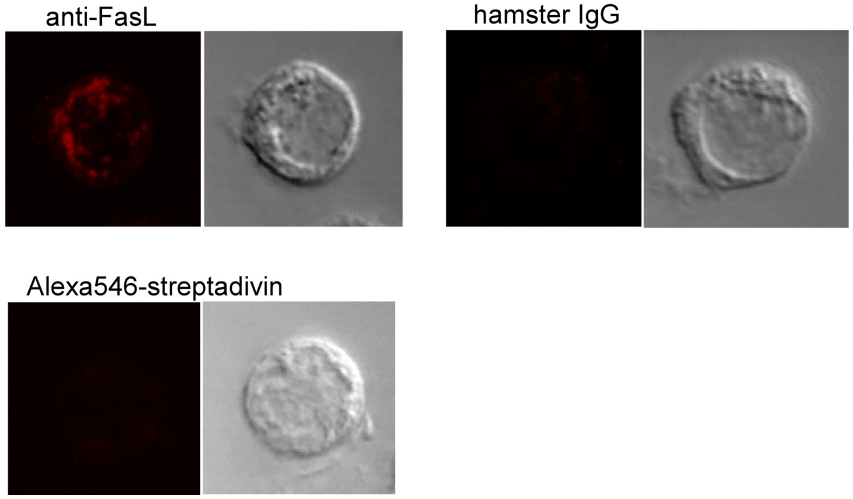
B


LAMP1
LAMP1
LAMP1
LAMP1
Cathepsin D
LAMP1
cathepsin D
LAMP1

C


COS-1/mFasL
COS-1

D


anti-FasL
hamster IgG
Alexa546-streptadivin

positive control (Figure 3-7B), demonstrated that the cytolytic granule structure was preserved, and that the majority of this staining colocalized to the same granules. The anti-FasL (MFL3) stained the mouse FasL transfected COS-1 but not the untransfected cells (Figure 3-7C), confirming the specificity of the FasL antibody used for the staining. Furthermore, no staining of cells was detected with biotinylated isotype control antibody (Figure 3-7D). These confocal data demonstrate that stored FasL does not localize with the cytolytic granules. I also stained cells two hours after stimulation and detected no colocalization of FasL with the cytolytic granules (Figure 4-5E), suggesting that the newly synthesized FasL also does not colocalize with the granules, however in these experiments I was unable to distinguish stored from newly synthesized FasL.

FasL vesicles segregate from cytolytic granules in human CD8$^+$ T cells

I have demonstrated that degranulation and FasL lytic mechanisms are differentially regulated with respect to stored component localization and regulation using murine CTL clones. It is not clear why my results differ from those of Bossi and Griffiths, who suggested that pre-existing FasL being stored solely in the lytic granules (Bossi and Griffiths 1999). One possibility could be that they used human CD8$^+$ T cells, whereas my experiments were conducted using murine CTLs. Therefore, I further examined a human CD8$^+$ T cell line hCD8. Confocal microscopy revealed that, similar to murine CTL, hCD8 has prestored FasL which segregates from cytolytic granules (Figure 3-8A), indicating the discrepancy is not an issue of species difference. Further, I wanted

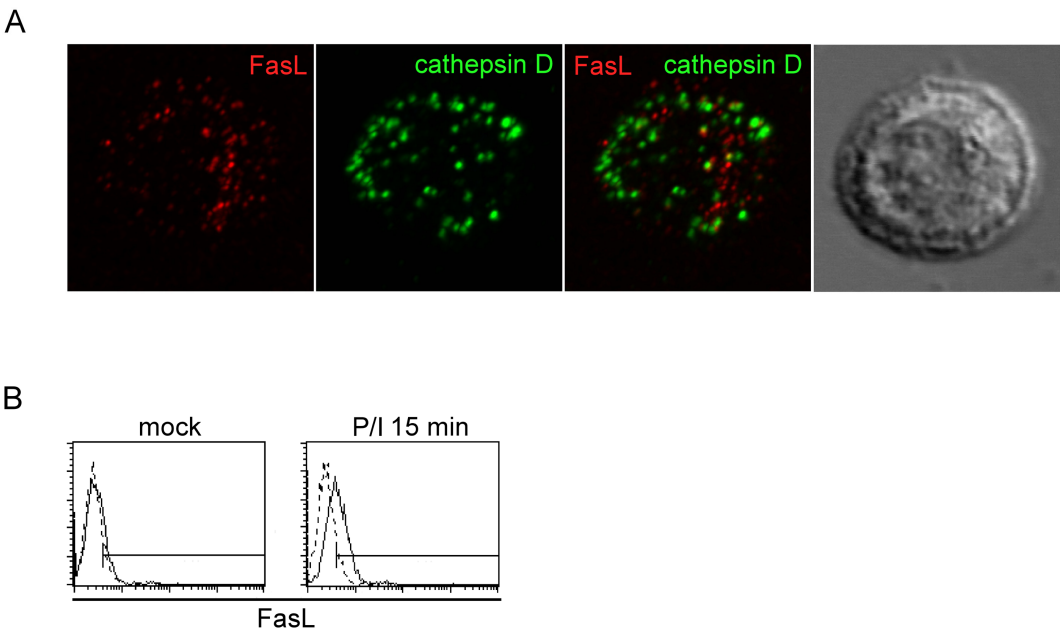


Figure 3-8. Stored FasL segregates from cytolytic granules in human CTL. *A*, Human CTL line hCD8 cells were stained with Abs specific for FasL (red) and the cytolytic granule marker cathepsin D (green) and the appropriate secondary Abs and analyzed by confocal microscopy. Z-stack images were acquired (interval, 0.2 μm) and subjected to deconvolution and three-dimensional reconstruction. Representative projection of the reconstructed three-dimensional images is shown. *B*, hCD8 were stimulated with 10 ng/ml PMA plus 0.5 μM ionomycin (P/I) or carrier control DMSO for 15 min. The expression of cell surface FasL upon stimulation (——) was examined by flow cytometry. Background (– – –) was secondary Ab staining control. Data are representative of two independent experiments.

to examine if this prestored FasL can be translocated to the cell surface upon stimulation. I found hCD8 exhibited rapid FasL cell surface expression upon stimulation with PMA plus ionomycin (Fig 3-8B), suggesting that preformed FasL, which is not stored in cytolytic granules, can be mobilized and detectable on the cell surface by human $CD8^+$ T cells.

Not all CTL are equivalent in their ability to rapidly mobilize stored FasL to the cell surface in response to target cells

We have observed that not all CTL clones are competent to lyse Fas^+ EL4 in the absence of extracellular Ca^{2+} ((Ostergaard and Clark 1989) and data not shown). Cl11 is one such clone, and I hypothesized that this CTL clone would be deficient in the rapid mobilization of stored FasL to the cell surface. Upon conjugation with L1210/K^b target cells, there is no immediate, Ca^{2+}-independent FasL cell surface expression on Cl11 (Figure 3-9A). Nevertheless, the Ca^{2+}-dependent, *de novo* FasL cell surface expression is as robust as that of AB.1 (Figure 3-9A). The possibility that there was no preformed FasL in Cl11 was excluded by the detection of intracellular FasL by FACS analysis (Figure 3-9B) and by immunoblotting (Figure 3-9C). However, the expression level of intracellular FasL in Cl11 is only about less than half that of AB.1 (Figure 3-9B and Figure 3-9C), which could be below a threshold for sufficient expression for Ca^{2+}- independent killing. Target cell apoptosis, measured as caspase 3 cleavage, in the presence of EGTA revealed that the preformed pool of FasL in Cl11 was not able to efficiently induce lysis of the EL4 Fas-expressing target cell

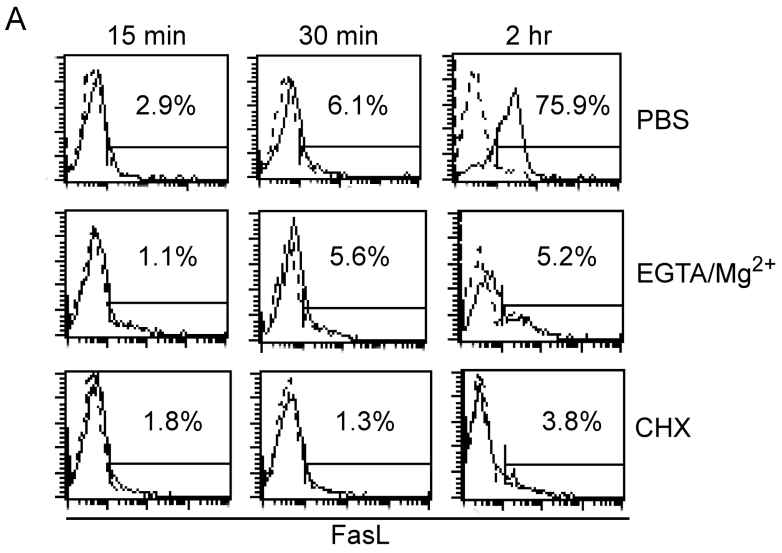
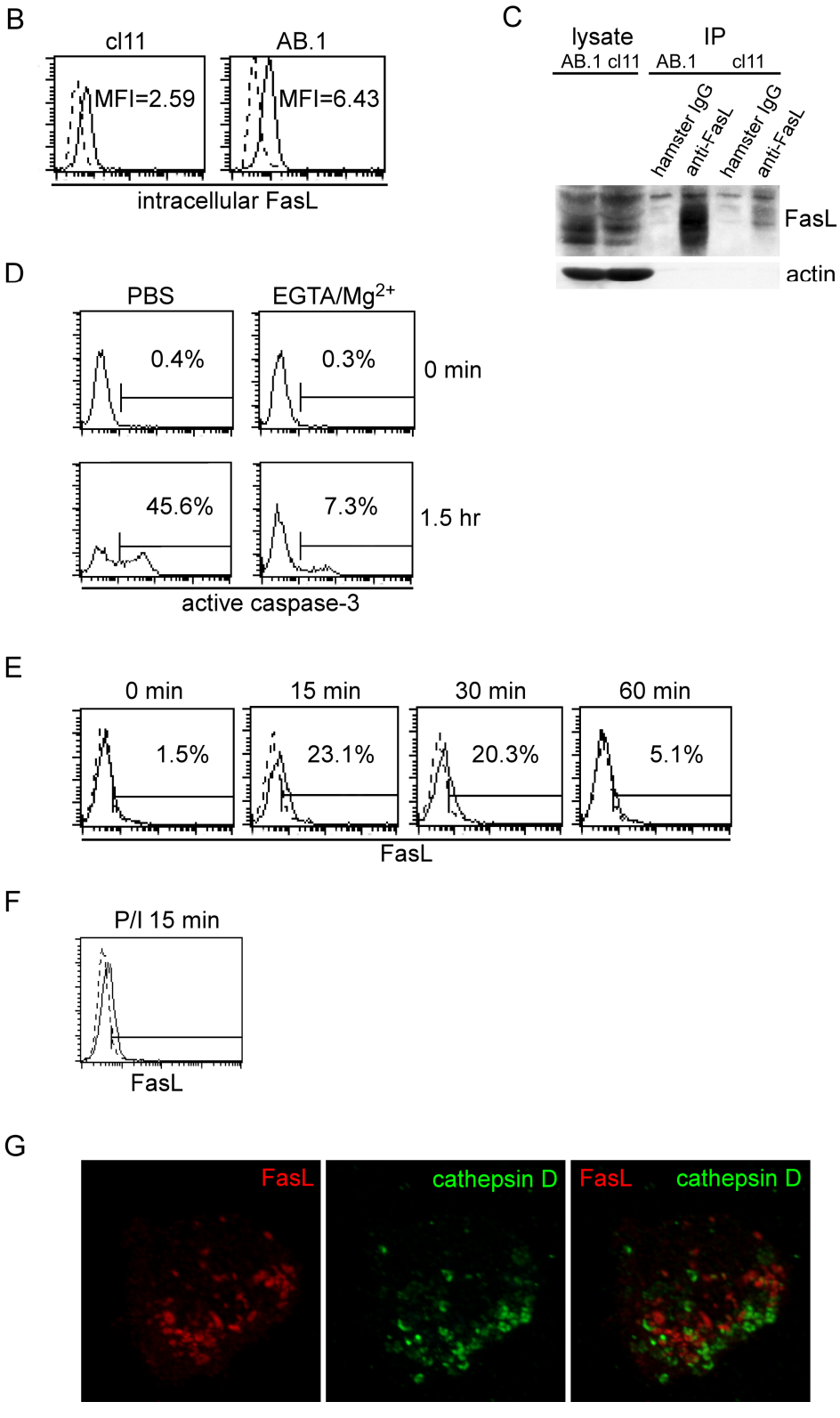


Figure 3-9. CTL clone Cl11 contains stored FasL but expresses primarily newly synthesized FasL on the cell surface in response to cognate target cells and exhibits limited extracellular Ca^{2+}-independent killing. *A*, CTL clone Cl11 was stimulated with L1210/K^b (——) or L1210 (– – –) target cells for the indicated time in the presence or absence of EGTA/Mg^{2+} or cycloheximide and then stained for surface FasL. Indicated values are the percentage of cell surface FasL-positive AB.1 upon L1210/K^b engagement subtracted by that of L1210 engagement. *B*, Unstimulated Cl11 and AB.1 were subjected to intracellular FasL staining (——) or isotype control Ab staining (– – –). Indicated values are mean fluorescent intensity (MFI) of FasL-PE staining subtracted by background staining. *C*, FasL IP of unstimulated AB.1 and Cl11 was carried out. Immunoblot of FasL IP and postnuclear lysate was carried out to detect FasL and actin. *D*, Cl11 was incubated for the indicated time with EL4 (Fas^+) target cells in the presence or absence of EGTA/$MgCl_2$. Cells were stained with an Ab specific for activated caspase-3. Indicated values represent expression of intracellular active caspase-3 on the gated target cell population. *E*, Cl11 was treated for the indicated time with soluble cross-linked anti-CD3 (145-2C11). Cell surface FasL expression was measured. Indicated values are percentage of cells with FasL-PE staining (——) subtracted by isotype-PE staining (– – –). *F*, Cl11 were stimulated with 10 ng/ml PMA plus 0.5 μM ionomycin (P/I) or carrier control DMSO for 15 min. The expression of cell surface FasL upon stimulation (——) was examined by flow cytometry. Background (– – –) was Cl11 treated with carrier control DMSO. *G*, Cl11 were stained with Abs specific for FasL (red) and the cytolytic granule marker cathepsin D (green) and the appropriate secondary Abs and analyzed by confocal microscopy. Z-stack images were acquired (interval, 0.2 μm) and subjected to deconvolution and three-dimensional reconstruction. Representative projection of the reconstructed three-dimensional images is shown. Data are representative of at least three independent experiments.


B
cl11
AB.1
MFI=2.59
MFI=6.43
intracellular FasL
C
lysate
IP
AB.1 cl11
AB.1
cl11
hamster IgG
anti-FasL
hamster IgG
anti-FasL
FasL
actin
D
PBS
EGTA/Mg2+
0.4%
0.3%
0 min
45.6%
7.3%
1.5 hr
active caspase-3
E
0 min
15 min
30 min
60 min
1.5%
23.1%
20.3%
5.1%
FasL
F
P/I 15 min
FasL
G
FasL
cathepsin D
FasL
cathepsin D

(Figure 3-9D). However, I was able to detect rapid and transient expression of FasL in response to crosslinked anti- CD3 (Figure 3-9E). Furthermore, stimulation with PMA plus ionomycin can elicit rapid FasL cell surface translocation (Figure 3-9F). These data demonstrated that FasL from the stored pool can be mobilized and detectable on the cell surface by Cl11. Confocal microscopy showed no co-localization of stored FasL with cytolytic granule markers in Cl11 (Figure 3-9G). These results reveal that not all CTL are equivalent in their ability to rapidly mobilize stored FasL to the cell surface in response to target cells in the absence of extracellular Ca^{2+}.

Stored FasL that is rapidly transported to the cell surface is found in ex vivo CTL

I next wanted to ascertain whether the observations obtained with AB.1 actually reflect FasL expression in *in vivo* derived CTL. To this aim, isolated $CD8^+$ T cells from peritoneal exudate lymphocytes (PEL) from BALB/c mice on day 10-11 after intraperitoneal injection of the EL4 lymphoma were assessed for target cell-induced FasL expression. I elected to examine PEL-derived CTL, as these can be easily examined *ex vivo* without any potential influence of culture in the presence of cytokines. Similar results to those found above for CTL clone AB.1 were obtained with *in vivo*-derived CTL (Figure 3-10). Namely, the *ex vivo* $CD8^+$ T cell population showed no basal FasL cell surface expression, but rapidly expressed pre-existing FasL at 15 min that was Ca^{2+}-independent for its induction. Furthermore, as with the CTL clones, there is later expression of Ca^{2+}-dependent,

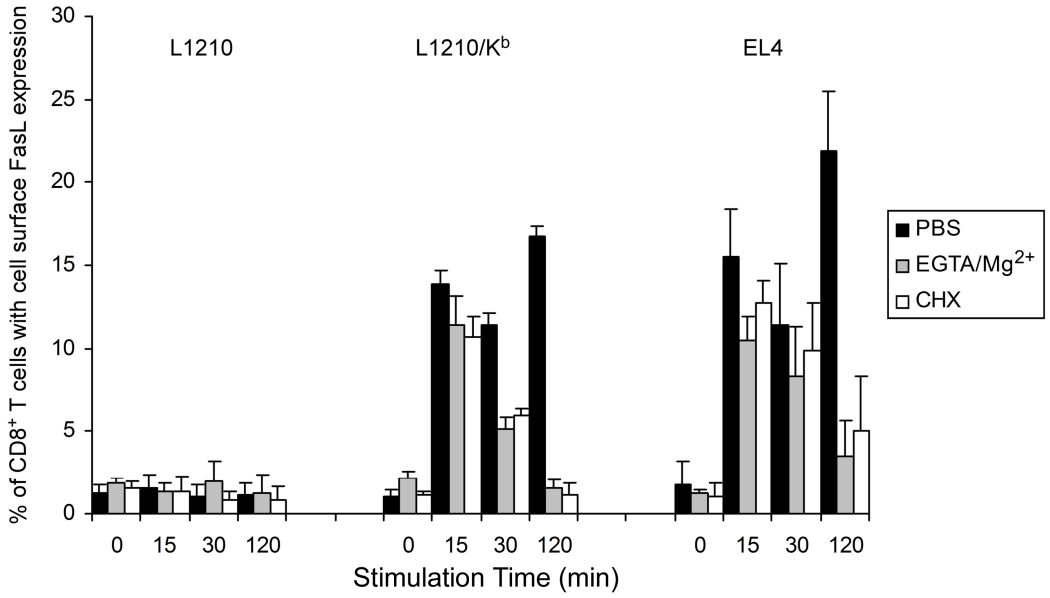


Figure 3-10. Purified $CD8^+$ CTL from *in vivo*-primed PELs exhibit rapid FasL expression independent of extracellular Ca^{2+} or new protein synthesis. *In vivo*-primed alloreactive $CD8^+$ T cells from BALB/c mice were isolated from the peritoneum and stimulated for the indicated time with specific target cells L1210/K^b or EL4 or the negative control target cell L1210 in the presence or absence of EGTA or CHX. Cell surface FasL expression on the gated $CD8^+$ CTL population was analyzed by flow cytometry. The percentage of FasL-positive $CD8^+$ T cell population was shown. Data are the average of three independent experiments; error bars represent SEM.

newly synthesized FasL at 2 hr after cognate target cell L1210/K^b or EL4 conjugation (Figure 3-10). These results demonstrated that *in vivo* derived CTL are triggered to rapidly express internal stores of FasL on the cell surface via an extracellular Ca^{2+}-independent pathway and confirm that *in vivo* derived CTL express two distinct waves of FasL at the cell surface after target cell engagement. I also observed intracellular pools of FasL in *ex vivo* effector $CD8^+$ T cells ($CD11a^{hi}/CD62L^{lo}$), but not naïve $CD8^+$ T cells ($CD11a^{lo}/CD62L^{hi}$) from spleen of BALB/c mice on day 12 after intraperitoneal injection of EL4 cells (Appendix Figure 1), which suggested that CTL expressing stored pools of FasL are also detected in effector but not naïve splenic T cells *in vivo*.

C. Discussion

The primary finding of this chapter is that CTL contain intracellular pools of FasL that are rapidly mobilized to the cell surface to trigger degranulation-independent, FasL-mediated lysis. This intracellular pool of FasL does not localize to the cytolytic granules and mobilization of this stored FasL to the cell surface occurs independently of degranulation. I provide substantial experimental evidence to support my conclusion that FasL is stored and regulated independently of the cytolytic granules. First, I could not detect degranulation by cells stimulated with crosslinked antibody even though these cells mobilized pre-existing FasL to the cell surface (Figure 3-3). Second, target cell stimulated degranulation was inhibited by extracellular Ca^{2+}-depletion, whereas the rapid FasL surface expression occurred under these same conditions (Figure 3-1).

Third, FasL translocation from the stored pool does not require calcineurin (Figure 3-5) or microtubule rearrangement (Figure 3-4), which are indispensible for degranulation upon target cell engagement. Fourth, CTL requires both phorbol ester PMA and calcium ionophore ionomycin for triggering degranulation, whereas either PMA alone or ionomycin alone is sufficient to elicit FasL translocation from the stored pool (Figure 3-6). Fifth, I detected Fas-dependent target cell apoptosis in the absence of granule-dependent lysis (Figure 3-2). Finally, I did not detect co-staining of FasL with the cytolytic granules in both the murine and human CTLs that I examined (Figure 3-7 & 3-8).

It is not clear why my results differ from those of Bossi and Griffiths (Bossi and Griffiths 1999). Several possibilities may reconcile my conclusions with the results of Bossi and Griffiths (Bossi and Griffiths 1999), who based their argument mainly on confocal imaging and GFP-tagged FasL overexpression. The first possibility is that FasL can be stored in cytolytic granules, but not exclusively. However my functional data indicate that the significant stored pool of FasL resides primarily outside of the lytic granules in the murine and human CTLs I examined. Close inspection of the confocal images of CTL obtained by Bossi and Griffiths reveals that there are several regions of non-overlap between FasL and granule staining, which the authors attributed to the localization of FasL along the secretory pathway (Bossi and Griffiths 1999). Second, the confocal images shown by Bossi and Griffiths were obtained from a single confocal plane rather than of the entire. However, in my study, Z-stack images were acquired and subjected to deconvolution and 3-D reconstruction, in order to minimize the

possibility of FasL and cytolytic granule markers being on top of each other and giving the illusion of being colocalized. Furthermore, confocal microscopy can benefit greatly from the advantages of deconvolution, such as increasing the signal-to-noise ratio and compensating for the decrease in resolution, thus creating clearer images. Therefore, it is possible that different resolutions of the confocal images lead to different interpretation of the results between our two groups. Third, confocal analysis of rat basophilic leukemia cell line RBL transfectants expressing FasL with a N-terminal GFP tag (FasL-GFP) demonstrated that FasL is sorted to the secretory lysosomes of these cells (Bossi and Griffiths 1999). However, the effects of GFP, a large protein with about 220 amino acids, on FasL processing and trafficking cannot be ruled out. Indeed, it has been reported that, without GFP tag, retention of PRD-containing FasL in the cytoplasm was not detected (Xiao et al. 2004), inconsistent with that observed by Bossi and Griffiths (Bossi and Griffiths 1999). Therefore, further study is required to verify the reliability of the tagged FasL construct for localization study, which may provide an important tool for live cell imaging and immunoisolation of FasL storage compartments. In addition, it is possible that overexpressed FasL in the transfectants might be sorted to the lysosome to undergo degradation and is therefore no longer bioactive.

My data clearly indicate that FasL expression and degranulation are under distinct controls and likely represent two independent pathways rather than FasL being a component of granule-mediated lysis. This is consistent with, and provides further insight to, a number of previous studies. Degranulation, but not

all CTL-mediated killing, is inhibited with extracellular Ca^{2+} depletion (Ostergaard et al. 1987; Trenn et al. 1987). In another study, a CTL clone that failed to mobilize intracellular Ca^{2+} after TCR engagement was unable to kill via the degranulation mechanism of killing, yet was still able to kill via the FasL/Fas mechanism in an antigen specific manner (Esser et al. 1996). Furthermore, CTL from Ashen mice are defective in degranulation, but are still able to kill via the FasL pathway (Haddad et al. 2001). These data support my conclusion that FasL is stored in vesicles distinct from the perforin/granzyme containing granules.

An increasing body of evidence indicates the presence of pre-existing pools of FasL in activated mouse and human $CD8^+$ T cells and NK cells (Li et al. 1998; Bossi and Griffiths 1999; Kojima et al. 2002). However, the exact nature of the stored FasL is largely unknown. Bossi and Griffiths showed that pre-existing, along with newly synthesized FasL, is sorted to the cytolytic perforin-containing granules in a human CTL clone and that upon degranulation FasL is delivered to the cell surface (Bossi and Griffith 1999). However, my data suggest a distinct FasL localization. There is precedent for such a vesicle type, as the chemokine RANTES is found in distinct storage granules in human CTL (Catalfamo et al. 2004). These vesicles rapidly released the stored RANTES upon T cell activation, similar to what I detect with FasL, however, the nature of the storage vesicles was not further characterized. Our CTL clones do not store RANTES (data not shown), so I could not determine if RANTES and FasL are stored in the same compartment in CTL. Further studies will be required to identify the TCR regulated compartment in which the FasL is stored.

I further demonstrated in this chapter that there are two waves of FasL cell surface expression in CTL clones and *in vivo* primed $CD8^+$ T cells, at the population level, in response to cognate target cell binding; a rapid mobilization of internal stores of FasL and a delayed *de novo* synthesis of FasL. There are conflicting studies in the literature describing the control of FasL expression on the CTL cell surface. Anti-TCR-induced FasL upregulation was shown to require protein synthesis and Ca^{2+} (Vignaux et al. 1995; Glass et al. 1996). In contrast, upregulation of surface FasL expression on *ex vivo* PEL CTL did not require protein synthesis but was derived from pre-existing pools of FasL (Li et al. 1998). My studies reconcile these apparently disparate data since I showed that cell surface FasL is both expressed from existing pools and newly synthesized after TCR engagement (Figure 3-1), similar to what has been reported after phorbol ester and calcium ionophore treatment of human CTL (Lettau et al. 2004). Cell surface expression of stored FasL or *de novo* synthesized FasL depends on the presence of extracellular Ca^{2+}, time of analysis after TCR stimulation, and potentially the strength of the TCR generated signal.

In this chapter, I showed that CTL clone 11 (Cl11), derived from allo-stimulation of *in vivo* primed PEL, contains pre-existing FasL that does not undergo cell surface transport upon cognate target cell stimulation (Figure 3-9). However, anti-TCR crosslinking or PMA plus ionomycin induced rapid preformed FasL cell surface transport in Cl11, suggesting that these cells are capable of moving their stores to the cell surface. These data suggest that there are complexities regarding CTL activation leading to relocalization of the internal

stores that are not currently understood. It is possible that the FasL is transported to the cell surface but is rapidly cleaved by MMP or internalized thereby limiting detection. Yet another scenario might be that there is an inhibitory receptor existing in Cl11 that is concomitantly engaged by certain target cells to block preformed FasL cell surface transport. It is also conceivable that L1210/K^b or EL4 do not provide a proper level of TCR stimulation to elicit preformed FasL cell surface transport in Cl11, given that TCR crosslinking could elicit early FasL cell surface translocation in Cl11.

Consistent with this hypothesis, I observed that TCR crosslinking can only induce preformed FasL cell surface expression, whereas plate bound anti-TCR can only induce *de novo* FasL expression, revealing that FasL cell surface expression might be finely tuned in CTL, perhaps by differential TCR signaling strength or kinetics. Crosslinked anti-TCR stimulates a shorter duration of signaling and a generally less robust signal compared to plate-bound anti-TCR (Berg et al. 1998). I examined cells stimulated with plate-bound anti-CD3 at early time points, as early as 5 minutes after stimulation, and at no time do I detect FasL translocation from internal stores (Figure 3-3C). I therefore think it is likely that signaling thresholds are key in determining if FasL is translocated or synthesized. This low level signaling requirement for translocation of stored FasL may also explain why some CTL are unable to mediate Ca^{2+}-independent killing of Fas expressing cells (e.g. Cl11 in Figure 3-9). Even though all CTL clones and *in vivo*-derived CTL that I have examined store FasL, not all are able to mobilize the FasL to the cell surface in response to cognate antigen on target cells. It is

possible that some of these CTL, such as Cl11, signal very strongly in response to target cells such that only degranulation and *de novo* FasL expression is triggered. Consistent with this Cl11 are triggered to degranulate with very low concentrations of purified class I MHC alloantigen compared to a number of other CTL clones (Kane et al. 1989). Interestingly, previous reports also indicated that activation of FasL-dependent cytotoxicity required weaker TCR signaling than the induction of degranulation or cytokine production (Cao et al. 1995; Kessler et al. 1998). These results are consistent with my hypothesis that a low level of signal preferentially induces surface translocation of stored FasL compared to degranulation and *de novo* FasL expression, which will be investigated in chapter 4.

In summary, I demonstrated that there are two waves of FasL expression that are differentially controlled in CTL. I further showed that CTL degranulation and FasL lytic mechanisms are fully independent with respect to stored component localization and regulation and suggest that CTL contain distinct storage vesicles that are independently regulated downstream of the T cell receptor. Further studies will need to be done to determine why CTL express two distinct waves of FasL expression after TCR stimulation and to uncover the nature of the FasL storage compartment.

CHAPTER 4 : Stored FasL Translocation Has a Lower Threshold of Activation Than *de novo* FasL Synthesis and Degranulation

A. Introduction

FasL is expressed on CTL and functions by engaging the death receptor Fas on target cells and triggering apoptosis. Fas is constitutively expressed on the surface of many cells, with cells of liver, heart, lung, kidney and ovary expressing the highest levels of Fas (Watanabe-Fukunaga et al. 1992; Leithauser et al. 1993). Since Fas is so ubiquitously expressed, the expression of FasL on CTL must be tightly regulated. I have demonstrated that CTLs undergo two waves of FasL cell surface expression after TCR engagement (He and Ostergaard 2007). The first wave is from a pre-existing pool of FasL, and the second wave requires new protein synthesis. However, the biological significance of the two waves of FasL expression remains unknown.

Fas and FasL are known to play important regulatory roles in the immune system (Nagata and Golstein 1995; Askenasy et al. 2005). Initial studies suggested that FasL was largely dispensable for viral clearance in the relatively few systems that were examined (Russell and Ley 2002). However more recent studies suggest that FasL may be important for clearing persistent infections (Rode et al. 2004; Zelinskyy et al. 2004) and may contribute, along with the perforin pathway, to the shaping of the diversity of escape variants of influenza (Price et al. 2005). Although FasL is not required for clearance of viruses that induce hepatitis in mice, it appears to contribute to viral pathogenesis due to

significant bystander killing of hepatic cells (Ando et al. 1997; Balkow et al. 2001; Gremion et al. 2004). Thus, FasL may contribute to virus clearance or pathogenesis, particularly in chronic infections.

Previous studies have suggested that FasL-mediated target cell killing has a lower signaling threshold for activation compared to degranulation, though the source of FasL (stored or *de novo*) was not specifically examined. For instance, a self-derived peptide was shown to selectively activate the FasL pathway (Cao et al. 1995) and a low threshold signal preferentially allows for FasL mediated killing (Kessler et al. 1998). I have found that signaling for FasL expression appears to be finely tuned, as weak TCR signal in the form of crosslinked anti-CD3 elicited stored FasL translocation without subsequent FasL synthesis; however if a strong stimulus is provided as plate bound anti-CD3, *de novo*-synthesized FasL is expressed with little or no stored FasL cell surface expression (He and Ostergaard 2007).

In this chapter, I quantitatively compared the signaling strength required for stored FasL translocation, *de novo* FasL cell surface expression and degranulation by CTL. I revealed that stored FasL translocation has a lower threshold of activation than *de novo* FasL synthesis and degranulation. Also, my data indicate that different from *de novo* FasL synthesis and degranulation, release of intracellular stores of Ca^{2+} is sufficient for FasL translocation from stored pools, further confirming the lower threshold of signal required for FasL translocation relative to degranulation and *de novo* FasL synthesis. Furthermore, I provide evidence to suggest that the stored, translocated FasL mediates specific,

directional CTL-mediated killing, whereas the *de novo* synthesized FasL, which is distributed around the cell surface, induced significant bystander killing. These data imply that FasL from these two sources may perform distinct roles in CTL mediated responses.

B. Results

Stored FasL cell surface translocation has a lower threshold of activation than does de novo FasL expression and degranulation

When the allo-specific CTL clone AB.1 (H-2^d anti-H-2^b) was stimulated with L1210 lymphoma target cells sorted for expression of different levels of H-$2K^b$ class I MHC (L1210/K^b), I found that target cells expressing a lower level of H-$2K^b$ induced less *de novo* FasL cell surface expression measured at 2 hrs, at which time only *de novo*, but not stored FasL, is expressed on the cell surface (He and Ostergaard 2007) and target cells expressing a higher level of H-$2K^b$ induced more *de novo* FasL cell surface expression (Figure 4-1A). However both high and low H-$2K^b$ expressing target cells induced a similar level of FasL translocation from the stored pool at 15 min (Figure 4-1A). The expression at 15 min is entirely from a presynthesized pool of FasL, since these cells were pretreated and assayed in the presence of the protein synthesis inhibitor CHX (He and Ostergaard 2007). These data suggested that FasL translocation has a lower signaling threshold than *de novo* synthesized FasL.

Given that it is difficult to control the antigen levels on the transfected target cell since the cell lines are not stable with respect to levels of class I MHC

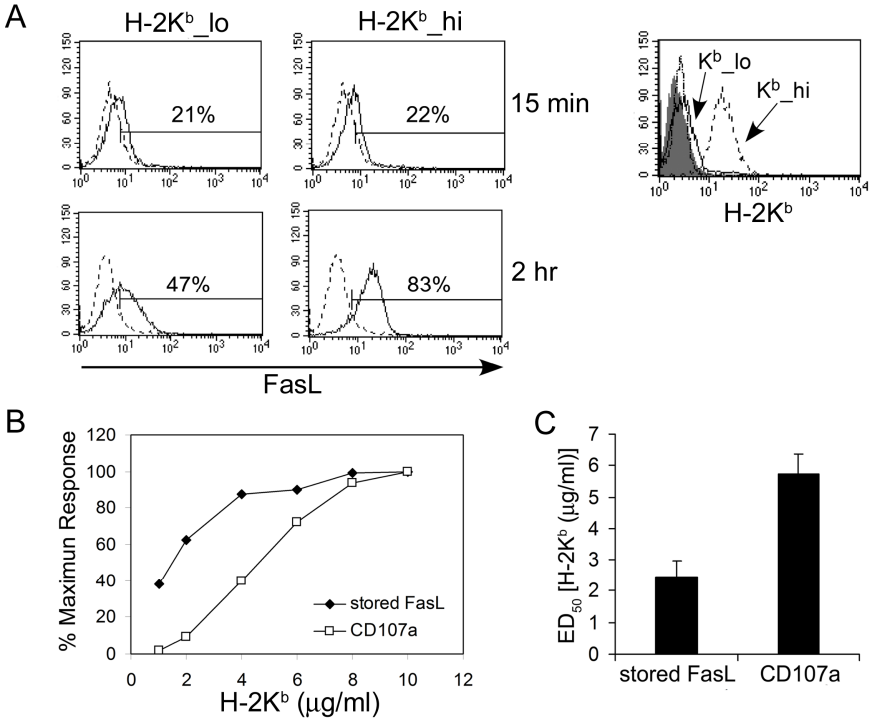


Figure 4-1. Stored FasL translocation is preferentially triggered at lower concentration of antigenic H-2K^b over *de novo* FasL cell surface expression and degranulation. *A*, allo-specific CTL clone AB.1 cells were stimulated with cognate target cells L1210/K^b expressing different levels of H-2K^b indicated as K^b-lo (solid line) and K^b-hi (dashed line), as measured by flow cytometry (top right panel). Also shown is the isotype control (shaded) and the untransfected L1210 (dotted/dashed line). After AB.1 were stimulated with the indicated H-2K^b transfected L1210 (solid line) or L1210 negative control (dashed line), FasL cell surface expression from the stored pool (15 min) and *de novo* synthesized pool (2 hr) was examined by flow cytometry. For the stored pool, cells were treated with CHX. Indicated values are FasL positive AB.1 stimulated with L1210/K^b subtracted by that of AB.1 mixed with the L1210 negative control. Data are representative of three independent experiments. *B*, CHX pretreated allo-specific CTL clone AB.1 was stimulated with latex beads coated with various concentrations of purified class I MHC or BSA as a control and a fixed concentration of ICAM-1. After 30 min, the bead/cell conjugates were separated with 5 mM EDTA and the percentage of surface FasL or CD107a positive AB.1 was determined by flow cytometry. The results were shown as the percentage of the maximum response obtained for stored FasL tranlocation or degranulation at plateau stimulation for 30 min. Data are representative of four independent experiments. *C*, Compiled data of class I MHC titrations from four independent experiments. The average H-2K^b concentrations for a 50% response (ED_{50}) to trigger stored FasL translocation and CD107a cell surface expression are shown. Error bars indicate SEM. Statistical significance was assessed by one-way ANOVA ($p<0.01$).

[H-2K^b] (μg/ml)	stored FasL 15min	CD107a 15min	stored FasL 30min	CD107a 30min
1	3.9	0.5	7.1	0.5
2	9.5	2.1	11.7	2.3
4	15.7	10.1	16.4	10.3
6	20.8	15.9	16.8	18.5
8	18.6	21.0	18.5	24.1
10	17.3	22.6	18.7	25.7

Table 4-1. Stored FasL translocation is preferentially triggered at lower concentration of antigenic H-2K^b over degranulation. CTL clone AB.1 was treated and stimulated as described in Fig. 1. The percentages of AB.1 with stored FasL cell surface translocation (15 min & 30 min), and degranulation (15 min & 30 min) upon conjugate formation with latex beads coated with different concentrations of H-2K^b are shown. Indicated values are the percentages of cell surface FasL-positive or CD107a positive AB.1 upon H-2K^b-coated latex beads engagement subtracted by that of BSA coated latex beads engagement. Data are representative of four independent experiments.

expression, I next decided to quantitatively compare the stored FasL translocation and degranulation under different stimulation conditions by titrating antigen concentration. To directly measure the effect of class I MHC density on stored FasL translocation, AB.1 was stimulated with latex beads coated with different concentrations of H-2K^b and a level of ICAM-1 which is similar to that expressed on the EL4 target cell as assessed by flow cytometry of the beads (data not shown). I found that AB.1 was capable of translocating stored FasL at low H-2K^b concentration (2 μg/ml), a condition where degranulation was barely detectable (Figure 4-1B & Table 4-1). Furthermore, stored FasL translocation approached its maximum at a lower H-2K^b concentration than that required for degranulation (Figure 4-1B & Table 4-1), indicating stored FasL translocation has a lower signaling threshold of activation than degranulation by AB.1. This experiment was repeated four times and the average concentration of class I MHC for a 50% response to trigger stored FasL translocation was 2.5±0.5 μg/ml while that required for degranulation was 5.7±0.6 μg/ml (Figure 4-1C). This difference was significant ($p<0.01$) (Figure 4-1C) and shows that the signaling threshold for FasL translocation is lower than that required for degranulation for an allo-specific CTL clone.

I next examined peptide-specific CTL clones which allowed a better comparison of the effects of TCR signaling strength on the induction of individual cytolytic functions by CTL through simple peptide titrations. To this aim, CTL clone 3/4, which is specific for the H-2D^b restricted nucleoprotein $NP_{366–374}$ epitope (ASNENMETM) derived from the influenza virus A/PR/8/34 was

employed. I found that clone 3/4 was capable of translocating stored FasL at low $NP_{366–374}$ concentration (30-60 ng/ml) where degranulation was barely detectable (Figure 4-2A & Table 4-2). Furthermore, stored FasL translocation approached its maximum at a lower $NP_{366–374}$ concentration than that required for degranulation (Figure 4-2A & Table 4-2), indicating stored FasL translocation has a lower signaling threshold of activation than degranulation by clone 3/4. Also, I observed that at low concentration of $NP_{366–374}$ (125 ng/ml), stored FasL translocation reached 80% of the maximum whereas *de novo* FasL only reached 20% of the maximum response (Figure 4-2B & Table 4-3), indicating stored FasL translocation to the cell surface has a lower signaling threshold of activation than *de novo* FasL synthesis by clone 3/4. The differences between the amount of peptide required for a 50% response is significantly different between stored FasL translocation compared with degranulation and *de novo* FasL synthesis (Figure 4-2C). These data demonstrate that stored FasL has a lower threshold for induction of translocation than for degranulation or FasL synthesis in a peptide specific CTL clone.

Both AB.1 and clone 3/4 are *in vitro* cultured untransformed CTL clones. I next wanted to examine whether the observations with these cultured CTLs can be repeated with the freshly generated CTL. To this aim, OT-I CTL, which is specific for H-$2K^b$ restricted SIINFEKL peptide (OVAp), were generated by co-culture of purified splenic$CD8^+$ T cells derived from OT-I (RAG-/-) transgenic mice with LPS-matured bone marrow-derived dendritic cells (BMDCs). By day 11 post coculture, FasL disappeared from the cell surface and was stored

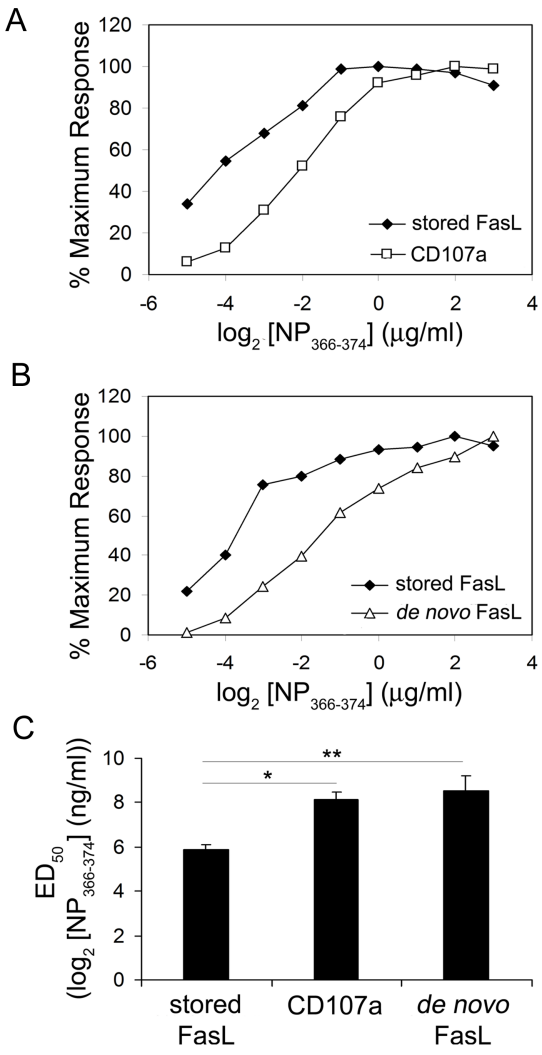


Figure 4-2. FasL translocation is preferentially triggered at a lower influenza antigen concentration over degranulation and *de novo* FasL synthesis. CHX pretreated CTL clone 3/4 was stimulated with EL4 target pulsed with various concentrations of $NP_{366-374}$ peptide or unpulsed EL4. After 15 min, the conjugates were separated with EDTA and the percentage of surface FasL or CD107a positive CTL was determined by flow cytometry (*A*). Meanwhile, CHX-untreated CTL was stimulated with peptide-pulsed target cells for 2 hr to examine *de novo* FasL cell surface expression and compared with stored FasL translocation at 15 min (*B*). The results were shown as the relative percentage of the maximum response obtained for each response at plateau upon target cell stimulation. Data are representative of at least four independent experiments. *C*, Compiled data of the peptide titrations from independent experiments. The average concentrations of $NP_{366-374}$ for a 50% response (ED_{50}) to trigger stored FasL translocation (n=6), CD107a cell surface expression (n=6), and *de novo* FasL cell surface expression (n=4) are shown. Error bars indicate SEM. Statistical significance was assessed by one-way ANOVA (* $p<0.0001$, ** $p=0.001$).

$\log_2[NP_{366-374}]$ (μg/ml)	stored FasL		CD107a	
	15min	30min	15min	30min
-5	12.0	18.8	2.2	2.9
-4	19.3	18.7	4.4	6.0
-3	24.0	26.0	10.7	12.2
-2	28.7	22.8	18.7	18.6
-1	35.0	27.7	26.2	26.4
0	35.3	24.2	31.7	31.6
1	35.0	27.6	33.0	34.4
2	34.2	25.1	34.4	34.6
3	32.1	24.2	34.1	35.3

Table 4-2. FasL translocation is preferentially triggered at a lower influenza antigen concentration over degranulation. CTL clone 3/4 was treated and stimulated as described in Fig. 2. The percentages of clone 3/4 with stored FasL cell surface translocation (15 min & 30 min), and degranulation (15 min & 30 min), upon conjugate with EL4 target pulsed with different concentrations of $NP_{366-374}$ peptides are shown. Indicated values are the percentages of cell surface FasL-positive or CD107a positive clone 3/4 upon $NP_{366-374}$ peptide pulsed EL4 engagement subtracted by that of unpulsed EL4 engagement. Data are representative of six independent experiments.

$\log_2[NP_{366-374}]$ (μg/ml)	stored FasL	*de novo* FasL
-5	7.1	0.9
-4	13.0	4.8
-3	24.5	14.2
-2	25.5	22.8
-1	28.6	35.6
0	30.3	42.7
1	30.7	48.8
2	32.4	51.8
3	30.9	57.9

Table 4-3. FasL translocation is preferentially triggered at a lower influenza antigen concentration over *de novo* FasL cell surface expression. CTL clone 3/4 was treated and stimulated as described in Fig. 2. The percentage of clone 3/4 with stored FasL cell surface translocation (15 min), and *de novo* FasL cell surface expression (2 hr), upon conjugate with EL4 target pulsed with different concentrations of $NP_{366-374}$ peptides were shown. Indicated values are the percentage of cell surface FasL-positive clone 3/4 upon $NP_{366-374}$ peptide pulsed EL4 engagement subtracted by that of unpulsed EL4 engagement. Data are representative of four independent experiments.

intracellularly (Figure 4-3A), similar to the *in vivo* primed allo-specific splenic $CD8^+$ T cells (Appendix Figure1). I also detected a significant amount of granzyme B stored in these cells (Figure 4-3A), indicating both effector mechanisms are developed in the freshly generated OT-I CTL. Similar to the results from AB.1 and clone 3/4, I found in OT-I CTL, stored FasL was preferentially translocated over degranulation (Figure 4-3B & Table 4-4) and *de novo* FasL synthesis (Figure 4-3C & Table 4-4) at low SIINFEKL concentration (0.1 nM). The differences between the amount of peptide required for a 50% response is significantly different between stored FasL translocation compared with degranulation and *de novo* FasL synthesis (Figure 4-3D), implying it is an inherent property of CTL that stored FasL has a lower threshold for induction of translocation than for degranulation or FasL synthesis.

SIINFEKL (OVAp) is a high affinity agonist for OT-I TCR, whereas SIIGFEKL (G4) is a low affinity agonist for OT-I TCR (Alam et al. 1999; Rosette et al. 2001), which provided an additional method to determine the effect of decreasing TCR signaling strength on the induction of individual effector mechanisms. I found that 1 μM G4 elicits stored FasL translocation to about 75% of the level that is induced by 1 μM OVAp (Figure 4-3E). *De novo* FasL cell surface expression was also mediated by G4, with less sensitivity than translocation from intracellular stores (Figure 4-3E). Cells exhibited only minimal degranulation in response to G4 (Figure 4-3E). Overall, these results indicate that stored FasL is preferentially translocated over *de novo* FasL cell surface expression and degranulation at low antigen level, further confirming that

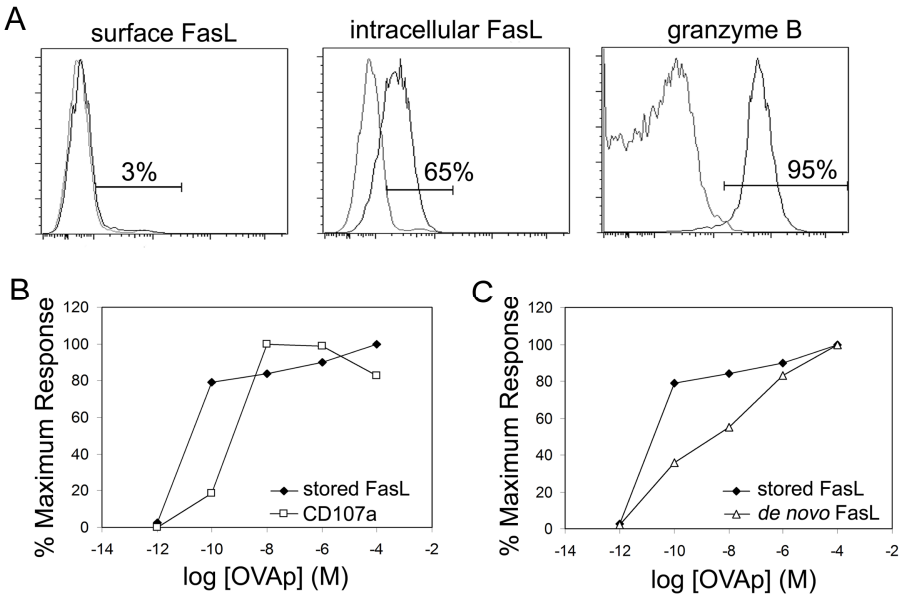
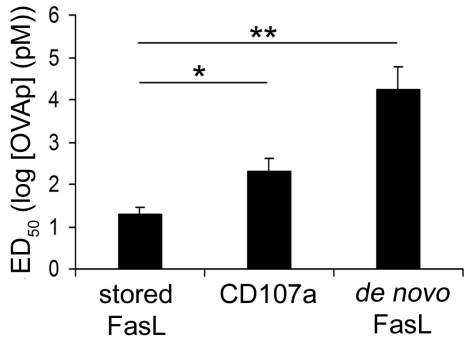
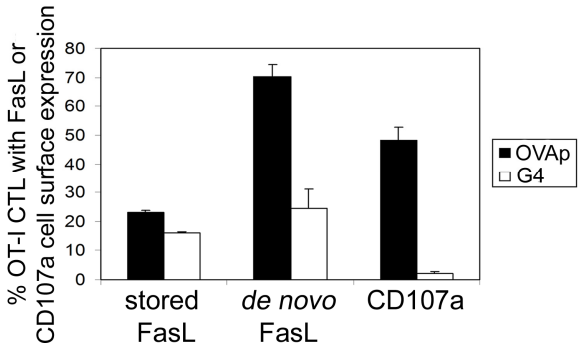


Figure 4-3. FasL translocation is preferentially triggered at a lower OVA peptide concentration than degranulation and *de novo* FasL synthesis. *A*, Purified $CD8^+$ OT-I T cells derived from naïve OT-I (RAG1-/-) Tg mice were cocultured with mature BMDCs pulsed with OVAp (SIINFEKL). The cell surface and intracellular FasL expression and intracellular granzyme B expression on OT-I $CD8^+$ T cells on day 11 post stimulation were shown (black line). Indicated values are the percentages of FasL or granzyme B positive OT-I CTL. Isotype control staining was shown as background (grey line). *B*, OT-I CTL were pretreated with CHX, then stimulated with EL4 target pulsed with various concentrations of OVAp SIINFEKL or unpulsed EL4 as negative control. After 30 min, the conjugates were separated with EDTA and the percentage of surface FasL or CD107a positive CTL was determined by flow cytometry. Meanwhile, *de novo* FasL cell surface expression were compared with stored FasL translocation (*C*). The results are shown as the relative percentages of the maximum response obtained for each response at plateau upon target cell stimulation. (Note: data presented in *B* and *C* are from the same experiment.) Data presented in *A*-*C* are representative of three independent experiments. *D*, Compiled data of the peptide titrations from three independent experiments. The average concentrations of OVAp for a 50% response (ED_{50}) to trigger stored FasL translocation, CD107a cell surface expression, and *de novo* FasL cell surface expression are shown. Error bars indicate SEM. Statistical significance was assessed by one-way ANOVA (* $p<0.05$, ** $p<0.01$). *E*, OT-I CTL was stimulated with EL4 target cells pulsed with 1 μM agonist peptide SIINFEKL (OVAp) or weak agonist peptide SIIGFEKL (G4). Stored FasL translocation at 30 min, *de novo* FasL cell surface expression at 2 hr and degranulation at 30 min were examined by flow cytometry. The percentages of OT-I CTL with cell surface FasL or CD107a expression are shown. Averages of three independent experiments are shown; error bars represent SEM.

D


ED50 (log [OVAp] (pM))
0
1
2
3
4
5
6
*
**
stored FasL
CD107a
de novo FasL

E


% OT-I CTL with FasL or CD107a cell surface expression
0
10
20
30
40
50
60
70
80
OVAp
G4
stored FasL
de novo FasL
CD107a

log[OVAp] (M)	stored FasL	CD107a	stored FasL	CD107a	*de novo* FasL
	15min		30min		2hr
-12	3.4	0	0.7	0	1.7
-10	12.6	4.6	23.4	10.0	28.1
-8	22.4	37.9	22.0	54.5	42.8
-6	22.5	43.6	25.1	53.9	65.0
-4	22.7	36.5	27.9	45.5	77.9

Table 4-4. FasL translocation is preferentially triggered at a lower OVA peptide concentration over degranulation and *de novo* FasL synthesis. OT-I CTL was treated and stimulated as described in Fig. 3. The absolute value of the percentage of OT-I CTL with stored FasL cell surface translocation (15 min & 30 min), *de novo* FasL cell surface expression (2 hr), and degranulation (15 min & 30 min), upon conjugate with EL4 target pulsed with different concentrations of SIINFEKL peptides were shown. Indicated values are the percentages of cell surface FasL-positive OT-I CTL upon OVAp pulsed EL4 engagement subtracted by that of unpulsed EL4 engagement. Data are representative of three independent experiments.

FasL translocation has a lower threshold for triggering than *de novo* FasL cell surface expression or degranulation.

A limited intracellular calcium signal is required for stored FasL translocation

Degranulation depends on both intracellular and extracellular Ca^{2+} (Takayama and Sitkovsky 1987; Esser et al. 1998). In chapter 3, I showed that *de novo* FasL cell surface expression requires extracellular Ca^{2+}, whereas stored FasL cell surface translocation is extracellular Ca^{2+}-independent (He and Ostergaard 2007). In this chapter, I sought to further address if Ca^{2+} flux from intracellular stores is necessary for these processes. To this aim, the membrane permeant intracellular Ca^{2+} chelator BAPTA-AM was employed. Once loaded into the cells, BAPTA-AM is hydrolyzed by cytosolic esterases and is trapped intracellularly as the active chelator BAPTA. AB.1 cells were prereated with BAPTA-AM, then excess BAPTA-AM in the medium was washed out after the preincubation and its effects on the stored FasL cell surface translocation and *de novo* FasL cell surface expression were examined upon target cell engagement. I found that, as with *de novo* FasL cell surface expression and degranulation, BAPTA-AM does inhibit stored FasL cell surface expression (Figure 4-4), suggesting that Ca^{2+} flux from intracellular stores is necessary and sufficient for cell surface translocation the stored pool of FasL.

FasL vesicles do not strongly polarize to the target cell and FasL does not appear to be enriched at the immunological synapse

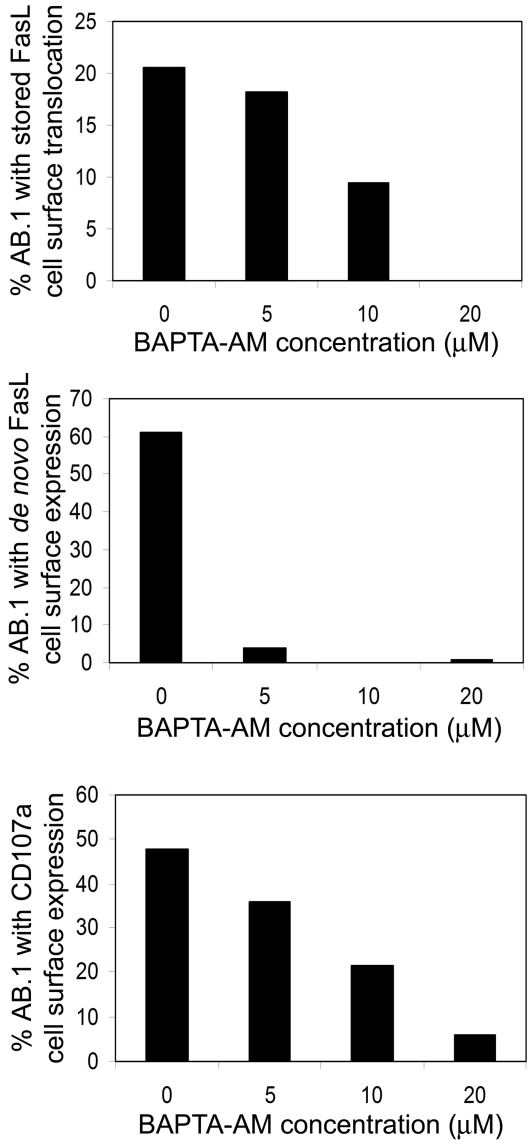


Figure 4-4. A limited intracellular calcium signal is required for FasL translocation. AB.1 were pretreated with carrier control or the indicated concentrations of BAPTA-AM, then stimulated with cognate target cell L1210/K^b or negative control L1210 for either 15 min or 2 hr. Cells were stained with CD8a (PE.Cy5), FasL (PE) and CD107a (FITC). After gating on the $CD8^+$ cells to examine only the CTL, the expression of FasL and CD107a was assessed. The percentage of AB.1 positive for stored FasL, *de novo* FasL or CD107a expression upon L1210/K^b stimulation was determined at different drug concentrations with value subtracted by that of L1210 engagement alone, which was never more than 10% for FasL and 0% for CD107a. Representative data are shown from two independent experiments where excess BAPTA-AM washed out after the preincubation. Three independent experiments with BAPTA-AM present during stimulation were also conducted, with similar results.

Since stored and *de novo* FasL appear to have different thresholds for activation, this might imply that FasL from these two pools may perform distinct roles in CTL mediated responses. I speculated that stored FasL would translocate to the contact point between the CTL and its target cell to mediate specific target cell killing, while *de novo* FasL would be uniformly expressed over the entire cell surface and be less specific in target cell killing. I therefore decided to examine FasL localization after target cell engagement by confocal microscopy. CTL clone AB.1 cells were pretreated with CHX to block *de novo* FasL synthesis, to allow for the exclusive examination of stored FasL translocation upon target cell EL4 engagement. I found that in some circumstances FasL vesicles and secretory lysosomes polarized towards the CTL-target cell contact area, but they were not overlapping (Figure 4-5A). I could also faintly detect FasL localization at the CTL-target cell synapse area in some CTL (Figure 4-5A). However, the position of the FasL vesicles varied among individual CTL cells upon target cell engagement. Some were unpolarized (Figure 4-5B) and some were polarized towards the contact site but did not accumulate at the target cell contact zone (Figure 4-5C). Only in around 10% of CTL, I found a portion of FasL vesicles close to the CTL membrane at the immunological synapse (Figure 4-5A). Therefore, in the majority of cases, I could not detect FasL localization at the immunological synapse, indicating overall FasL vesicle movement towards the target cell is unlikely. The studies shown were performed on permeabilized cells. I also tried to stain for cell surface FasL 15 min after target cell binding, but was unable to detect any surface FasL staining (data not shown). This could be

because the level of surface FasL is below the level of detection. This may not be surprising given that I also could not detect CD107a localization at the synapse area either in permeabilized cells (Figure 4-5A), as has been shown in T cells stimulated with antigen coated planar lipid bilayers (Beal et al. 2008), or unpermeabilized cells (data not shown). It should also be noted that the anti-FasL antibody is a very low affinity Ab, so it is difficult to detect low levels of protein over background.

I speculated that *de novo* FasL cell surface expression might follow the constitutive pathway of protein transport, thereby being distributed around CTL cell surface. Two hours after target cell engagement, FasL cell surface expression on non-permeabilized CTL was examined. I did observe FasL around the CTL cell surface with no localization towards the target cell, however this staining was not uniformly distributed as predicted, instead it occurred in clusters over the entire surface (Figure 4-5D). I also performed intracellular FasL staining, however, because of the much higher levels of expression inside the cell, I was unable to discriminate between surface and intracellular expression (Figure 4-5E). However, I was able to demonstrate that *de novo* synthesized FasL is not stored in cytolytic granules (Figure 4-5E). Taken together, based on my imaging data, I was unable to make any conclusions regarding the localization of stored FasL translocated to the cell surface after target cell engagement, but did demonstrate that stored FasL does not polarize with the cytolytic granules. The *de novo* FasL appears to translocate to the CTL cell surface without directionality after target cell engagement.

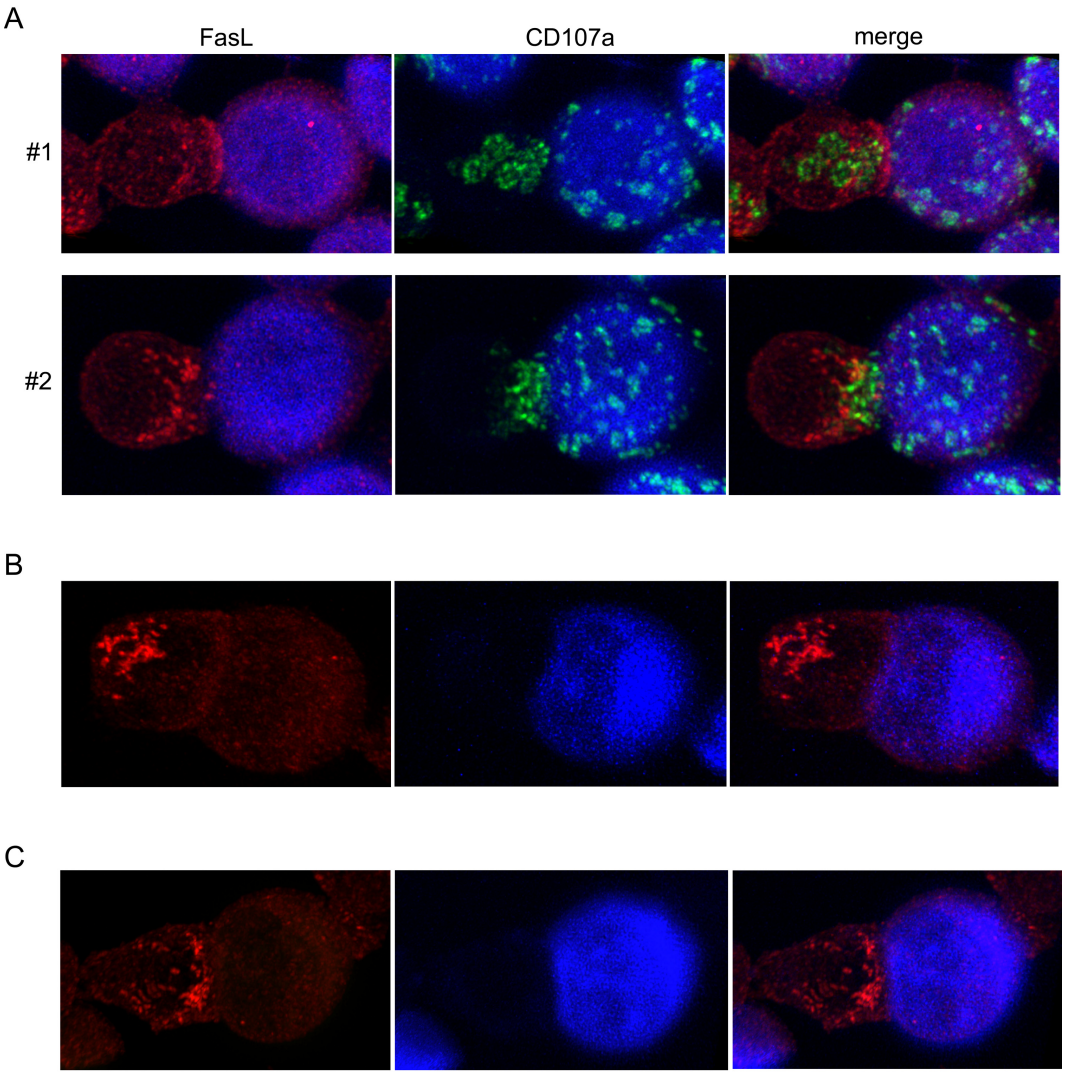
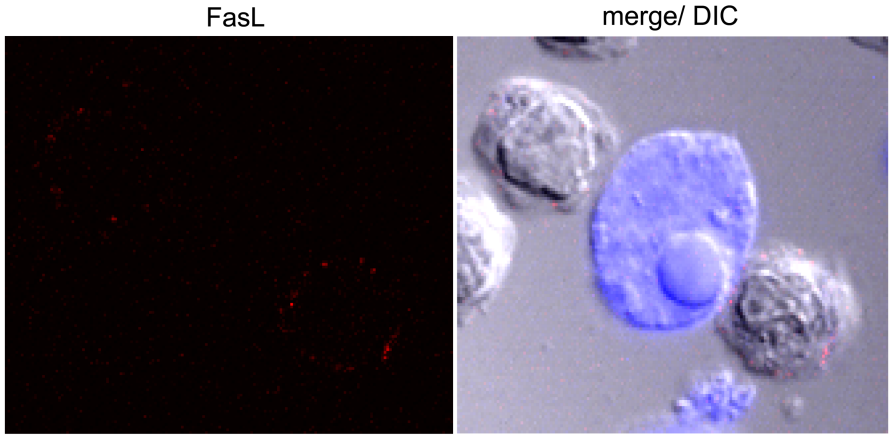
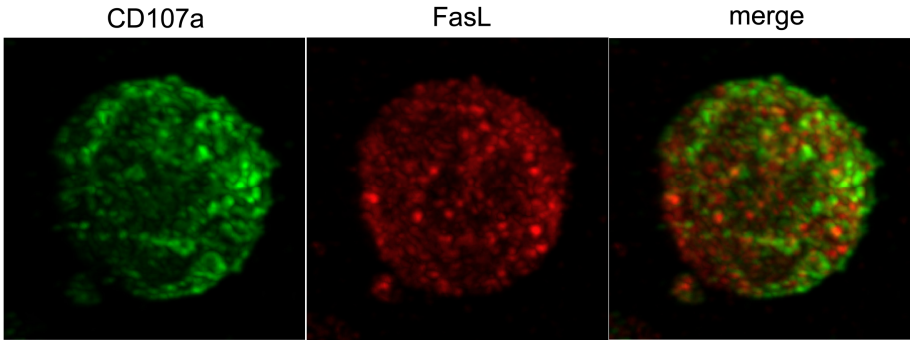


Figure 4-5. Localization of FasL after 15 min and 2 hr stimulation with target cells. EL4 target cells were stained with CellTracker Blue and conjugated with CHX pretreated CTL AB.1, then applied to poly-L-lysine coverslip and incubated at 37°C for 15 min. After fixation and permeablization with methanol, FasL and CD107a localization (*A*), or FasL localization (*B*, *C*) were examined by confocal microscopy. Two representative images are shown in *A*. *D*, AB.1 were stimulated with CellTracker Blue stained EL4 target cells for 2 hr, then either stained to examine cell surface FasL expression, or fixed, permeabilized and then stained to examine overall FasL along with CD107a (*E*) by confocal microscopy. For intracellular staining, Z-stack images were acquired (interval, 0.2 μm) and subjected to deconvolution and three-dimensional reconstruction. Representative projections of the reconstructed three-dimensional images are shown. Data are representative of at least three independent experiments.

D



E



Translocated FasL expression has a fast turnover rate

Since I was unable to gain significant insight into the localization of surface FasL, I sought to characterize FasL expression by examing the kinetics of cell surface FasL expression. I found that kinetics of the stored FasL cell surface translocation depends on TCR signaling strength. Target cells expressing higher levels of class I MHC induced faster stored FasL translocation in CTL AB.1, which peaked at 15min then underwent a rapid turnover, whereas target cells expressing lower levels of class I MHC elicited slower FasL kinetics (Figure 4-6A & 6B). Nonetheless, FasL disappeared from the cell surface at one hour after target cell conjugation (Fig 4-6B).

It is clear that FasL surface expression occurs quickly after target cell conjugation. However, I found there is still a significant population of CTL in conjugation with the target cell one hour after engagement at the population level (Fig 4-6C). Given that CTL-target cell conjugation is such a dynamic process in which CTL may continue to search and conjugate with new target cells after dissociation from a conjugate, it is difficult to compare the kinetics of decreased FasL cell surface expression with that of CTL-target cell conjugate dissociation at the population level. Our lab previously observed in AB.1 that tyrosine phosphorylation upon L1210/K^b stimulation peaked at 5 min and remained strong for the 45 min of assay, and the continued tyrosine phosphorylation required an intact cytoskeleton (Shen et al. 2005). Cytoskeleton is necessary for the formation and maintenance of CTL-target cell conjugate. Disruption of cytoskeleton by adding cytochalasine E 30min after target cell conjugation still significantly inhibited

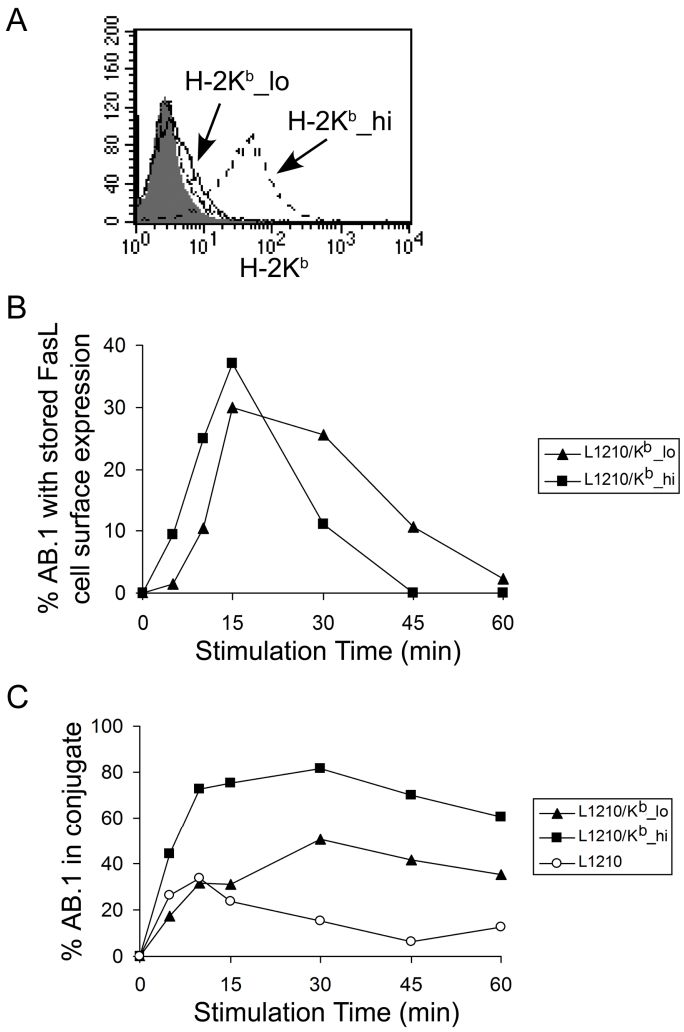


Figure 4-6. Translocated FasL expression has a fast turnover rate. *A*, Flow cytometry shows different H-2K^b level on L1210/K^b target cells. Background is isotype control staining. *B*, Stored FasL cell surface expression kinetics upon target cell engagement. CHX pretreated AB.1 was stimulated with cognate target cell L1210/K^b or negative control L1210 for indicated time. The percentage of cell surface FasL positive AB.1 upon L1210/K^b engagement was assessed, with the value subtracted by that of L1210 engagement. *C*, Cell tracker orange stained, CHX pretreated AB.1 were stimulated with unstained L1210 or L1210/K^b as in *B*. After indicated time of stimulation, cells were vortexed to eliminate nonspecific binding and fixed. The percentage of AB.1 in conjugates was assessed by focusing on red fluorescence-positive cells and analyzing forward scatter. Data presented in *B* and *C* were conducted in parallel from the same experiment. Data are representative of at least three independent experiments.

some protein tyrosine phosphorylation (Shen et al. 2005), indicating there might be still a significant population of CTL continuely conjugated with its target. Taken together, I found FasL cell surface expression from the stored pool has a fast turnover rate, and the natural kinetics of translocated FasL on the cell surface mirrors conjugation and might ensure the directional mobilized FasL is being presented exclusively to the specific target cells that initiate its translocation.

De novo synthesized FasL, but not stored translocated FasL, mediates bystander killing

Previous studies have shown that once FasL is expressed on the cell surface, it can mediate killing of any target that expresses cell surface Fas (Rouvier et al. 1993; Vignaux 1995; Glass et al. 1996), however the rapid turnover of the FasL from the internal stores makes it unlikely that this would mediate efficient bystander lysis. In chapter 3, I showed that FasL is both preformed and synthesized *de novo*, suggesting there are two phases of FasL expression that could contribute to the death of Fas-expressing target cells in a short-term (4 hour) killing assay, so the relative contribution of the stored or *de novo* FasL to various aspects of CTL-mediated lysis is unknown. I have also shown that the transient FasL translocation to the cell surface is sufficient to induce target cell killing (He and Ostergaard 2007). Since stored FasL only transiently remains on the cell surface during conjugation, I wanted to determine if translocated FasL from intracellular stores mediates cognate target cell killing or bystander killing of non-Ag-bearing cells.

To this aim, allo-specific clone AB.1 (anti-H-2^b) CTL were mixed with ^{51}Cr-labeled antigen bearing L1210/K^b or EL4 (H-2^b) target cells to determine cognate target cell killing. Also, AB.1 was mixed with ^{51}Cr-labeled Fas expressing L.Fas (H-2^d) bystanders together with unlabeled L1210/K^b or EL4 to examine bystander killing of innocent target cells. ^{51}Cr-labeled L1210 bystander (with very low Fas) was used as a negative control. I found that cognate target cells induced significant bystander killing of Fas^+ L.Fas but not Fas^- L1210 by AB.1 (Figure 4-7B & 4-7D), indicating the bystander killing was mediated by FasL in the current *in vitro* system. To further determine the source of FasL that contributed to the bystander killing, I employed extracellular Ca^{2+} chelator EGTA and protein synthesis inhibitor cycloheximide (CHX) to discriminate the different sources of FasL and degranulation. First, in the absence of extracellular Ca^{2+}, which is not required for stored FasL cell surface translocation but indispensable for degranulation and *de novo* FasL cell surface expression, stored FasL is sufficient to induce Fas^+ cognate target cell EL4 killing (Figure 4-7A) but not Fas^+ bystander target L.Fas lysis (Figure 4-7B). Second, when CHX was used to pretreat AB.1 and included during the 4-hour killing assay, translocated FasL from the stored pool and degranulation of preformed cytolytic granule components can mediate significant Fas^+ cognate target cell EL4 and Fas^{lo} cognate target cell L1210/K^b killing (Figure 4-7C) but not Fas^+ bystander target L.Fas lysis (Figure 4-7D). These results indicated that stored FasL translocation induces Ag-specific but not bystander killing, whereas *de novo* synthesised FasL induces significant bystander killing. These results also suggested

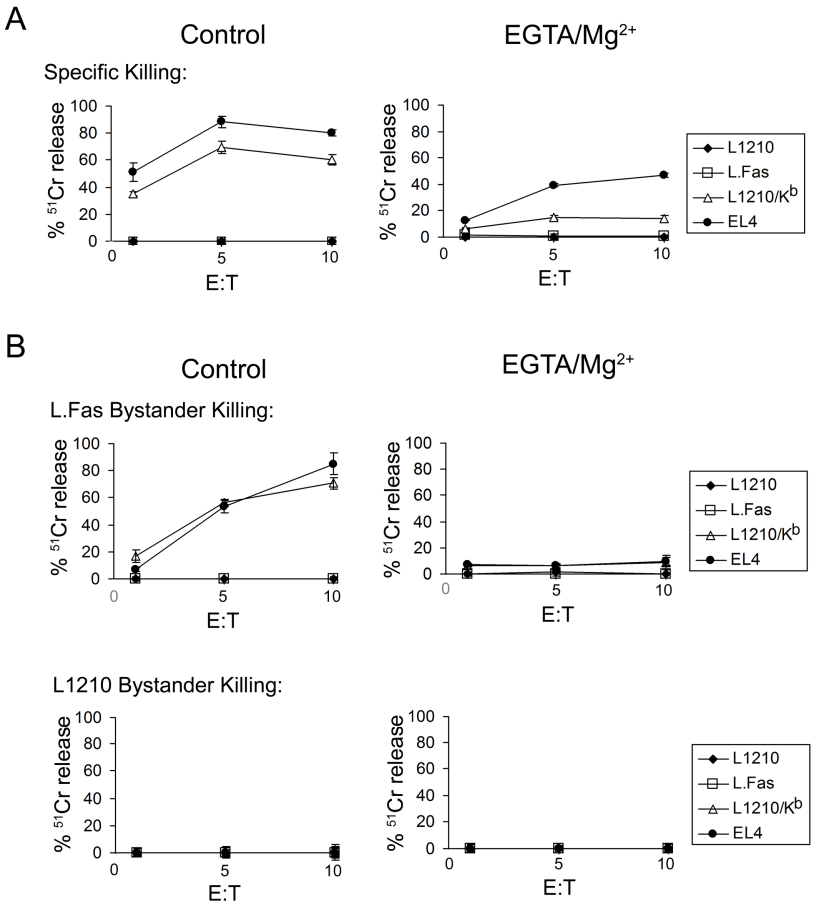
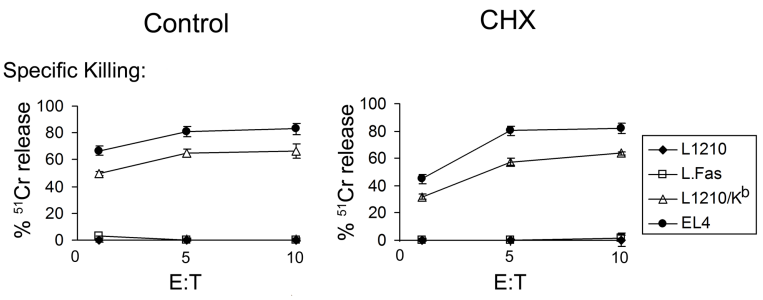
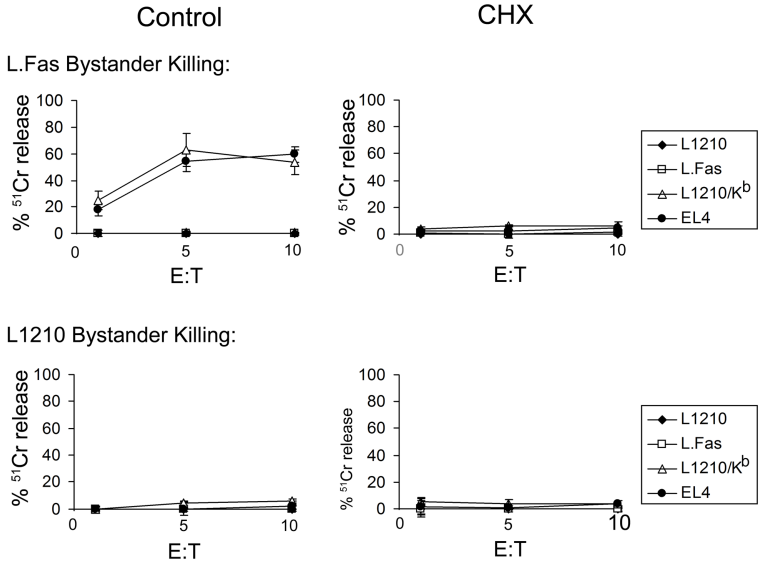


Figure 4-7. *De novo* synthesized FasL, but not stored translocated FasL, mediates bystander killing. CTL clone AB.1 was incubated in the presence or absence of 4 mM EGTA/ 3 mM Mg^{2+} (*A*) or left untreated or pretreated with 10 μg/ml cycloheximide (CHX) for 45 min (*C*), then stimulated with with ^{51}Cr-labelled congate target cell EL4 and L1210/K^b, or L1210 and L.Fas negative control for 4hr, then the specific target cell killing was assessed. Meanwhile, AB.1 was incubated in the presence or absence of 4 mM EGTA/ 3 mM Mg^{2+} (*B*) or left untreated or pretreated with 10 μg/ml cycloheximide (CHX) for 45 min (*D*) with ^{51}Cr-labelled non-Ag bearing L1210 and Fas-expressing L.Fas, along with unlabelled congate target cell EL4 and L1210/K^b, or L1210 and L.Fas negative control for 4hr, then the bystander killing by AB.1 through cytotoxicity induced by congate target cells was assessed. Data presented in *A* and *B* were conducted in parallel from the same experiment. Data presented in *C* and *D* were conducted in parallel from the same experiment. *E*, AB.1 was stimulated with immobilized anti-CD3 (IMM) for 2hr to elicit *de novo* FasL cell surface expression. After the induction phase, ^{51}Cr-labelled L1210 or L.Fas was introduced to AB.1 (E:T=10:1), and bystander killing was examined. Average of triplicated samples from a single experiment is shown; error bars represent SD. Data are representative of at least three independent experiments. (The data presented in this figure was generated with Dong-Er Gong.)

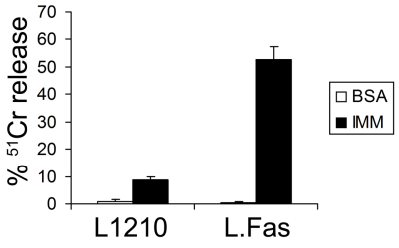
C


Control
CHX
Specific Killing:
% 51Cr release
E:T
L1210
L.Fas
L1210/Kb
EL4

D


Control
CHX
L.Fas Bystander Killing:
L1210 Bystander Killing:
% 51Cr release
E:T
L1210
L.Fas
L1210/Kb
EL4

E


% 51Cr release
BSA
IMM
L1210
L.Fas

that, in this system, degranulation does not contribute to bystander killing, for which conflicting data exists (Isaaz et al. 1995; Kuwano and Arai 1996).

I previously showed that CTL stimulation with plate-bound anti-CD3 elicits *de novo* FasL cell surface expression without detectable FasL translocation from intracellular stores (He and Ostergaard 2007). In this study, I extended that experiment by adding either Fas^+ or Fas^- non-Ag-bearing cells to CTL after *de novo* FasL induction with plate-bound anti-CD3, and observed significant bystander killing of Fas^+ but not Fas^- cells (Figure 4-7E) as has been previously shown (Vignaux et al. 1995). Taken together, these observations clearly demonstrate that translocated FasL from intracellular stores and degranulation mediate specific target cell killing, and, in contrast, significant bystander killing of innocent target cells is attributed to *de novo* synthesized FasL.

C. Discussion

In this study, I quantitatively determined if different thresholds of signal lead to stored FasL, *de novo* synthesized FasL expression and degranulation, and have shown that at a low level of signaling strength, stored FasL is preferentially translocated to the cell surface over *de novo* FasL synthesis and degranulation. I further observed that the stored, translocated FasL mediates specific, directional CTL-mediated killing, whereas the significant bystander killing that has been documented for FasL (Ando 1997; Smyth 1997; Smyth et al. 1998; Gremion et al. 2004) was attributed to the *de novo* synthesized FasL, which is expressed on the cell surface without directionality. This, combined with the observation of

differential signaling thresholds for individual killing mechanisms suggests that stored FasL might be employed under circumstances of low level infection and may be required to clear the final pathogens after infection to play a preferential role during low level chronic infections. In contrast, when there is a massive virus infection that has infected large numbers of cells, it would be advantageous for the CTL to kill all surrounding cells to quickly clear the virus. In this circumstance, a significant amount of bystander killing of innocent cells would occur, inducing inflammatory tissue damage because of the late, constitutively expressed FasL on the cell surface. Indeed, recent studies have suggested that FasL may be important for clearing chronic infections (Rode et al. 2004; Zelinskyy et al. 2004), and may contribute, along with the perforin pathway, to the shaping of the diversity of escape variants of influenza (Price et al. 2005). It would be of interest to investigate the contribution from particular FasL pools (stored versus *de novo* synthesised) for the antiviral immunity during these infections.

TCR signal strength plays a critical role in regulating various aspects of T cell biology, including linage fate decisions (Hayes et al 2005), differentiation (Rogers and Croft 1999; Lozza et al. 2008), cytokine production (Itoh and Germain 1997), cytotoxicity (Valitutti et al. 1996; Faroudi et al. 2003) and the induction of distinct tissue homing receptors (Svensson et al. 2008). At very low antigen level, subtle changes in antigen dose can be translated into significant biological effect. Purbhoo and colleagues showed that, CTLs are able to detect even a single peptide/MHC complex at the T cell-target interface but require as

few as three specific peptide/MHC complexes to induce cytotoxicity, whereas about ten complexes of peptide–major histocompatibility complex (pMHC) is required to achieve full calcium increase and to form a mature synapse (Purbhoo et al. 2004). It was shown that OT-1 CTLs induced target cell death accompanied by intense membrane blebbing, an early marker of apoptosis, 5-15 min upon conjugation (Purbhoo et al. 2004). Interestingly, consistent with my results, they found that blocking of Fas-mediated killing reduced the occurrence of membrane blebbing in target cells containing 3 to 30 peptide/MHC complexes at the CTL-target interface from 100% to 15%, implying that FasL induced killing may predominate at the lowest antigen density (3 peptide/MHC), whereas higher antigen loading may further elicit lytic granule exocytosis, thereby increasing the contribution of degranulation to cell death (Purbhoo et al. 2004). Cell membrane blebbing observed in that study was shortly after target cell engagement, and was presumably mediated by FasL from stored, but not the *de novo* pool. In the current study, I extend the observation by clearly showing that during low levels of antigen stimulation, stored FasL translocation preferentially occurs over degranulation by OT-I CTL and two other CTL clones, implying this is an inherent property of CTL. Interfering with antigen presentation is one strategy employed by some viruses and tumors (Restifo et al. 1993; Hengel and Koszinowski 1997; Ehrlich 1997; Matsui et al. 2002) to escape the host immune response. FasL from stored pools could contribute significantly to the anti-viral, anti-tumor immunity when class I MHC expression or the presented viral/tumor antigen level is reduced. Therefore, understanding the pathways regulating stored

FasL translocation is critical for developing new therapeutics. Recently, Jenkins and colleagues compared D^bNP_{366} and D^bPA_{224}-specific CTLs recovered directly from the lungs of mice with influenza pneumonia, and found that despite the higher avidity and longer duration of contact between D^bPA_{224}-specific CTLs and target cell, it took significantly more time to induce target cell apoptosis than lower avidity D^bNP_{366}-specific CTL (Jenkins et al. 2009). This observation suggests that lower TCR/epitope avidity may be more beneficial than higher epitope avidity for cell-mediated immunity. This lower avidity might result in a shorter contact time that may allow individual CTLs to lyse more targets. It is also possible that different TCR signaling strengths may induce qualitatively and quantitatively different killing mechanisms in different CTLs.

I previously found that stored FasL cell surface translocation is extracellular Ca^{2+} -independent (He and Ostergaard 2007). In the current study, I found that Ca^{2+} mobilization from intracellular stores is necessary to elicit this stored FasL translocation upon target cell engagement; in contrast, *de novo* FasL synthesis and degranulation requires Ca^{2+} flux from both intracellular stores and extracellular environment. Taking together these observations, and those from the signaling strength requirement, I conclude that a strong TCR signal will induce Ca^{2+} mobilization from both intracellular stores and extracellular environment, which are necessary for the induction of degranulation and *de novo* FasL cell surface expression; in contrast, weak TCR signal only induces low levels of intracellular flux of Ca^{2+}, which is sufficient for stored FasL cell surface translocation, suggesting that these killing mechanisms might be differentially

controlled and may have been selected to function under specific circumstances. Consistent with my observation, it was shown that impaired or low level Ca^{2+} mobilization is sufficient to induce FasL-mediated killing (Kessler et al. 1998; Guillaume et al. 2003; Purbhoo et al. 2004), presumably from the stored FasL pool. G4 is a weak agonist for OT-I TCR, which is unable to induce full TCRζ chain phosphorylation and calcium flux (Rosette et al. 2001). In the current study, I found that in OT-I CTL stored FasL translocation can be achieved by G4-pulsed target cell stimulation, although with a lower level response than did high affinity peptide SIINFEKL. This further supports my conclusion that a low level of intracellular Ca^{2+} mobilization is sufficient to induce stored FasL translocation.

Directional delivery of cytolytic granule contents toward antigen-bearing cells is considered to be an important property of the CTL mediated target cell killing. On the other hand, it appears that T cells use two distinct pathways for directional cytokine and chemokine secretion; some target to the immunological synapse to ensure a local antigen-specific effect and others deliver biological active molecules in a multidirectional way, which might facilitate the establishment of an inflammatory melieu to affect cells at a distance (Huse et al. 2005). In the current study, I provide evidence to suggest that FasL from a stored pool undergoes transient, antigen specific, directional delivery to the CTL/ target cell contact area, whereas *de novo* synthesized FasL is distributed around the CTL cell surface leading to significant bystander killing. There are precedents that one cytokine/ chemokine undergoes both directional polarization and less confined delivery depending on which pool it comes from (stored versus *de novo*

synthesized). Human $CD8^+$ T cells polarized prestored RANTES (CCL5) towards the target cell contact area (Catalfamo et al. 2004), whereas *de novo* synthesized RANTES underwent multidirectional secretion by murine T_H cells (Huse et al. 2005), although different cell sources might account for the disparate results observed. Also, it was shown in the same T_H1 cells that TNF was detected at the immunological synapse 30min after stimulation, but scattered throughout the cell 2-3h after initial stimulation, presumably from the *de novo* synthesized pool (Huse et al. 2005).

Different delivery patterns of FasL from different pools might result in unique biological effects. Given the low level of early FasL translocation from the stored pool, targeted delivery might facilitate more focused, efficient killing of the antigen-specific target cells where low avidity or affinity antigen is present on a limited number of cells. In another scenario, since FasL has been revealed to serve as a costimulatory molecule in T cells (Suzuki and Fink 1998; Suzuki et al. 2000), FasL localized in the proximity with TCR at the synaptic area might promote such costimulatory activity. In addition to target cell killing, FasL was also found to induce inflammation. When there is a massive virus infection that has infected large numbers of cells, it would be advantageous for the CTL to kill all cells around to quickly clear the virus. Multidirectional property of the late *de novo* synthesized FasL might facility this massive killing of innocent bystander cells. The production of neutrophil chemoattractants by Fas stimulated apoptotic cells would further induce local recruitment of inflammatory cells (O'Connell et al. 2001), precipitating inflammatory tissue damage. The bystander killing is

demonstrated to be damaging during long-term, chronic infections accompanied by the persistent presence of antigen-expressing targets. Although many tissues do not express Fas, cells of the liver and heart are among the most highly Fas-expressing cells (Watanabe-Fukunaga et al. 1992; Ogasawara et al. 1993), therefore, controlling *de novo* FasL expression might be important for limiting inflammation in these tissues.

I showed that FasL from a pre-existing intracellular pool is rapidly (15 to 30 min) expressed on the cell surface in response to TCR engagement (Figure 4-6). This rapidly expressed FasL also appears to quickly disappear from the cell surface before target cell release. Two possibilities might account for this rapid disappearance of early FasL. One is that FasL might be cleaved by matrix metalloprotease (MMP) since FasL has been shown to be cleaved from the cell surface (Kayagaki et al. 1995). However, MMP inhibitor did not substantially increase the amount of rapid FasL surface expression from stored pools and only slightly delayed the disappearance of FasL from the cell surface (Appendix Figure 2). This is in contrast to the cell surface expression of the later *de novo* synthesized FasL which was increased by 30-40% (Appendix Figure 2) in the presence of MMP inhibitors. This suggests that cleavage is not the primary means by which the rapidly expressed FasL is cleared from the cell surface. One might imagine that the tight contact between CTL and target cells might preclude MMP access to the directionally translocated FasL from stored pool, whereas the ubiquitously distributed *de novo* FasL is more susceptible to MMP cleavage. However, one study has shown that TPA/ionomycin induced early FasL

expression in human T cells was also not MMP-sensitive (Lettau et al. 2004). Therefore, it might be the different regulation mechanisms between the stored and *de novo* pool of FasL that control the fate of FasL once it is expressed on the cell surface. I speculate that FasL molecules from different pools might undergo different modification which endows their distinct MMP sensitivity, a possibility that needs to be investigated in the future. An alternative mechanism for the loss of FasL from the cell surface by endocytosis has been proposed (Nguyen and Russell 2001) since endocytosis inhibitors, such as cytochalasins, prevented the loss of surface FasL. Interestingly, I also found significantly increased and prolonged early FasL cell surface expression by crosslinked anti-CD3 stimulation in the presence of cytochalasin D (Appendix Figure 3), which might suggest that endocytosis could contribute to this rapid FasL disappearance. On the other hand, it has been shown that disruption of actin polymerization by cytochalasin D increased exocytosis in a granule-specific manner in neutrophils (Jog et al. 2007), indicating cytochalasin D might also directly regulate exocytosis of FasL vesicles in the current study. Further studies will need to be done to determine how the FasL transported to the cell surface from internal stores is so rapidly cleared from the cell surface. Overall, it seems that FasL from both pools has unique mechanisms for regulation of cell surface expression, which might ensure the specific killing mediated by stored FasL and limit the inflammatory tissue damage induced by *de novo* synthesized FasL.

So far, the molecular mechanisms that regulate the distinct storage and trafficking of FasL from these two pools are not yet known. It has been shown

that different sets of Rab proteins and SNARE proteins are required for trafficking of different secretory vesicles in T cells (Stinchcombe et al. 2001; Huse et al. 2005). Rab27a is important for the release of granules from the microtubules and membrane docking during degranulation (Stinchcombe et al. 2001; Haddad et al. 2001; Stinchcombe et al. 2004) but does not appear to be required for the lysis of Fas-expressing target cells (Haddad et al. 2001). However, in this study it is not clear if the lysis was mediated by stored or *de novo* FasL, since only the ability to lyse Fas^+ versus Fas^- target cells was assessed (Haddad et al. 2001). In this study, I showed that stored FasL polarization is not driven by degranulation since FasL vesicles were not colocalized with cytolytic granules at the synaptic junction. Therefore it is unknown if stored FasL employs Rab27 or alternate Rab proteins for cell surface translocation, which will be explored in future studies.

FasL on CTL may be important for the generation of autoimmune disease. Fas/FasL is required for the induction of diabetes in the non-obese diabetic (NOD) mice (Itoh et al. 1997; Su et al. 2000). Intriguingly, as NOD mice age, their islet-associated autoreactive CTL switch from FasL- to perforin-mediated killing (Qin et al. 2004), and this change is associated with an increase in the avidity of autoreactive CTL for a β-cell epitope (Amrani et al. 2000). Furthermore, it was demonstrated that despite the preferential use of the Fas/FasL pathway for cytolysis of β-cell targets, transgenic β-cell-specific CTL derived from NOD mice were able to kill targets via the perforin pathway when triggered by a higher affinity stimulus (Qin et al. 2004). These results indicate that TCR signaling strength might play an important role in determining which killing mechanisms

are elicited. In addition, Zelinskyy and colleagues showed that during acute Friend murine leukemia virus (F-MuLV) infection, the replication level of F-MuLV was the critical factor that determined the differential expression of cytotoxic molecules by CTL and the pathway of CTL cytotoxicity; the low-level infection induced CTL expressing solely FasL but not the cytotoxic molecules granzymes A and B, whereas the high-level infection resulted in induction of CTL secreting molecules of the granule exocytosis pathway (Zelinskyy et al. 2007). Although the mechanism is not yet known, one possibility is that CTL signaling strength determines differential activation of lytic mechanisms in F-MuLV-specific CTL, an interpretation favoured by my results. It is also possible that the threshold of initial stimulation for the generation of CTL could determine if the initial signal strength allows for killers of one type or another to be generated.

In summary, in this chapter I show that stored FasL has a lower signaling threshold of activation than *de novo* FasL synthesis and degranulation by titrating antigen concentrations and examination of TCR signaling requirement. I provide evidence to suggest that stored FasL translocation mediates directional killing and remains on the cell surface during conjugation, whereas *de novo* synthesized FasL, which appears to be expressed ubiquitously around the cell surface, mediates significant bystander killing. My observations imply that stored FasL might be employed during low level infection and may be required to clear the final pathogens after infection to play a preferential role during low level chronic infections. In contrast, when there is a massive virus infection, degranulation and *de novo* FasL synthesis will play crucial roles for virus clearance. In this

circumstance, a significant amount of bystander killing of innocent cells would occur, inducing inflammatory tissue damage because of the constitutively expressed FasL on the cell surface.

CHAPTER 5 : Identification and Characterization of the Storage Compartment of the Stored FasL in Cytotoxic T Lymphocytes

A. Introduction

An increasing body of evidence indicates the presence of pre-existing pools of FasL in activated mouse and human $CD8^+$ T cells, NK cells, monocytes and tumor cells (Li et al. 1998; Kessler et al. 1998; Bossi and Griffiths 1999; Martinez-Lorenzo et al. 1999; Kojima et al. 2002; He and Ostergaard 2007; Kassahn et al. 2009; Kiener et al. 1997; Andreola et al. 2002). However, the nature of the compartment containing the stored FasL is not fully established and remains controversial.

Li and colleagues reported that preformed FasL mediated cytotoxicity is CHX resistant but brefeldin A (BFA) sensitive in a CTL clone and PEL (Li et al. 1998). The authors therefore speculated that preformed FasL is either constitutively expressed on the cell surface or stored in the Golgi apparatus (Li et al. 1998). Based on confocal microscopy and FasL over-expression studies, the group led by Griffiths suggested that pre-existing, along with newly synthesized FasL, is sorted to the cytolytic perforin-containing granules in a human CTL clone and NK cells, and that upon degranulation FasL is delivered to the cell surface (Bossi and Griffiths 1999). An intracellular FasL localization confined to multivesicular body (MVB) has also been described to occur in melanoma cells (Andreola et al. 2002) and PHA-stimulated human T cells (Martinez-Lorenzo et al. 1999), which undergoes exosome regulated secretion. In contrast, my confocal

and functional data are consistent with FasL storage vesicles distinct from the cytolytic granules in CTL (He and Ostergaard 2007). Recently, the group led by Brunner further confirmed my observations (Kassahn et al. 2009). Therefore, further studies will be required to identify the compartment in which the FasL is stored in CTL.

In this chapter, I characterized the FasL storage compartment in CTL by subcellular fractionation, confocal microscopy, and biochemical assays. In contrast to the reported ER/ Golgi or lysosomal associated compartment localization, I described that FasL is stored in BFA-sensitive compartment which may associate with recycling endosomes in CTL. I further suggested that FasL vesicles might be located in a special niche between ER and mitochondria to readily sense the Ca^{2+} flux upon T cell activation.

B. Results

Stored FasL cell surface translocation is BFA sensitive

A previous study by Li and colleagues using our CTL clone AB.1 and PEL suggested that FasL-mediated lysis was not blocked with protein synthesis inhibitors but was inhibited with the fungal toxin BFA (Li et al. 1998). The most characteristic effect of BFA is the rapid disassembly of the Golgi complex, inhibition of secretion and the redistribution of resident Golgi proteins to the ER. Thus, the authors hypothesized that FasL is either stored in an ER/ Golgi compartment or constitutively expressed on the cell surface (Li et al. 1998). In this chapter, I further confirmed their observations by showing that stored FasL

translocation induced by either target cell engagement (Figure 5-1A), or Ca^{2+} ionophore ionomycin, phorbol ester PMA stimulation, either alone or in combination (Figure 5-1B), was barely affected by CHX treatment but was significantly blocked by BFA. The level of blocking by BFA in combination with CHX was similar to BFA treatment alone (Figure 5-1A & 1B), indicating the BFA-sensitive FasL is from the prestored pool. Furthermore, target cell lysis of Fas-expressing cells, in the absence of extracellular Ca^{2+} to exclusively examine the stored FasL translocation, is impaired by BFA (Figure 5-1C). Taken together, I further confirmed the observations by Li and colleagues by showing that stored FasL translocation to the cell surface upon CTL activation and the subsequent target cell killing are substantially BFA-sensitive.

Stored FasL is fully mature N-glycoprotein

Given the sensitivity of the vesicular trafficking between or from the ER and Golgi apparatus to BFA, I next wanted to analyze the glycosylation status of the stored FasL, which would provide additional insight into the storage location of the stored FasL. Since all of the FasL expressed in resting CTL is stored intracellularly, I was able to examine the total FasL isolated from resting cells to examine the glycosylation status. Mouse FasL has four potential N-glycosylation sites, and human FasL has three potential N-glycosylation sites (Figure 1-1). N-glycosidase F (PNGase F) digestion of preformed FasL immunoprecipitates demonstrates that stored FasL has N-linked glycans (Figure 5-2A). Endoglycosidase H (endo H) is a highly specific endoglycosidase which cleaves

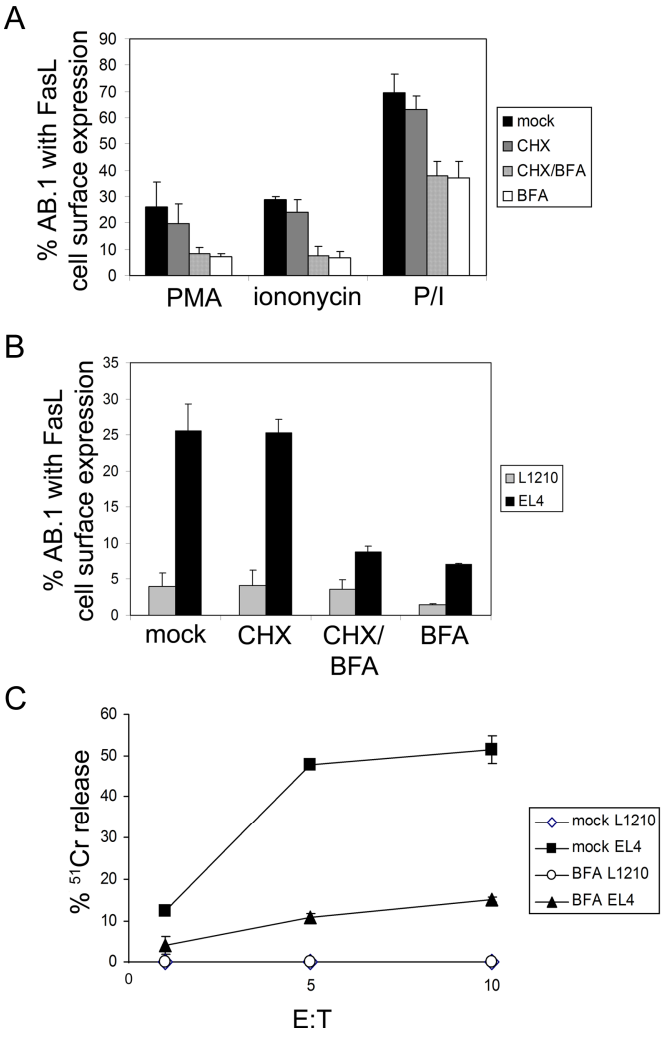


Figure 5-1. Stored FasL cell surface translocation is BFA sensitive. *A*, CTL clone AB.1 was pretreated with 10 μg/ml CHX, or 4 μg/ml BFA, or CHX plus BFA or mock control for 30 min, then stimulated with 10 ng/ml PMA, or 2.5 μM ionomycin, or 10 ng/ml PMA plus 0.5 μM ionomycin (P/I), or left unstimulated for 15 min, cell surface FasL expression upon stimulation was examined by flow cytometry. The data shown are the percentage of AB.1 with cell surface FasL expression subtracted with that of the unstimulated control. *B*, AB.1 was pretreated the same as in *A*, and stimulated with either cognate target cell EL4 or negative target L1210 for 15 min, then cell surface FasL expression upon stimulation was examined by flow cytometry. The E:T ratio is 1:1. Data in *A* and *B* are the average of three independent experiments; error bars represent SEM. *C*, AB.1 was pretreated with 4 μg/ml BFA or mock control, then mixed with either cognate target cell EL4 or negative target L1210 in the presence of EGTA/Mg^{2+} to exclusively examine the stored FasL mediated cytotoxicity by the 4-hour ^{51}Cr release assay. Average of triplicated samples is shown; error bars represent SD. Data are representative of three independent experiments. (Data presented in *C* was generated with Dong-Er Gong.)

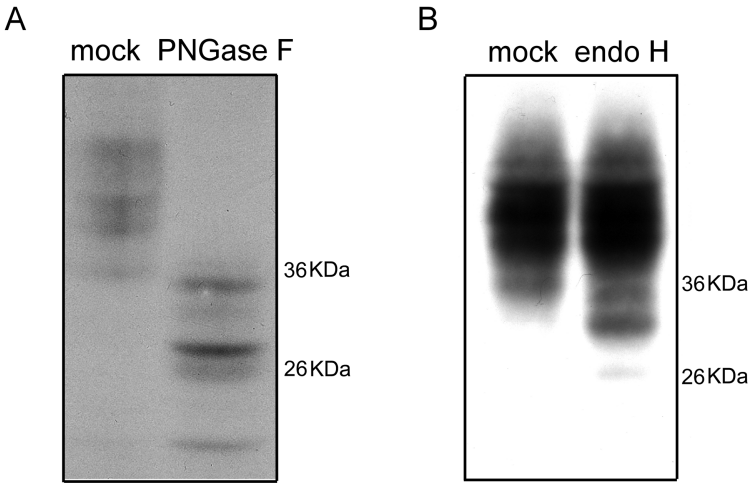


Figure 5-2. Stored FasL is fully mature N-glycoprotein. FasL was immunoprecipitated from the postnuclear lysate of resting AB.1, then subjected to N-glycosidase F (PNGase F) (*A*), or Endoglycosidase H (endo H) (*B*) digestion or mock treatment overnight at 37°C prior to resolving by SDS-PAGE. Immunoblotting of FasL are shown. Data are representative of three independent experiments.

immature asparagine-linked high-mannose type glycans, but not highly processed complex oligosaccharides from glycoproteins. I found the majority of the stored FasL has fully mature Endo H resistant N-linked glycans (Figure 5-2B), suggesting that FasL has already passed through the late ER along the secretory pathway.

FasL is not stored in the ER or Golgi apparatus

To directly visualize the intracellular compartment in which FasL is stored, confocal microscopy studies were conducted. Consistent with the glycosylation data, I observed that FasL segregated from the ER marker calnexin (Figure 5-3A), indicating FasL is stored outside of the ER. β-COP is the major component of the COPI protein complex, which is critical for vesicular traffic between the ER and Golgi and is useful as a marker for the vesicular-tubular clusters found at the cis face of the Golgi stack, as well as the cis-Golgi itself (Oprins et al. 1993). Golgi 58K, a formiminotransferase cyclodeaminase, is a marker for the membrane of the Golgi apparatus (Bloom and Brashear 1989; Gao et al. 1998). I found that FasL vesicles segregate from β-COP (Figure 5-3B), indicating FasL is not located between the ER and the Golgi apparatus. I further found that FasL is located very close to Golgi 58K (Figure 5-3C). However, subcellular fractionation carried out by Optiprep® density gradient showed that FasL vesicles are not co-enriched with the Golgi apparatus, indicating FasL vesicles might only have a peri-Golgi distribution, rather than being stored in the Golgi (Figure 5-4). Subcellular fractionation also showed that FasL vesicles are not completely co-enriched with

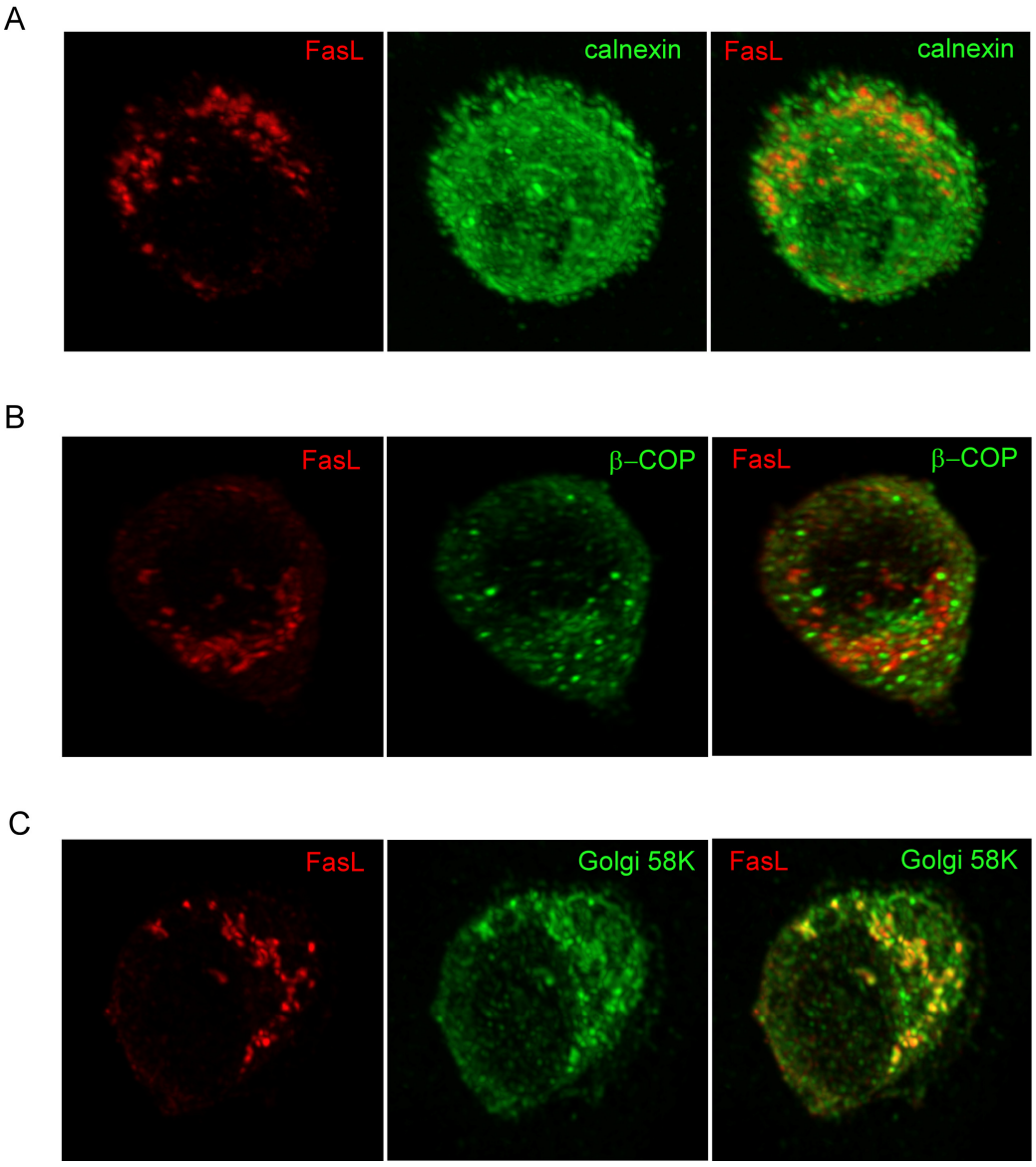


Figure 5-3. The preformed FasL is not stored in the ER and Golgi apparatus. AB.1 cells were stained with Abs specific for FasL (red) and the ER marker calnexin (green) (*A*), or cis-Golgi/ ER-GIC marker β-COP (green) (*B*), or Golgi 58K (green) (*C*), and the appropriate secondary Abs and analyzed by confocal microscopy. Z-stack images were acquired (interval, 0.2 μm) and subjected to deconvolution and three-dimensional reconstruction. Representative projections of the reconstructed three-dimensional images are shown. Data are representative of at least three independent experiments.

the markers for the ER, confirming that FasL is not stored in the ER (Figure 5-4). These observations suggest that stored FasL has already passed the ER and the Golgi apparatus along the secretory pathway, in contrast to the previously reported hypothesis that FasL is stored in the ER/ Golgi compartment (Li et al. 1998).

FasL is stored in a subcompartment that might associate with recycling endosomes

I next further examined the intracellular distribution of stored FasL by subcellular fractionation. I found that FasL-enriched fractions do not completely overlap with markers for the ER (calnexin and Glucosidase II), Golgi apparatus (GM-130 and β-COP), mitochondria (cytochrome C), lysosomes (LAMP1) and plasma membrane (LFA-1) (Figure 5-4), suggesting that FasL is not stored in these compartments. Surprisingly, although the majority of early endosome marker EEA1 (Early endosomal antigen 1) does not overlap with FasL, FasL fractions do overlap with another early endosome marker Rab4 (Figure 5-4). Further, FasL fractions overlap with the late recycling endosome marker Rab11 (Figure 5-4). It has been known that Rab4 is involved in membrane recycling from early endosomes to the late recycling endosomes (van der Sluijs et al. 1992), and there is a dynamic overlap or continuity of late recycling endosomes with early endosomes, as demonstrated by the sequential but overlapping distributions of Rab11 and Rab4 (Sönnichsen et al. 2000). Therefore, FasL might be stored in a subcompartment that associates with recycling endosomes. I therefore examined

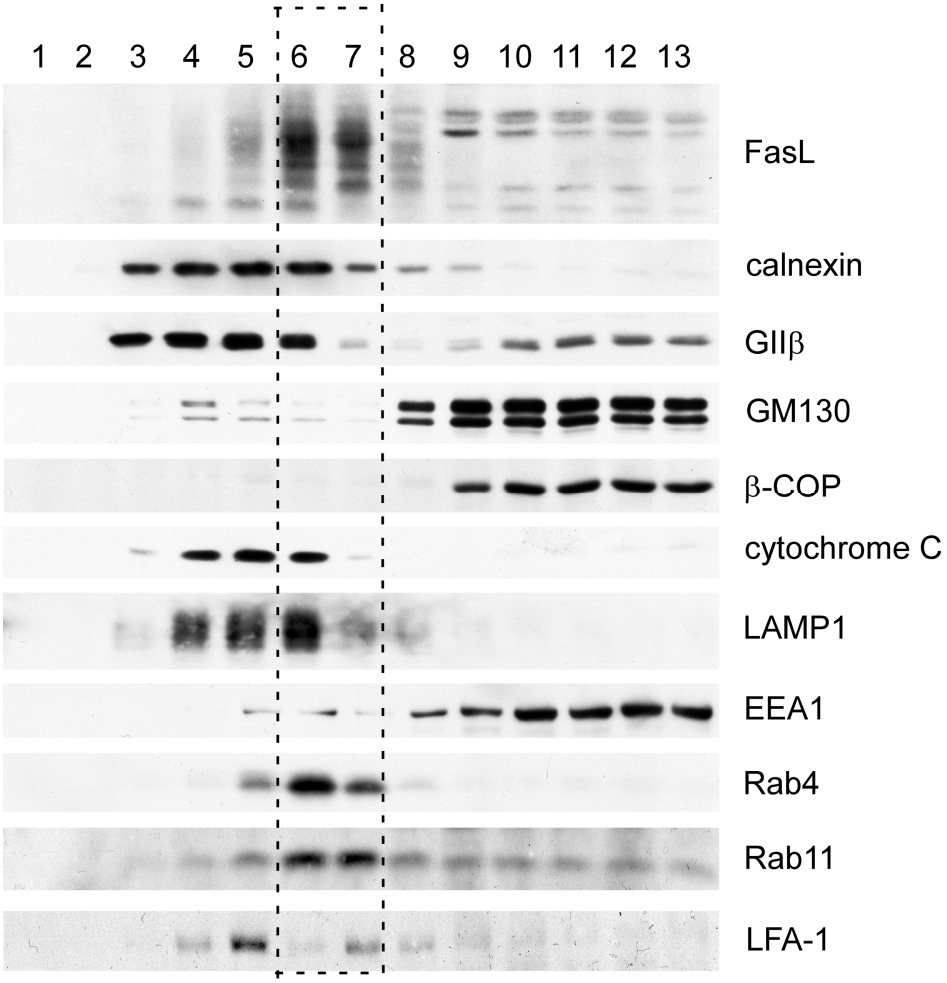


Figure 5-4. FasL is co-enriched with recycling endosomes by subcellular fractionation. Postnuclear homogenate from 1×10^8 AB.1 cells was subjected to subcellular fractionation by 30% - 0% continuous OptiPrep density gradient. 1ml fractions were collected, and 40 μl of each fraction was subjected to 10% SDS-PAGE gel separation and examined by immunoblotting. Fractions from 1 to 13 represent decreased OptiPrep density. Immunoblot for FasL, ER markers calnexin and glucosidase II β (GIIβ), Golgi markers GM130 and β-COP, mitochondria marker cytochrome C, lysosome marker LAMP1, early endosome markers EEA1 and Rab4, recycling endosome marker Rab11, and plasma membrane marker LFA-1 are shown. Box with dotted line highlights the FasL enriched fractions.

the relevant localization of FasL and early/ recycling endosomes by confocal microscopy. I found FasL segregated from the early endosome marker EEA1, and the early/ recycling endosome marker transferrin receptor (TfR) (Figure 5-5A). I observed partial overlap in staining between FasL and Rab4 (Figure 5-5A). However, the majority of FasL and Rab4 appear to have a non-overlapping distribution (Figure 5-5A). Accordingly, the percentage of Rab4 colocalized with FasL, or the percentage of FasL colocalized with Rab4 was higher than that of EEA1 and FasL as well as TfR and FasL (Figure 5-5B). I also tried to compare FasL localization to Rab11 by confocal microscopy. However, I was unable to detect obvious Rab11 staining because of low protein abundance or the lack of a high affinity antibody recognizing native conformation of the protein.

FasL vesicles are located adjacent to mitochondria and the ER

Intriguingly, I found that FasL vesicles are located adjacent to a fraction of mitochondria and an ER resident protein glucosidase II (GII) by confocal microscopy (Figure 5-6A), although no complete overlap with the ER marker calnexin was observed (Figure 5-3A). Therefore, FasL vesicles might have close contact with a subcompartment of the ER. When three-color staining for FasL, cytochrome C and calnexin was carried out, I did observe some areas of close contact between these three markers (Figure 5-6B). So far, the physiological significance of this close proximity of localization is unclear. I hypothesize that FasL vesicles are located in a special niche between the ER and mitochondria to readily sense the Ca^{2+} flux from the ER upon T cell activation since that low levels

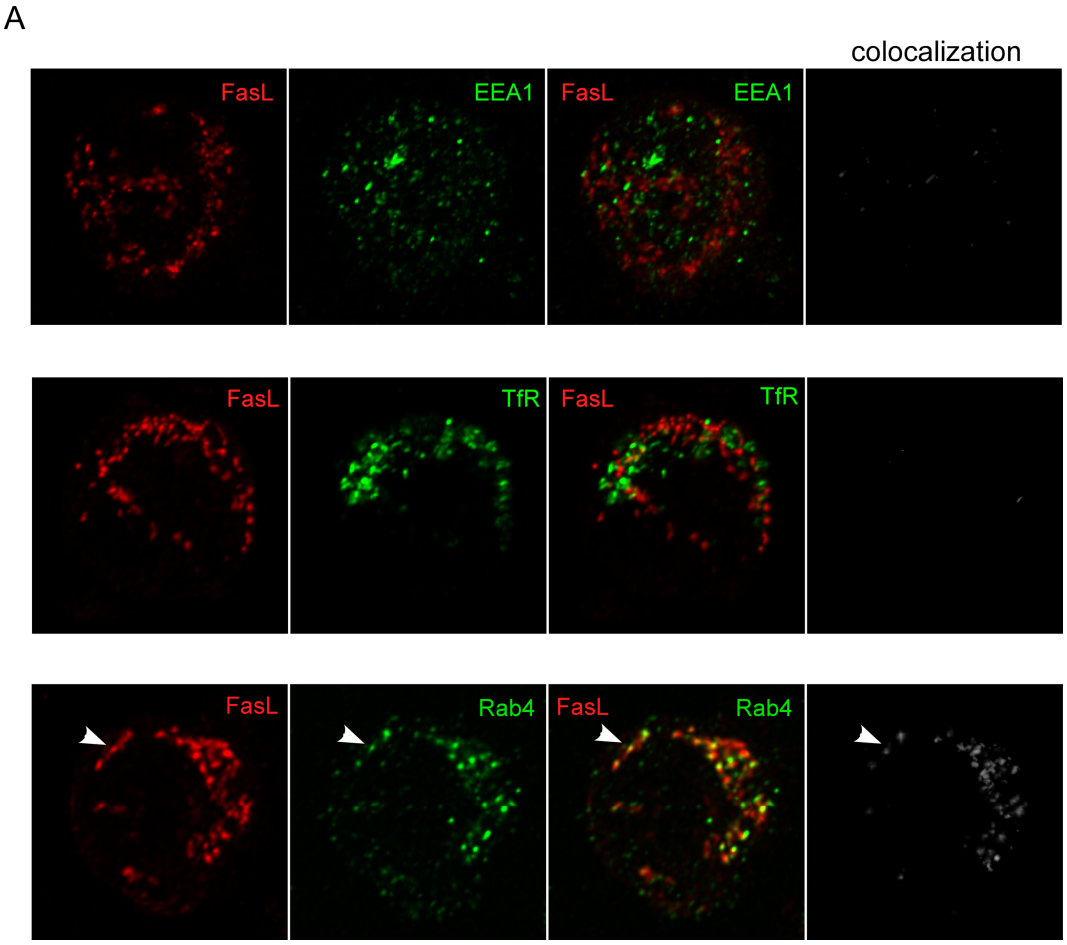
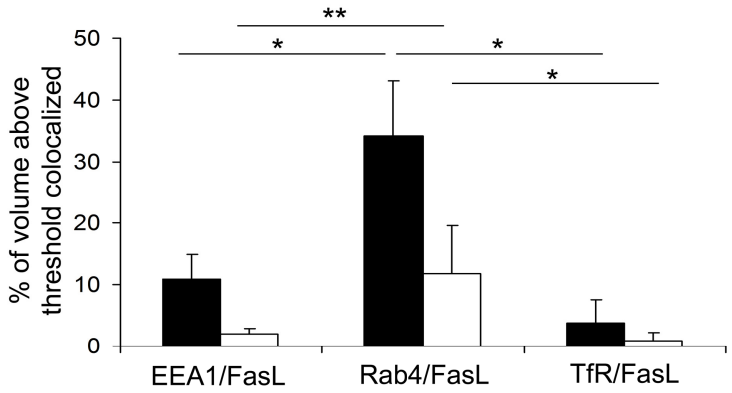


Figure 5-5. The relevant localization of FasL and early/ recycling endosomes by confocal microscopy. AB.1 cells were stained with Abs specific for FasL (red) and the endosome markers EEA1 (green), or transferrin receptor (TfR) (green) or Rab4 (green), and the appropriate secondary Abs and analyzed by confocal microscopy. Z-stack images were acquired (interval, 0.2 μm) and subjected to deconvolution and three-dimensional reconstruction. *A*, Representative projections of the reconstructed three-dimensional images are shown. Additionally, a colocalization channel in black and white is shown. Arrowhead indicates a site of colocalization between FasL and Rab4. *B*, The percentage of channel A (green) volume above threshold that is colocalized with channel B (red) (black bar), or the percentage of channel B (red) volume above threshold that is colocalized with channel A (green) (white bar) was calculated by Imaris software. The average value of the molecule pairs: EEA1 and FasL (n=6), TfR and FasL (n=9), Rab4 and FasL (n=12) is shown; error bars represent SD. *, $P < 0.001$, **, $P < 0.01$ (unpaired two-tailed Student's t-test).

B


**
*
*
*
50
40
30
20
10
0
% of volume above threshold colocalized
EEA1/FasL
Rab4/FasL
TfR/FasL

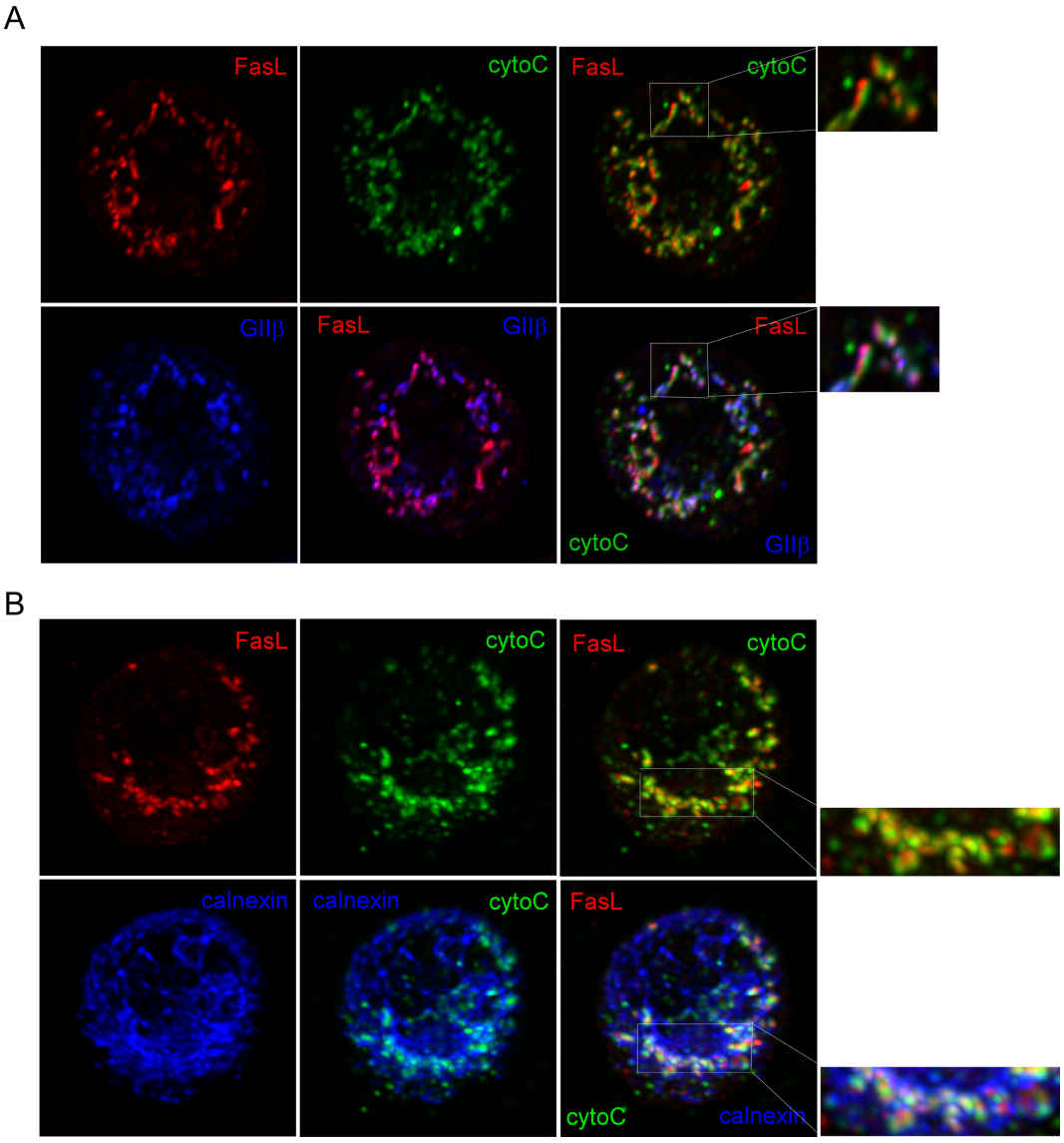


Figure 5-6. FasL vesicles are located adjacent to mitochondria and the ER. AB.1 cells were stained with Abs specific for FasL (red), mitochondria marker cytochrome C (green) and ER marker GIIβ (blue) (*A*), or ER marker calnexin (blue) (*B*), and the appropriate secondary Abs and analyzed by confocal microscopy. Z-stack images were acquired (interval, 0.2 μm) and subjected to deconvolution and three-dimensional reconstruction. Representative projections, as well as the enlargement of indicated area, of the reconstructed three-dimensional images are shown. Data are representative of at least three independent experiments.

of intracellular Ca^{2+} flux alone is sufficient to induce rapid FasL translocation to the cell surface (Figure 4-4). Interestingly, similar to what I observed with FasL, our lab has found that GII is released from the cell after TCR stimulation and with Ca^{2+} ionophore treatment in a BFA-dependent fashion (S. Broomfield & H. Ostergaard, unpublished data), suggesting there might be either a overlapping distribution or a similar signaling requirement for mobilization between the stored FasL and GII.

FasL vesicles do not have close contact with STIM1 by confocal microscopy

I described in chapter 3 that the stored FasL translocation to the cell surface is microtubule independent. However, the mechanism underlying this microtubule-independent, TCR signal regulated FasL translocation is unknown. Recent studies have revealed that the depletion of Ca^{2+} from the ER triggers the oligomerization of the ER Ca^{2+} sensor STIM1 and its redistribution in ER regions closely associated with the plasma membrane, where the CRAC channel subunit ORAI1 accumulates in the plasma membrane and CRAC channels open (Roos et al. 2005; Wu et al. 2006; Luik et al. 2006; Luik et al. 2008; Park et al. 2009). Given the special localization of FasL in relation to the ER and mitochondria, and the high sensitivity of FasL translocation to intracellular Ca^{2+}, I wanted to examine if FasL vesicles are located close to the ER subcompartment where STIM1 proteins reside, and translocate to the cell surface along with STIM redistribution to the ER- plasma membrane junctions upon T cell activation by confocal microscopy. Unfortunately, I could not detect a close contact between

the stored FasL and STIM1 in the CTL before and after treatment with thapsigargin (TG) (Figure 5-7), an ER Ca^{2+} pump inhibitor which results in gradual ER Ca^{2+} store depletion (Thastrup et al. 1990), although I could not exclude a possible functional relationship between these two proteins.

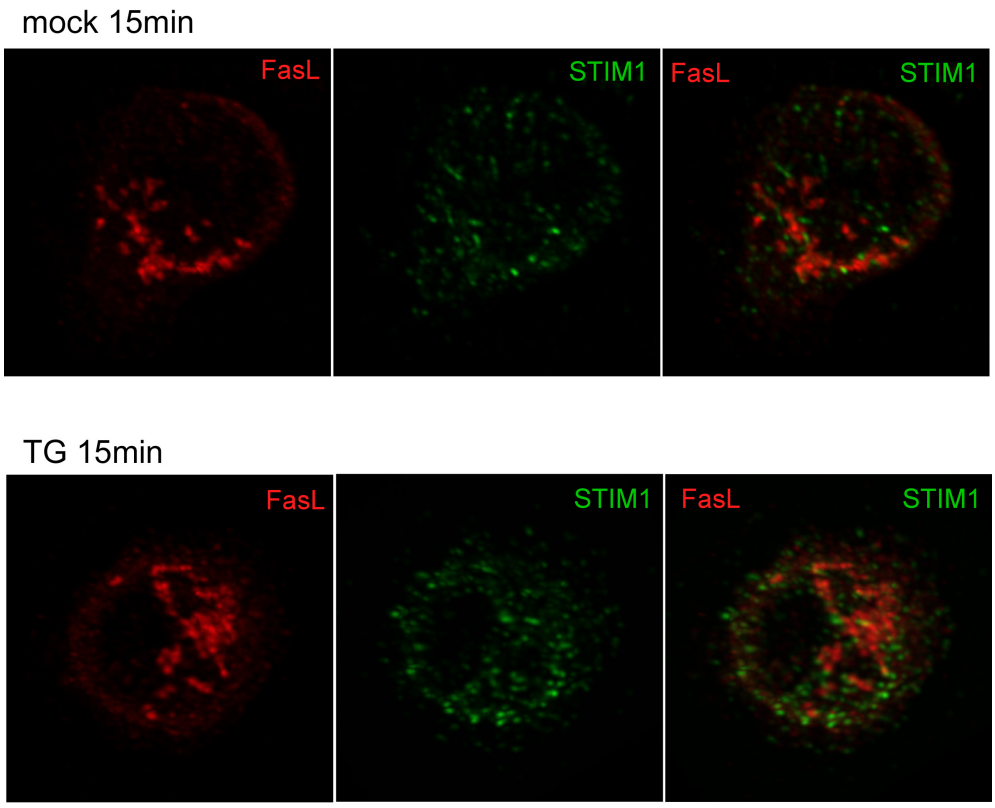


Figure 5-7. FasL vesicles do not have close contact with STIM1 by confocal microscopy. AB.1 cells were treated with 2 μM thapsigargin (TG) or left untreated for 15 min, then stained with Abs specific for FasL (red) and the ER calcium sensor STIM1 (green), and the appropriate secondary Abs and analyzed by confocal microscopy. Z-stack images were acquired (interval, 0.2 μm) and subjected to deconvolution and three-dimensional reconstruction. Representative projections of the reconstructed three-dimensional images are shown. Data are from a single experiment.

C. Discussion

In this chapter, I characterized the FasL storage compartment by subcellular fractionation, confocal microscopy, and biochemical assays. In contrast to the reported Golgi or lysosomal associated compartment localization, I described here, for the first time, that FasL is stored in BFA-sensitive vesicles that might associate with late recycling endosomes in CTL. Furthermore, I demonstrated that FasL vesicles are located adjacent to mitochondria and an ER marker. Given that low levels of intracellular Ca^{2+} mobilization is sufficient to elicit stored FasL cell surface translocation, I hypothesize that FasL vesicles is located in a special niche between ER and mitochondria to readily sense the Ca^{2+} flux upon T cell activation.

My data that stored FasL translocation to the cell surface is BFA sensitive, is consistent with the observations by Li and colleagues (Li et al. 1998) but with different interpretation according to the recent clarification of the target of BFA. The nucleation of coat protein assembly at a variety of intracellular locations requires members of the ARF family of small GTPases. Like other small GTPase, ARF has a GTP-bound active form and a GDP-bound inactive form, whose activation is regulated by guanine nucleotide exchange factors (GEFs), called Sec-7 domain proteins (Jackson and Casanova 2000; Casanova 2007). The target of BFA is a subset of Sec-7 domain proteins. Genetic screens uncovered a 35-amino acid region of the Sec7 domain that harbors residues conferring BFA-resistance (Jackson and Casanova 2000). BFA blocks coat protein assembly by binding tightly to the ARF–GDP–Sec7 domain complex, sequestering the GEFs in an

abortive reaction intermediate (Anders and Jürgens 2008). Mammals express three BFA-sensitive ARF GEFs, GBF1 (Golgi BFA-resistance factor 1) (Claude et al. 1999), BIG1 (BFA-inhibited GEF1) and BIG2 (Togawa et al. 1999). Although all three isoforms localize largely to the Golgi apparatus and regulate ER and Golgi associated vesicle trafficking, BIG2 has also been found in association with recycling endosomes and are involved in the regulation of recycling endosome morphology and functionality (Casavona 2007). Consistent with these studies, it has been reported that knockdown of BIG2 by RNAi affects the morphology of recycling endosomes and slows recycling of transferrin (Shen et al. 2006). Also, $Rab4^+$ and $Rab11^+$ vesicles are sensitive to BFA treatment (Daro et al. 1996; Sönnichsen et al. 2000). It would be of interest to examine whether BIG2 is involved in FasL vesicle trafficking. The recycling endosome is a complex of vesicles and tubules, which are often located close to the TGN. In this chapter, I showed that the FasL vesicle has a close contact with Golgi 58K, a marker for the membrane of the Golgi apparatus (Figure 5-3), which might resemble the recycling endosome localization with a peri-Golgi distribution. Taken together, the observations from the subcellular fractionation, which shows FasL is coenriched with a $Rab4^+$ or $Rab11^+$ compartment, and the sensitivity of the stored FasL trafficking to BFA appears to provide evidence suggesting that FasL is stored in a compartment associated with recycling endosomes.

The endosome system, which consists of early, late and recycling endosomes, has a central function in membrane dynamics by sorting and delivering cargo to the plasma membrane, TGN or lysosomes (Trowbridge et al.

1993; Pfeffer 2003; Russell et al. 2006) (Figure 5-8). Internalized molecules can be degraded after sorting into the late endosomes/ lysosomes or be recycled back to the cell surface (Maxfield and McGraw, 2004). Recycling to the cell surface can occur either directly from the early endosomes or the late recycling endosomes (Maxfield and McGraw 2004). On the other hand, recycling endosomes can also serve as an intermediate during transport en route from the Golgi to the plasma membrane. Biochemically, late recycling endosomes are enriched in Rab11 and the v-SNARE vesicle-associated membrane protein 3 (VAMP-3) (Daro et al. 1996; Ullrich et al. 1996; Bajno et al. 2000). Early endosomes are enriched in Rab4, Rab5, and EEA-1 (Sönnichsen et al. 2000). Rab4 is involved in membrane recycling from early endosomes to the late recycling endosomes, and there is a sequential but overlapping distribution of Rab11 and Rab4 (Sönnichsen et al. 2000), which is consistent with the subcellular fractionation data that I showed in this chapter. I found incomplete overlap between EEA1 and Rab4 or Rab11 by subcellular fractionation in the CTL that I examined (Figure 5-4). I also found FasL vesicles segregate from EEA1 labeled early endosomes by both subcellular fractionation (Figure 5-4) and confocal microscopy (Figure 5-5). These observations suggest that FasL vesicles might be associated with the late recycling endosomes in CTL. So far, it is unclear whether Rab4 or Rab11 contributes to FasL trafficking, which needs to be addressed in the future.

Recently, accumulating evidence has suggested that the recycling endosome has a dynamic trafficking activity, which serves as an active distribution

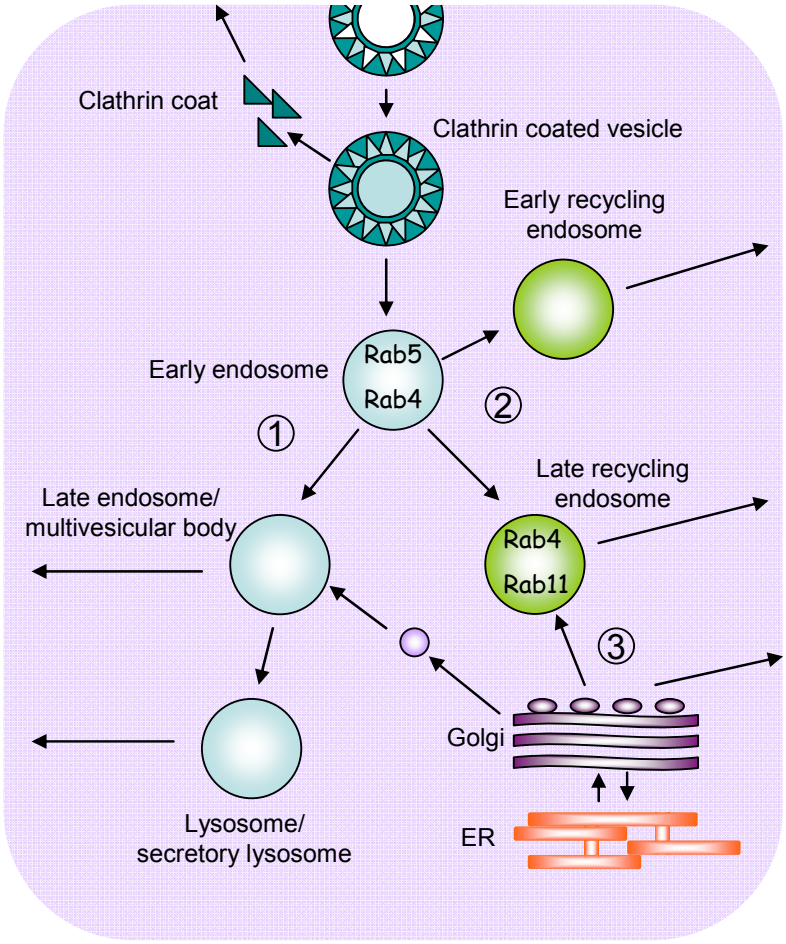


Figure 5-8. Vesicular trafficking within the endosomal membrane system. Endocytic vesicles that are formed by invagination and pinching of clathrin-coated pits become uncoated in the cytoplasm and fuse with early endosomes, from which endocytosed molecules are sorted to various intracellular destinations. 1) Internalized molecules can be transported to late endosomes and lysosomes for degradation. 2) Internalized molecules can also be recycled back from early endosomes or a late recycling endosomes to the plasma membrane. 3) Newly synthesized molecules are delivered from TGN directly to the cell surface, or via late recycling endosomes to the cell surface. Early endosomes are enriched in Rab5 and late recycling endosomes are enriched in Rab11. Rab4 is involved in membrane recycling from early endosomes to the late recycling endosomes.

center involved in a variety of cellular activities such as the maintenance of cell polarity, directional cytokine secretion and cytolytic granule maturation (Ang et al. 2004; Murray et al. 2005; Manderson et al. 2007; Ménager et al. 2007). In macrophages, TNFα is trafficked from the TGN to the recycling endosome, then polarized to the cell surface at the site of phagocytic cup formation (Murray et al. 2005). Interestingly, although IL-6 is also sorted from the TGN to the cell surface via the recycling endosomes in macrophages, it is not delivered to phagocytic cups as does TNFα (Manderson et al. 2007). Therefore, the recycling endosome may have a central role in orchestrating secretion with differential directionality. It was reported that in $CD4^+$ T cells the stored TNFα is polarized towards the immunological synapse upon target cell engagement, whereas the *de novo* synthesized TNFα secretes without directionality (Huse et al. 2006). It is unclear whether the recycling endosome is involved in targeted trafficking of TNFα in T cells, and whether FasL is stored in the same compartment as TNFα in CTL, a possibility that needs to be addressed in the future.

It was suggested recently that upon target cell engagement, polarized cytotoxic granules in CTLs require final maturation steps, which involve the fusion of perforin/ granzyme-containing granules with an "exocytic vesicle" at the immunologic synapse, so that exocytosis of cytolytic contents can occur (Ménager et al. 2007). Interestingly, the "exocytic vesicle" is formed by Munc13-4 mediated fusion of $Rab27a^+$ late lysosomes with the Munc13-4^+ recycling endosomes (Ménager et al. 2007), indicating the involvement of recycling endosomes in CTL granule effector function. It was shown that the ability of

Munc13-4 to assemble recycling and late endosomal structures occurred independently of cell activation and did not require Munc13-4–Rab27a interaction (Ménager et al. 2007). In this chapter, I suggested that FasL is stored in a compartment associated with recycling endosomes. Although Rab27a does not appear to be required for the lysis of Fas-expressing target cells, it is not clear if the lysis was mediated by stored or *de novo* FasL, since only the ability to lyse Fas^+ versus Fas^- target cells was assessed (Haddad et al. 2001). Therefore it is unknown if stored FasL employs Rab27 for cell surface translocation. It would be of interest to determine if the stored FasL colocalizes with Munc13-4, and whether Rab27a or/and Munc13-4 is involved in FasL trafficking towards the immunological synapse. Under this scenario, FasL vesicles might not require the final "maturation" step for delivery as cytolytic granules do, which could partially explain why FasL cell surface translocation has a minimum TCR signaling requirement compared to degranulation, although the signaling requirement for the final maturation of cytolytic granules has not been elucidated. In chapter 3, I showed that FasL is not colocalized with cytolytic granules in both murine and human CTLs that I examined, an observation inconsistent with that of Bossi and Griffiths (Bossi and Griffiths 1999). Given the close association of FasL with the recycling endosomes, it is possible that at some point, FasL vesicles do meet cytolytic granules, presumably depending on the type and the activation/maturation state of the cell.

Since the recycling endosome handles cargo from both the exocytic pathway and endocytic/ recycling pathways, it is unclear through which route

FasL is sorted to the recycling endosome associated subcompartment. One possibility is that FasL is sorted from TGN to the recycling endosomes, from which it undergoes TCR regulated cell surface translocation upon target cell engagement. The other possibility is that FasL is recycled from the cell surface. However, Griffiths and colleagues showed that endogenous FasL cannot internalize extracellular antibody, and suggested that FasL does not transit the plasma membrane en route to the intracellular stores, though they hypothesize FasL is stored in secretory lysosomes (Blott et al. 2001). It has been shown that blocking Rab11 activity interferes with recycling endosome functionality. For example, dominant-negative Rab11 blocked TNFα cell surface delivery without affecting newly synthesized TNFα at the Golgi complex (Murray et al. 2005). Similarly, it would be of interest to examine if blocking Rab11 activity, by either RNAi or a dominant negative mutant, could change stored FasL subcellular distribution, which could provide insight into the route by which FasL is sorted to the subcompartment associated with recycling endosomes.

The subcompartmentalization of cargo within the recycling endosomes and different patterns of cargo trafficking through the recycling endosomes have been described. The group led by Murray showed that although both TNFα and IL-6 are sorted from the TGN to the cell surface via the recycling endosomes in macrophages, TNFα is delivered to phagocytic cups whereas IL-6 is secreted without directionality (Murray et al. 2005; Manderson et al. 2007). Manderson further showed that subcompartments of the recycling endosome direct the differential secretion, in which IL-6 and TNFα converge with recycling

transferrin (Tfn) within the recycling endosomes, however all three of these cargo proteins appear to be partially segregated within this compartment based on imaging data (Manderson et al. 2007). In this chapter, I observed overlap of FasL, Rab4 and Rab11 by subcellular fractionation (Figure 5-4). However, I only observed partial overlap between FasL and Rab4 by confocal microscopy (Figure 5-5). And the majority of FasL and Rab4 appear to have a non-overlapping distribution (Figure 5-5). I further showed that FasL vesicles segregate from the recycling transferrin receptor (TfR) vesicles by confocal microscopy (Figure 5-5). These data indicate that FasL might be stored in a subcompartment associated with the recycling endosomes. In preliminary analyses I was unable to detect obvious Rab11 staining in CTL by confocal microscopy because of low protein abundance or the lack of a high affinity antibody recognizing native conformation of the protein. Therefore, the relative distribution of FasL compared to Rab11 is not clear, an issue required to be addressed in the future through transient transfection of tagged protein.

In this chapter, I showed that FasL vesicles are located adjacent to the mitochondria and ER (Figure 5-6). Juxtaposition between ER and mitochondria, which is known as the mitochondria-associated ER membrane (MAM) (Hayashi et al. 2009), is crucial for mitochondrial lipid biosynthesis (Vance 1990; Vance 1991) as well as provides the physical basis for intercommunication during Ca^{2+} signaling (Rizzuto et al. 1998; Rizzuto et al. 2009). Regions of the ER apposed to mitochondria are enriched with IP3 receptors, identifying these zones as 'hotspots' of Ca^{2+} transfer from the ER to the mitochondria (Rizzuto et al. 1998).

Therefore, I hypothesize that FasL vesicles might be located in a special niche between the ER and mitochondria to readily sense the Ca^{2+} flux from the ER upon T cell activation since I have shown that low levels of intracellular Ca^{2+} flux alone is sufficient to induce rapid FasL translocation to the cell surface in chapter 4. I could not decipher how FasL vesicle translocation to the cell surface is triggered upon intracellular Ca^{2+} mobilization. One candidate was ER Ca^{2+} sensor STIM1. However, I was unable to detect direct interaction between FasL and STIM1 by confocal microscopy (Figure 5-7), although I can not exclude a possible functional connection between the proteins.

In summary, in this chapter I showed that FasL is stored in a BFA-sensitive compartment which has the characteristics of recycling endosomes. I further demonstrated that FasL vesicles are located in a niche between mitochondria and the ER which might help FasL to readily sense the Ca^{2+} flux from the ER upon T cell activation. Further studies are required to systemically characterize the FasL storage compartment, which will provide insight into the trafficking machinery that controls the stored FasL trafficking.

CHAPTER 6 : General Discussion

A. Summary of results

CTL lyse target cells through the release of cytolytic granule contents and cell surface expression of FasL. The prevailing model at the time I initiated my studies was that FasL is stored in cytolytic granules and that FasL cell surface expression would be subject to the same controls as degranulation (Bossi and Griffiths 1999) (Figure 1-2). In this thesis, I investigated TCR-regulated FasL cell surface expression upon target cell engagement in untransformed CTL clones. I found that CTLs contain intracellular stores of FasL that are rapidly mobilized to the cell surface to trigger degranulation-independent, FasL-mediated lysis. This intracellular pool of FasL does not localize to the cytolytic granules, and mobilization of this stored FasL to the cell surface occurs independently of degranulation, as there were multiple circumstances whereby rapid FasL cell surface expression and FasL-dependent killing occurred in the absence of detectable degranulation. First, FasL can be rapidly expressed on CTL clones and *in vivo* primed CTL in the absence of extracellular Ca^{2+}, a condition that clearly inhibited degranulation. Second, I could not detect degranulation by cells stimulated with cross-linked anti-CD3 even though these cells mobilized stored FasL to the cell surface. Third, FasL translocation from the stored pool does not require calcineurin or microtubule rearrangement, which, however, are indispensible for degranulation upon target cell engagement. Fourth, CTL require both phorbol ester PMA and calcium ionophore ionomycin for triggering

degranulation, whereas either PMA alone or ionomycin alone is sufficient to elicit FasL translocation from the stored pool. Fifth, it is known that degranulation is BFA-resistant, whereas consistent with an earlier report (Li et al. 1998), I found that stored FasL cell surface expression is BFA-sensitive. Sixth, I detected rapid Fas-dependent target cell apoptosis in the absence of degranulation. Finally, I showed that FasL is stored in a subcompartment that has characteristics of recycling endosomes but are distinct from cytolytic granules, by both confocal microscopy and subcellular fractionation. Taken together, I provide substantial evidence to support my conclusion that FasL is stored and regulated independently of the cytolytic granules (Figure 6-1).

I further showed that there are two waves of FasL cell surface expression, at the population level, in response to cognate target cell binding: a rapid and transient, preformed FasL translocation, followed by a delayed, *de novo* FasL expression. This two-wave pattern of FasL expression occurs in both CTL clones and *in vivo* primed $CD8^+$ T cells. Further, I found that signaling for FasL expression appears to be finely tuned as a weak TCR signal in the form of crosslinked anti-CD3 elicited stored FasL translocation without subsequent FasL synthesis and degranulation; however if a strong stimulus was provided as plate bound anti-CD3, *de novo* FasL cell surface expression and degranulation occurred with little or no stored FasL cell surface translocation. I also found that Ca^{2+} flux from intracellular stores is necessary and sufficient for FasL cell surface expression from the stored pool, whereas *de novo* FasL cell surface expression requires Ca^{2+} flux from both intracellular and extracellular stores. These data

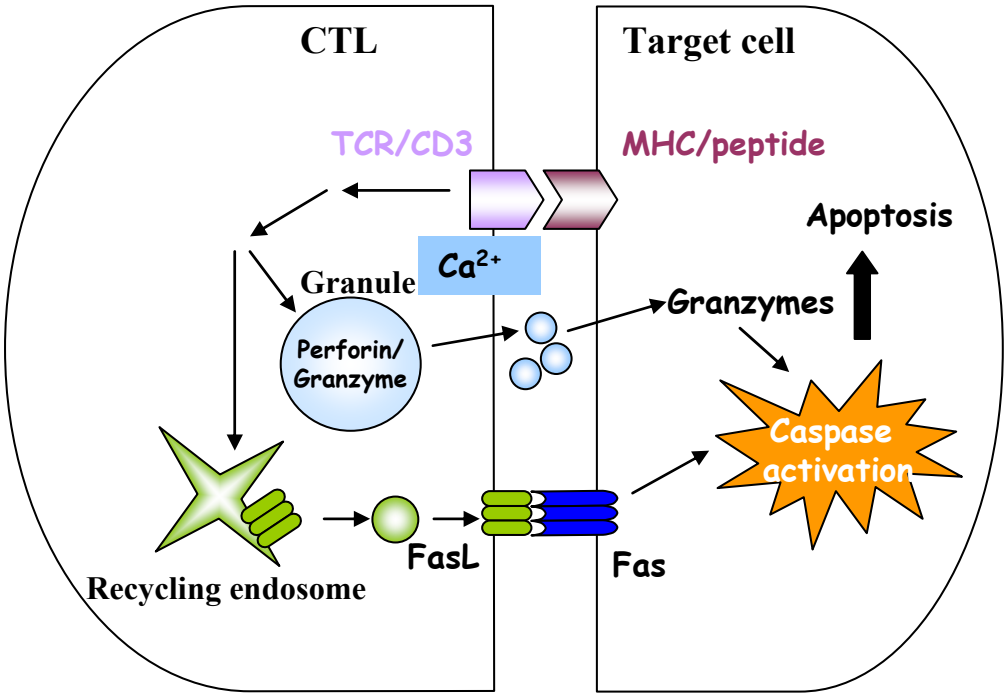


Figure 6-1. My model of the two mechanisms used by CTL to destroy target cell. In this model, FasL is stored in a subcompartment associated with the recycling endosomes, and TCR signaling regulated cell surface translocation of the stored FasL occurs independently of degranulation.

indicate that different from *de novo* FasL synthesis, minimum TCR signaling is required to elicit FasL translocation from stored pools. Furthermore, I quantitatively compared the signaling strength required for stored FasL translocation, *de novo* FasL cell surface expression and degranulation by directly titrating the levels of antigen presented by latex beads or target cells, and found that stored FasL was preferentially translocated over *de novo* FasL cell surface expression and degranulation at low levels of antigen stimulation. Of note, I found that an altered peptide ligand (APL) for OT-I TCR, which is unable to induce full TCRζ chain phosphorylation and calcium flux (Rosette et al. 2001), was capable of eliciting FasL expression primarily from the stored pool but limited degranulation. Taken together, these observations indicate that stored FasL translocation has a lower threshold of activation than *de novo* FasL synthesis and degranulation, suggesting that these killing mechanisms might be differentially controlled and have been selected to function under specific circumstances. Consistent with this hypothesis, I revealed that the stored, translocated FasL mediates specific, directional CTL-mediated killing, whereas the *de novo* synthesized FasL follows the constitutive secretory pathway and is distributed around the cell surface, inducing significant bystander killing. These data imply that FasL from these two pools may perform distinct roles in CTL mediated cytotoxicity.

An increasing body of evidence indicates the presence of pre-existing pools of FasL in activated mouse and human $CD8^+$ T cells and NK cells (Li et al. 1998; Bossi and Griffiths, 1999; Kojima et al. 2002; He and Ostergaard 2007;

Kassahn et al. 2009). However, the FasL storage compartment has remained elusive and even controversial. In this thesis, I characterized the FasL storage compartment by subcellular fractionation, confocal microscopy, and biochemical assays. In contrast to the reported ER/ Golgi or lysosomal associated compartment localization, I described here, for the first time, that FasL is stored in BFA-sensitive compartment which may associate with perinuclear recycling endosomes in CTL. Stored FasL has fully mature Endo H resistant N-linked glycans, suggesting that FasL has already passed through late ER along the secretory pathway. Furthermore, I demonstrated that FasL vesicles are located adjacent to mitochondria and ER. So far, the physiological significance of this close proximity of localization is unclear. Given that low levels of intracellular Ca^{2+} mobilization is sufficient to elicit stored FasL cell surface translocation, I hypothesize that FasL vesicles are located in a special niche between ER and mitochondria to readily sense the Ca^{2+} flux upon T cell activation.

I found that stored FasL translocation to the cell surface is microtubule-independent, which is indispensible for the polarization of cytolytic granules towards the immunological synapse. However, the position of the FasL vesicles varied among individual CTL cells upon target cell engagement. Some were unpolarized, some were polarized towards the contact site but distal from the target cell contact zone. Only in around 10% of CTL did I find a portion of FasL vesicles close to the CTL membrane at the immunological synapse. It is possible that FasL translocates by means other than docking of the entire FasL vesicle pool at the plasma membrane, a possibility that needs to be addressed in the future.

B. Major contributions

In contrast to the intense research on the function of FasL/Fas under both physiological and pathological conditions, the regulation of FasL cell surface expression, especially post-translational regulation, in CTL is largely unknown and even controversial. In this thesis, I revealed for the first time that there are two waves of FasL cell surface expression upon target cell engagement, which are differentially regulated by TCR signaling and perform distinct roles in CTL mediated responses. I provide substantial evidence supporting my conclusions that CTL degranulation and FasL lytic mechanisms are fully independent with respect to stored component localization and regulation, in contrast to the current model in which FasL is stored in cytolytic granules and degranulation controls FasL cell surface expression (Bossi and Griffiths 1999). Finally, I suggest that FasL is stored in a recycling endosome-associated compartment, which locates in a special niche between the ER and mitochondria, and use a novel microtubule-independent secretory mechanism to translocate to the plasma membrane.

Two waves of FasL cell surface expression

There are apparently conflicting studies in the literature describing the control of FasL expression on the CTL cell surface. FasL up-regulation was shown to require extracellular Ca^{2+} (Vignaux et al. 1995; Glass et al. 1996); however, the lytic phase of FasL-mediated lysis could occur in the absence of extracellular Ca^{2+}. FasL up-regulation was also shown to require protein synthesis (Walsh et al. 1994b; Vignaux et al. 1995; Glass et al. 1996); however,

Li et al. suggested that FasL expression on *ex vivo* CTL did not require protein synthesis (Li et al. 1998). In this thesis, I resolved this long-standing controversy by showing that cell surface FasL is both expressed from existing pools and newly synthesized protein after TCR stimulation (He and Ostergaard 2007), similar to what has been reported after phorbol ester and calcium ionophore treatment of human CTL (Lettau et al. 2004). I found that cell surface expression of stored FasL or *de novo* synthesized FasL depends on the presence of extracellular Ca^{2+}, time of analysis after TCR stimulation, and the strength of the TCR generated signal (He and Ostergaard 2007). I further revealed that not all CTLs are equal in their ability to mobilize preformed FasL to the cell surface upon target cell engagement, although they are capable of expressing significant level of *de novo* synthesized FasL, suggesting an additional level of regulation leading to cell surface transport of preformed FasL (He and Ostergaard).

CTL degranulation and FasL lytic mechanisms are fully independent

Functional studies suggest that FasL expression and degranulation are not purely redundant mechanisms for killing but may have been selected to function under specific circumstances. Consistent with this, distinct TCR signaling requirements for degranulation and FasL mediated cytotoxicity have been reported (Esser et al. 1996). Furthermore, CTL from Ashen mice are defective in degranulation, but are still able to kill via the FasL pathway (Haddad et al. 2001). However, based on confocal microscopy and FasL over-expression studies, Bossi and Griffiths suggested that pre-existing, along with newly synthesized FasL, is

sorted to the cytolytic perforin-containing granules in human CTL clone and NK cells, and that upon degranulation FasL is delivered to the cell surface (Bossi and Griffith 1999). In this thesis, I clearly show that the intracellular storage compartment for FasL is distinct from the previously described cytolytic granules in both murine and human CTL clones. Furthermore, functional data revealed that degranulation and surface FasL expression (from both stored pool and *de novo* synthesized pool) are under distinct control, further confirming the localization data. Therefore, I conclude that CTL degranulation and FasL lytic mechanisms are fully independent with respect to stored component localization and regulation. Recently, the group led by Brunner further confirmed my observations (Kassahn et al. 2009).

I showed that the translocation of the stored FasL to the cell surface is clearly signal regulated (He and Ostergaard 2007), in contrast to previous research suggesting that CTL express pre-existing FasL via the constitutive pathway of intracellular protein transport rather than by regulated secretion (Li et al. 1998). There is precedent for such a vesicle type. For example, the chemokine RANTES is found in distinct storage vesicles in human CTLs; these vesicles rapidly released the stored RANTES upon T cell activation, similar to what I detected with FasL (Catalfamo et al. 2004). However, the nature of the storage vesicles was not further characterized. Our CTL clone does not secrete RANTES upon TCR crosslinking (Appendix Figure 4), a situation under which rapid FasL translocation occurs, so I could not determine whether RANTES and FasL are stored in the same compartment in CTLs. Also, an intracellular compartment

containing the chemokine receptor CXCR1, which is distinct from both cytolytic granules and the RANTES storage compartment, has been described in human effector and memory $CD8^+$ T cells (Gasser et al. 2005). This CXCR1 storage compartment is mobilized rapidly to the cell surface upon exposure to neutrophil-derived inflammatory mediators but not TCR stimulation (Gasser et al. 2005). Therefore, CTL may contain distinct storage vesicles, with more diversity than initially anticipated, that are independently regulated downstream of the TCR and/or other cell surface receptors.

It is not clear why my results (He and Ostergaard 2007) differ from those of Bossi and Griffiths (Bossi and Griffiths 1999). It is possible that FasL could be stored in multiple locations within the cell. Close inspection of their images reveals that there are several regions of non-overlap between FasL and granule staining, which the authors attributed to the localization of FasL along the secretory pathway (Bossi and Griffiths 1999). It is also possible that there is heterogeneity of killer cells and some CTL populations have more FasL granule localization than others. I speculate it is not a species issue, given that FasL vesicles segregate from cytolytic granules in both murine and human CTL that I examined. Recently, it was reported that upon target cell engagement, polarized cytotoxic granules in CTLs need further maturation steps at the T cell-target cell contact point, in which perforin/ granzymes-containing granules fuse with an "exocytic vesicle", which in turn is formed by the fusion of late lysosomes with the recycling endosomes allowing for degranulation (Ménager et al. 2007). Given the close association of FasL with the recycling endosomes, it is possible that at

some point, FasL vesicles do meet cytolytic granules, presumably depending on the type and the activation/maturation state of the cell. Therefore, it is possible that FasL could be stored in multiple locations within the cell, however my functional data indicate that FasL that mediates target cell killing resides primarily outside of the cytolytic granules in the CTL I examined.

FasL translocation from intracellular stores has a lower threshold of activation than de novo FasL synthesis and degranulation

Previous reports indicated that activation of Fas-dependent cytotoxicity required weaker TCR signaling than activation of perforin-dependent killing or cytokine production. Cao et al. initially reported that an influenza-specific $CD8^+$ T cells partially activated by a self-derived peptide killed only by the FasL/Fas pathway but not degranulation (Cao et al. 1995). Also, epitope modification or blocking of CD8 binding results in decreased TCR-ligand binding and CD3 phophorylation and greatly impaired calcium mobilization, degranulation of CTL, and IFN-γ release with no effect on Fas-dependent cytotoxicity (Kessler et al. 1998; Guillaume et al. 2003). These studies indicate that under suboptimal antigen activation, FasL-dependent cytotoxicity might be preferentially induced in CTL mediated killing, although the source of FasL (stored versus *de novo* synthesized) contributing to such cytotoxicity was not investigated.

In this thesis, I clearly show that stored FasL cell surface translocation has a lower threshold of activation than *de novo* FasL synthesis and degranulation. Therefore, the reported FasL mediated cytotoxicity induced by suboptimal TCR

signaling might be mainly contributed by FasL from the stored pool. Interfering with antigen presentation is one strategy employed by some viruses and tumors (Restifo et al. 1993; Hengel and Koszinowski 1997; Ehrlich 1997; Matsui et al. 2002) to escape the host immune response. The sensitive nature of the stored FasL response upon suboptimal antigen presentation could thereofore contribute significantly to the anti-viral and anti-tumor immunity when class I MHC expression or antigen presentation is down-modulated.

Why do CTLs express two distinct waves of FasL after target cell engagement? I showed that the stored, translocated FasL mediates specific, directional CTL-mediated killing, whereas the *de novo* synthesized FasL, which followed the constitutive secretory pathway and was distributed around the cell surface and induced significant bystander killing. These data combined with the different signaling threshold observations indicate that stored FasL might be employed during low level infection and may be required to clear the final pathogens after infection to play a preferential role during low level chronic infections. In contrast, when there is a massive virus infection, degranulation and *de novo* FasL synthesis will play crucial roles in virus clearance. In this circumstance, a significant amount of bystander killing of innocent cells would occur, inducing inflammatory tissue damage because of the constitutively expressed FasL on the cell surface. Indeed, recent studies have suggested that FasL may be important for clearing chronic infections (Rode et al. 2004; Zelinskyy et al. 2004), and may contribute, along with the perforin pathway, to the shaping of the diversity of escape variants of influenza (Price et al. 2005). It

would be of interest to investigate the contribution from particular FasL pools (stored versus *de novo* synthesised) for the antiviral immunity during these infections.

C. Future directions

FasL expression and function in CTL during chronic infection

It was suggested that degranulation plays a key role in clearing acute infections. However, recent evidence demonstrated that FasL may be important for clearing chronic infections (Rode et al. 2004; Zelinskyy et al. 2004). In addition, increasing evidence has suggested a role of FasL in maintaining T cell homeostasis by eliminating chronically activated foreign antigen-specific or autoreactive T cells (Stranges et al. 2007; Hughes et al. 2008; Weant et al. 2008; Mabrouk et al. 2008). Taken together, these data support the involvement of FasL in chronically activated T cell responses.

A number of studies have reported that chronic viral infections often result in T cell exhaustion, in which the virus-specific T cells display impaired proliferation, cytokine secretion, and degranulation (Shin and Wherry 2007). Unfortunately, the expression profile of FasL was not systemically examined in this context. Recently, Wherry and colleagues reported that although exhausted $CD8^+$ T cells have a deficiency in perforin expression, FasL mRNA expression was substantially elevated by these T cells during chronic LCMV infection, however the protein level was not investigated (Wherry et al. 2007). Furthermore, CTL specific for immunodominant epitopes were shown to be prone to functional

exhaustion earlier than other less-immunogenic epitopes (Wherry et al. 2003), indicating persistent antigenic presence might cause the adaptive immune system to attenuate the CTL cytolytic response to high-affinity antigens. These data, combined with my observations that stored FasL has a lower threshold of activation than *de novo* FasL synthesis and degranulation, indicate that stored FasL might play key roles in CTL response during chronic infection. It has been reported that FasL is important for the clearance of chronic Friend retrovirus infection, during which activated $CD8^+$ T cells from infected mice were deficient in cytolytic molecule expression and degranulation (Zelinskyy et al. 2004). Therefore, it will be of interest in the future to determine how the expression of FasL (both stored and *de novo* synthesized) corresponds with different states of viral-specific $CD8^+$ T cell differentiation during acute and chronic viral infections, and to examine their potential involvement in pathogen clearance, T cell homeostasis, inflammation and co-stimulation, in the model systems such as the acute and chronic LCMV infection or Friend retrovirus infection. Such studies may also provide insight into the distinct functions of the stored and *de novo* synthesized FasL during chronic viral infections.

Molecular factors that dictate distinct FasL trafficking and localization

In this thesis, I showed that stored FasL undergoes TCR regulated exocytosis whereas *de novo* synthesized FasL cell surface expression upon target cell engagement follows the constitutive secretion pathway, demonstrating distinct intracellular trafficking mechanisms between these two pools in CTL.

Interestingly, we also found that when FasL is expressed in COS cells the FasL goes directly to the cell surface, however when it is expressed in CTL, the FasL localizes to the storage vesicles, indicating FasL may be differentially sorted in different cell types (He, Hu, Clementin and Ostergaard, unpublished data). So far, the molecular mechanisms that control the distinct FasL trafficking is undefined. In the current study, I found distinct MMP sensitivity between the two pools of FasL in CTL (Appendix Figure 2). I also observed FasL with different molecular weights before and after immobilized anti-CD3 stimulation of CTL by immunoblotting (Appendix Figure 5), which might either reflect the status of newly synthesized protein moving along the way towards maturation, or result from different modification such as glycosylation or phosphorylation. I further found that the transfected FasL in COS cells exhibit different molecular weight compared to the endogonous stored FasL in AB.1 (Appendix Figure 6). Also, murine CTL clone CTLL-2 does not translocate early FasL to the cell surface although it has stored FasL. I found that stored FasL from CTLL-2 also exhibits a different molecular weight compared to the stored FasL in AB.1 (Appendix Figure 6). Therefore, it is possible that FasL molecules from different pools in CTL or from different cell types might undergo different posttranslational modification which mediates their distinct trafficking patterns, a possibility that needs to be investigated in the future. Given the multifunctionality of the Fas/FasL system, it would also be worth examining if some posttranslational modification of FasL, especially the extracellular domain, will lead to distinct outcomes on Fas^+ target cells in terms of apoptosis versus activation.

To characterize the intracellular trafficking of the stored FasL in CTL, we have recently constructed a number of epitope-tagged FasL mutants and chimeras aimed at identifying the elements in FasL that direct its trafficking to its unique storage compartment, which will provide insight into the sequence/motifs that regulate intracellular trafficking of the stored FasL. So far, some studies have shown that the proline rich domain of the intracellular tail is important for FasL intracellular retention in the rat basophilic leukaemia cell line RBL and 293T cells (Blott et al. 2001; Baum et al. 2005; Lettau et al. 2006). It is necessary to examine if this domain is also involved in FasL trafficking in CTL. It would also be worth examining if FasL extracellular domain is involved in FasL intracellular trafficking in CTL, which has not been described so far. Taken together, this information will provide insight into the molecular aspects of FasL that might dictate its trafficking.

Characterization of FasL storage compartment and regulatory molecules involved in its trafficking

The localization of stored FasL in CTL has remained elusive and even controversial. I recently described FasL might be stored in a subcompartment associated with recycling endosomes. One of the future directions will be aimed at characterizing the FasL storage compartment, and further deciphering the molecular and cellular mechanisms that regulate intracellular trafficking of the stored FasL. To characterize the FasL storage compartment, we could purify the FasL vesicles by immunoisolation, and identify proteins, for example, SNAREs

and effector proteins, that are found in the FasL compartment by proteomics, which may provide insight into the regulation of trafficking of these vesicles. A functional RNAi screen, which focuses on the genes such as that encode ARF proteins, ARF GEFs, Rab proteins, SNAREs and effector proteins, would provide further insight into the regulation of stored FasL cell surface delivery upon target cell engagement. If we can identify a regulatory element that controls FasL translocation such as a particular Rab protein, that does not impact *de novo* FasL expression, we could use this as a tool to probe the *in vivo* function of stored FasL without interfering with other FasL functions.

Cellular mechanism of stored FasL cell surface delivery

In this thesis, I provide evidence to suggest that stored FasL translocation mediates directional killing. However, I did not observe significant FasL vesicle accumulation at the immunological synapse upon target cell engagement. Therefore, I was unable to decipher the cellular mechanisms underlying FasL translocation from the stored pool. During regulated exocytosis, the entire secretory granule can fuse directly with the plasma membrane for release. This pathway, for example, is used by exocytosis of cytolytic granules in CTL. Alternatively, cargo can be transported through piecemeal exocytosis, in which small secretory vesicles bud from the larger secretory granule, and fuse with the plasma membrane, resulting in selective release. Piecemeal exocytosis has been described in cells such as eosinophils, mast cells, basophils and other types of secretory cells (Crivellato et al. 2003; Melo et al. 2008). There is recent evidence

to suggest a mechanism of differential mobilization and membrane fusion of secretory vesicles versus granules; these two compartments possess a different set of SNARE and Rab molecules as vesicle fusion and transport-docking proteins, respectively (Moqbel and Coughlin 2006). Exocytosis of cytolytic granules is BFA-resistant in CTL. In contrast, it was shown that BFA inhibits vesicular transport-mediated piecemeal exocytosis by human eosinophils (Melo et al. 2005), a finding resembling FasL cell surface translocation that I described in chapter 5. So far, it is unknown whether piecemeal exocytosis is a general secretory mechanism, and, if so, whether it is involved in vesicular trafficking in T lymphocytes. It will be of interest to examine if FasL cell surface translocation employs a mechanism similar to piecemeal exocytosis, which could explain why there is no obvious FasL vesicle located close to the contact point between T cell and target cell.

In summary, the current study is aimed at understanding the regulation of FasL expression in CTL mediated response. Progress has been made with important findings in this thesis. However, there are many questions that remain unanswered. Future studies are required to fully understand the regulation of FasL expression, intracellular trafficking, cell surface translocation and potential biological functions by CTL mediated responses.

CHAPTER 7 : Bibliography

Advani RJ, Bae HR, Bock JB, Chao DS, Doung YC, Prekeris R, Yoo JS and Scheller RH. (1998). Seven novel mammalian SNARE proteins localize to distinct membrane compartments. J Biol Chem. 273: 10317-10324.

Alam SM, Davies GM, Lin CM, Zal T, Nasholds W, Jameson SC, Hogquist KA, Gascoigne NR and Travers PJ. (1999). Qualitative and quantitative differences in T cell receptor binding of agonist and antagonist ligands. Immunity. 10: 227-237.

Algeciras-Schimnich A, Shen L, Barnhart BC, Murmann AE, Burkhardt JK and Peter ME. (2002). Molecular ordering of the initial signaling events of CD95. Mol Cell Biol. 22: 207-220.

Amrani A, Verdaguer J, Serra P, Tafuro S, Tan R and Santamaria P. (2000). Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. Nature. 406: 739-742.

Anders N and Jürgens G. (2008). Large ARF guanine nucleotide exchange factors in membrane trafficking. Cell Mol Life Sci. 65: 3433-3445.

Ando K, Hiroishi K, Kaneko T, Moriyama T, Muto Y, Kayagaki N, Yagita H, Okumura K and Imawari M. (1997). Perforin, Fas/Fas ligand, and TNF-alpha pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. J Immunol. 158: 5283-5291.

Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, Squarcina P, Accornero P, Lozupone F, Lugini L, Stringaro A, Molinari A, Arancia G, Gentile M, Parmiani G and Fais S. (2002). Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. J Exp Med. 195: 1303-1316.

Anel A, Buferne M, Boyer C, Schmitt-Verhulst AM and Golstein P. (1994). T cell receptor-induced Fas ligand expression in cytotoxic T lymphocyte clones is blocked by protein tyrosine kinase inhibitors and cyclosporin A. Eur J Immunol. 24: 2469-2476.

Ang AL, Taguchi T, Francis S, Fölsch H, Murrells LJ, Pypaert M, Warren G and Mellman I. (2004). Recycling endosomes can serve as intermediates during transport from the Golgi to the plasma membrane of MDCK cells. J Cell Biol. 167: 531-543.

Ansel KM, Djuretic I, Tanasa B and Rao A. (2006). Regulation of Th2 differentiation and Il4 locus accessibility. Annu Rev Immunol. 24: 607-656.

Aoki K, Kurooka M, Chen JJ, Petryniak J, Nabel EG and Nabel GJ. (2001). Extracellular matrix interacts with soluble CD95L: retention and enhancement of cytotoxicity. Nat Immunol. 2: 333-337.

Askenasy N, Yolcu ES, Yaniv I and Shirwan H. (2005). Induction of tolerance using Fas ligand: a double-edged immunomodulator. Blood. 105: 1396-1404.

Atkinson EA, Barry M, Darmon AJ, Shostak I, Turner PC, Moyer RW and Bleackley RC. (1998). Cytotoxic T lymphocyte-assisted suicide. Caspase 3 activation is primarily the result of the direct action of granzyme B. J Biol Chem. 273: 21261-21266.

Aujla SJ, Dubin PJ and Kolls JK. (2007). Th17 cells and mucosal host defense. Semin Immunol. 19: 377-382.

Bajno L, Peng XR, Schreiber AD, Moore HP, Trimble WS and Grinstein S. (2000). Focal exocytosis of VAMP3-containing vesicles at sites of phagosome formation. J Cell Biol. 149: 697-706.

Balkow S, Kersten A, Tran TT, Stehle T, Grosse P, Museteanu C, Utermöhlen O, Pircher H, von Weizsäcker F, Wallich R, Müllbacher A and Simon MM. (2001). Concerted action of the FasL/Fas and perforin/granzyme A and B pathways is mandatory for the development of early viral hepatitis but not for recovery from viral infection. J Virol. 75: 8781-8891.

Barry M and Bleackley RC. (2002). Cytotoxic T lymphocytes: all roads lead to death. Nat Rev Immunol. 2: 401-409.

Barry M, Heibein JA, Pinkoski MJ, Lee SF, Moyer RW, Green DR and Bleackley

RC. (2000). Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol Cell Biol. 20: 3781-3794.

Baum W, Kirkin V, Fernández SB, Pick R, Lettau M, Janssen O and Zörnig M. (2005). Binding of the intracellular Fas ligand (FasL) domain to the adaptor protein PSTPIP results in a cytoplasmic localization of FasL. J Biol Chem. 280: 40012-40024.

Beal AM, Anikeeva N, Varma R, Cameron TO, Norris PJ, Dustin ML and Sykulev Y. (2008). Protein kinase C theta regulates stability of the peripheral adhesion ring junction and contributes to the sensitivity of target cell lysis by CTL. J Immunol. 181: 4815-4824.

Bentele M, Lavrik I, Ulrich M, Stösser S, Heermann DW, Kalthoff H, Krammer PH and Eils R. (2004). Mathematical modeling reveals threshold mechanism in CD95-induced apoptosis. J Cell Biol. 166: 839-851.

Beresford PJ, Xia Z, Greenberg AH and Lieberman J. (1999). Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. Immunity. 10: 585-594.

Berg NN, Puente LG, Dawicki W and Ostergaard HL. (1998). Sustained TCR signaling is required for mitogen-activated protein kinase activation and degranulation by cytotoxic T lymphocytes. J Immunol. 161: 2919-2924.

Bergmann-Leitner ES and Abrams SI. (2000). Differential role of Fas/Fas ligand interactions in cytolysis of primary and metastatic colon carcinoma cell lines by human antigen-specific $CD8^+$ CTL. J Immunol. 164: 4941-4954.

Berke G, Rosen D and Ronen D. (1993). Mechanism of lymphocyte-mediated cytolysis: functional cytolytic T cells lacking perforin and granzymes. Immunology. 78: 105-112.

Berrebi G, Takayama H and Sitkovsky MV. (1987). Antigen-receptor interaction requirement for conjugate formation and lethal-hit triggering by cytotoxic T lymphocytes can be bypassed by protein kinase C activators and Ca^{2+} ionophores. Proc Natl Acad Sci USA. 84: 1364-1368.

Blakely A, Gorman K, Ostergaard H, Svoboda K, Liu CC, Young JD and Clark

WR. (1987). Resistance of cloned cytotoxic T lymphocytes to cell-mediated cytotoxicity. J Exp Med. 166: 1070-1083.

Bloom GS and Brashear TA. (1989). A novel 58-kDa protein associates with the Golgi apparatus and microtubules. J Biol Chem. 264: 16083-16092.

Blott EJ, Bossi G, Clark R, Zvelebil M and Griffiths GM. (2001). Fas ligand is targeted to secretory lysosomes via a proline-rich domain in its cytoplasmic tail. J Cell Sci. 114: 2405-2416.

Blott EJ and Griffiths GM. (2002). Secretory lysosomes. Nat Rev Mol Cell Biol. 3: 122-131.

Bock JB, Matern HT, Peden AA and Scheller RH. (2001). A genomic perspective on membrane compartment organization. Nature. 409: 839-841.

Bodmer JL, Schneider P and Tschopp J. (2002). The molecular architecture of the TNF superfamily. Trends Biochem Sci. 27: 19-26.

Bodor J, Bodorova J, Bare C, Hodge DL, Young HA and Gress RE. (2002). Differential inducibility of the transcriptional repressor ICER and its role in modulation of Fas ligand expression in T and NK lymphocytes. Eur J Immunol. 32: 203-212.

Bonardelle D, Benihoud K, Kiger N and Bobé P. (2005). B lymphocytes mediate Fas-dependent cytotoxicity in MRL/lpr mice. J Leukoc Biol. 78: 1052-1059.

Bonifacino JS and Glick BS. (2004). The mechanisms of vesicle budding and fusion. Cell. 116: 153-166.

Bossi G and Griffiths GM. (1999). Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. Nat Med. 5: 90-96.

Bossi G and Griffiths GM. (2005). CTL secretory lysosomes: biogenesis and secretion of a harmful organelle. Semin Immunol. 17: 87-94.

Bossi G, Trambas C, Booth S, Clark R, Stinchcombe J and Griffiths GM. (2002). The secretory synapse: the secrets of a serial killer. Immunol Rev. 189: 152-160.

Boursalian TE and Fink PJ. (2003). Mutation in fas ligand impairs maturation of

thymocytes bearing moderate affinity T cell receptors. J Exp Med. 198: 349-360.

Burgess TL and Kelly RB. (1987). Constitutive and regulated secretion of proteins. Annu Rev Cell Biol. 3: 243-293.

Burkhardt JK, Hester S, Lapham CK and Argon Y. (1990). The lytic granules of natural killer cells are dual-function organelles combining secretory and prelysosomal compartments. J Cell Biol. 111:2327-40.

Caldwell SA, Ryan MH, McDuffie E and Abrams SI. (2003). The Fas/Fas ligand pathway is important for optimal tumor regression in a mouse model of CTL adoptive immunotherapy of experimental CMS4 lung metastases. J Immunol. 171: 2402-2412.

Cao W, Tykodi SS, Esser MT, Braciale VL and Braciale TJ. (1995). Partial activation of $CD8^+$ T cells by a self-derived peptide. Nature. 378: 295-298.

Casanova JE. (2007). Regulation of Arf activation: the Sec7 family of guanine nucleotide exchange factors. Traffic. 8: 1476-1485.

Catalfamo M, Karpova T, McNally J, Costes SV, Lockett SJ, Bos E, Peters PJ and Henkart PA. (2004). Human $CD8^+$ T cells store RANTES in a unique secretory compartment and release it rapidly after TcR stimulation. Immunity. 20: 219-230.

Chakraborty M, Abrams SI, Camphausen K, Liu K, Scott T, Coleman CN and Hodge JW. (2003). Irradiation of tumor cells up-regulates Fas and enhances CTL lytic activity and CTL adoptive immunotherapy. J Immunol. 170: 6338-6347.

Chinnaiyan AM, O'Rourke K, Tewari M and Dixit VM. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell. 81: 505-512.

Chow WA, Fang JJ and Yee JK. (2000). The IFN regulatory factor family participates in regulation of Fas ligand gene expression in T cells. J Immunol. 164: 3512-3518.

Claude A, Zhao BP, Kuziemsky CE, Dahan S, Berger SJ, Yan JP, Armold AD,

Sullivan EM and Melancon P. (1999). GBF1: a novel Golgi-associated BFA resistant guanine nucleotide exchange factor that displays specificity for ADP-ribosylation factor 5. J Cell Biol. 146: 71-84.

Crivellato E, Nico B, Mallardi F, Beltrami CA and Ribatti D. (2003). Piecemeal degranulation as a general secretory mechanism? Anat Rec A Discov Mol Cell Evol Biol. 274: 778-784.

Curtsinger JM, Lins DC, Johnson CM, Mescher MF. (2005). Signal 3 tolerant CD8 T cells degranulate in response to antigen but lack granzyme B to mediate cytolysis. J Immunol. 175: 4392-4399.

Daniel PT and Krammer PH. (1994). Activation induces sensitivity towards APO-1 (CD95)-mediated apoptosis in human B cells. J Immunol. 152: 5624-5632.

Daro E, van der Sluijs P, Galli T and Mellman I. (1996). Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. Proc Natl Acad Sci USA. 93: 9559-9564.

Davis MM, Krogsgaard M, Huse M, Huppa J, Lillemeier BF and Li QJ. (2007). T cells as a self-referential, sensory organ. Annu Rev Immunol. 25: 681-695.

Desbarats J, Duke RC and Newell MK. (1998). Newly discovered role for Fas ligand in the cell-cycle arrest of $CD4^+$ T cells. Nat Med. 4: 1377-1382.

Dhein J, Walczak H, Baumler C, Debatin KM and Krammer PH. (1995). Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature. 373: 438-441.

Dobrzanski MJ, Reome JB, Hollenbaugh JA, Hylind JC and Dutton RW. (2004). Effector cell-derived lymphotoxin alpha and Fas ligand, but not perforin, promote Tc1 and Tc2 effector cell-mediated tumor therapy in established pulmonary metastases. Cancer Res. 64: 406-414.

D'Souza-Schorey C and Chavrier P. (2006). ARF proteins: roles in membrane traffic and beyond. Nat Rev Mol Cell Biol. 7: 347-358.

Durairaj M, Sharma R, Varghese JC and Kane KP. (2003). Requirement for Q226, but not multiple charged residues, in the class I MHC CD loop/D strand for TCR-activated CD8 accessory function. Eur J Immunol. 33: 676-684.

Dutz JP, Fruman DA, Burakoff SJ and Bierer BE. (1993). A role for calcineurin in degranulation of murine cytotoxic T lymphocytes. J Immunol. 150: 2591-2598.

Ehrlich R. (1997). Modulation of antigen processing and presentation by persistent virus infections and in tumors. Hum Immunol. 54: 104-116.

Eischen CM, Williams BL, Zhang W, Samelson LE, Lynch DH, Abraham RT and Leibson PJ. (1997). ZAP-70 tyrosine kinase is required for the up-regulation of Fas ligand in activation-induced T cell apoptosis. J Immunol. 159: 1135-1139.

Esser MT, Haverstick DM, Fuller CL, Gullo CA and Braciale VL. (1998). Ca^{2+} signaling modulates cytolytic T lymphocyte effector functions. J Exp Med. 187: 1057-1067.

Esser MT, Krishnamurthy B and Braciale VL. (1996). Distinct T cell receptor signaling requirements for perforin- or FasL-mediated cytotoxicity. J Exp Med. 183: 1697-1706.

Faris M, Latinis KM, Kempiak SJ, Koretzky GA and Nel A. (1998). Stress-induced Fas ligand expression in T cells is mediated through a MEK kinase 1-regulated response element in the Fas ligand promoter. Mol Cell Biol. 18: 5414-5424.

Faroudi M, Utzny C, Salio M, Cerundolo V, Guiraud M, Muller S and Valitutti S. (2003). Lytic versus stimulatory synapse in cytotoxic T lymphocyte/target cell interaction: manifestation of a dual activation threshold. Proc Natl Acad Sci USA. 100: 14145–14150.

Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, Lambert N, Ouachée-Chardin M, Chedeville G, Tamary H, Minard-Colin V, Vilmer E, Blanche S, Le Deist F, Fischer A and de Saint Basile G. (2003). Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell. 115: 461-473.

Gao YS, Alvarez C, Nelson DS and Sztul E. (1998). Molecular cloning,

characterization, and dynamics of rat formiminotransferase cyclodeaminase, a Golgi-associated 58-kDa protein. J Biol Chem. 273: 33825-33834.

Gasser O, Missiou A, Eken C and Hess C. (2005). Human $CD8^+$ T cells store CXCR1 in a distinct intracellular compartment and up-regulate it rapidly to the cell surface upon activation. Blood. 106: 3718-3724.

Germain RN and Stefanová I. (1999). The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. Annu Rev Immunol. 17: 467-522.

Ghadimi MP, Sanzenbacher R, Thiede B, Wenzel J, Jing Q, Plomann M, Borkhardt A, Kabelitz D and Janssen O. (2002). Identification of interaction partners of the cytosolic polyproline region of CD95 ligand (CD178). FEBS Lett. 519: 50-58.

Giese T and Davidson WF. (1994). Chronic treatment of C3H-lpr/lpr and C3H-gld/gld mice with anti-CD8 monoclonal antibody prevents the accumulation of double negative T cells but not autoantibody production. J Immunol. 152: 2000-2010.

Gismondi A, Cifaldi L, Mazza C, Giliani S, Parolini S, Morrone S, Jacobelli J, Bandiera E, Notarangelo L and Santoni A. (2004). Impaired natural and CD16-mediated NK cell cytotoxicity in patients with WAS and XLT: ability of IL-2 to correct NK cell functional defect. Blood. 104: 436-443.

Glass A, Walsh CM, Lynch DH and Clark WR. (1996). Regulation of the Fas lytic pathway in cloned CTL. J Immunol. 156: 3638-3644.

Gonzalez-Garcia A, R-Borlado L, Leonardo E, Merida I, Martinez-A C and Carrera AC. (1997). Lck is necessary and sufficient for Fas-ligand expression and apoptotic cell death in mature cycling T cells. J Immunol. 158: 4104-4112.

Gremion C, Grabscheid B, Wölk B, Moradpour D, Reichen J, Pichler W and Cerny A. (2004). Cytotoxic T lymphocytes derived from patients with chronic hepatitis C virus infection kill bystander cells via Fas-FasL interaction. J Virol. 78: 2152-2157.

Griffith TS, Brunner T, Fletcher SM, Green DR and Ferguson TA. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. Science. 270: 1189-1192.

Griffith TS and Ferguson TA. (1997). The role of FasL-induced apoptosis in immune privilege. Immunol Today. 18: 240-244.

Guillaume P, Legler DF, Boucheron N, Doucey MA, Cerottini JC and Luescher IF. (2003). Soluble major histocompatibility complex-peptide octamers with impaired CD8 binding selectively induce Fas-dependent apoptosis. J Biol Chem. 278: 4500-4509.

Guo Z, Zhang M, Tang H and Cao X. (2005). Fas signal links innate and adaptive immunity by promoting dendritic-cell secretion of CC and CXC chemokines. Blood. 106: 2033-2041.

Gupta M, Greer P, Mahanty S, Shieh WJ, Zaki SR, Ahmed R and Rollin PE. (2005). CD8-mediated protection against Ebola virus infection is perforin dependent. J Immunol. 174: 4198-4202.

Haddad EK, Wu X, Hammer JA 3rd and Henkart PA. (2001). Defective granule exocytosis in Rab27a-deficient lymphocytes from Ashen mice. J Cell Biol. 152: 835-842.

Hahne M, Rimoldi D, Schroter M, Romero P, Schreier M, French LE, Schneider P, Bornand T, Fontana A, Lienard D et al. (1996). Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. Science. 274: 1363-1366.

Hane M, Lowin B, Peitsch M, Becker K and Tschopp J. (1995). Interaction of peptides derived from the Fas ligand with the Fyn-SH3 domain. FEBS Lett. 373: 265-268.

Hao Z, Hampel B, Yagita H and Rajewsky K. (2004). T cell-specific ablation of Fas leads to Fas ligand-mediated lymphocyte depletion and inflammatory pulmonary fibrosis. J Exp Med. 199: 1355-1365.

Harty JT and Badovinac VP. (2008). Shaping and reshaping $CD8^+$ T-cell memory. Nat Rev Immunol. 8: 107-119.

Hayashi T, Rizzuto R, Hajnoczky G and Su TP. (2009). MAM: more than just a

housekeeper. Trends Cell Biol. 19: 81-88.

Hayes SM, Li L and Love PE. (2005). TCR signal strength influences alphabeta/ gammadelta lineage fate. Immunity. 22: 583-593.

He JS and Ostergaard HL. (2007). CTLs contain and use intracellular stores of FasL distinct from cytolytic granules. J Immunol. 179: 2339-2348.

Helgason CD, Prendergast JA, Berke G and Bleackley RC. (1992). Peritoneal exudate lymphocyte and mixed lymphocyte culture hybridomas are cytolytic in the absence of cytotoxic cell proteinases and perforin. Eur J Immunol. 22: 3187-3190.

Hengel H and Koszinowski UH. (1997). Interference with antigen processing by viruses. Curr Opin Immunol. 9: 470-476.

Henkart PA. (1994). Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. Immunity. 1: 343-346.

Hohlbaum AM, Moe S and Marshak-Rothstein A. (2000). Opposing effects of transmembrane and soluble Fas ligand expression on inflammation and tumor cell survival. J Exp Med. 191: 1209-1220.

Huang DC, Tschopp J and Strasser A. (2000). Bcl-2 does not inhibit cell death induced by the physiological Fas ligand: implications for the existence of type I and type II cells. Cell Death Differ. 7: 754-755.

Hughes PD, Belz GT, Fortner KA, Budd RC, Strasser A and Bouillet P. (2008). Apoptosis regulators Fas and Bim cooperate in shutdown of chronic immune responses and prevention of autoimmunity. Immunity. 28: 197-205.

Huse M, Lillemeier BF, Kuhns MS, Chen DS and Davis MM. (2006). T cells use two directionally distinct pathways for cytokine secretion. Nat Immunol. 7: 247-255.

Ibuki N, Yamamoto K, Yabushita K, Okano N, Okamoto R, Shimada N, Hakoda T, Mizuno M, Higashi T and Tsuji T (2002). In situ expression of Granzyme B and Fas-ligand in the liver of viral hepatitis. Liver. 22: 198-204.

Igney FH and Krammer PH. (2005). Tumor counterattack: fact or fiction? Cancer

Immunol Immunother. 54: 1127-1136.

Isaaz S, Baetz K, Olsen K, Podack E and Griffiths GM. (1995). Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway. Eur J Immunol. 25: 1071-1079.

Itoh N, Imagawa A, Hanafusa T, Waguri M, Yamamoto K, Iwahashi H, Moriwaki M, Nakajima H, Miyagawa J, Namba M, Makino S, Nagata S, Kono N and Matsuzawa Y. (1997). Requirement of Fas for the development of autoimmune diabetes in nonobese diabetic mice. J Exp Med. 186: 613-618.

Itoh Y and Germain RN. (1997). Single cell analysis reveals regulated hierarchical T cell antigen receptor signaling thresholds and intraclonal heterogeneity for individual cytokine responses of $CD4^+$ T cells. J Exp Med. 186: 757-766.

Jackson CL and Casanova JE. (2000). Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors. Trends Cell Biol. 10: 60-67.

Janssen O, Qian J, Linkermann A and Kabelitz D. (2003). CD95 ligand-death factor and costimulatory molecule? Cell Death Differ. 10: 1215-1225.

Jenkins MR, La Gruta NL, Doherty PC, Trapani JA, Turner SJ and Waterhouse NJ. (2009). Visualizing CTL activity for different $CD8^+$ effector T cells supports the idea that lower TCR/epitope avidity may be advantageous for target cell killing. Cell Death Differ. 16: 537-542.

Jerome KR, Sloan DD and Aubert M. (2003). Measuring T-cell-mediated cytotoxicity using antibody to activated caspase 3. Nat Med. 9: 4-5.

Jog NR, Rane MJ, Lominadze G, Luerman GC, Ward RA and McLeish KR. (2007). The actin cytoskeleton regulates exocytosis of all neutrophil granule subsets. Am J Physiol Cell Physiol. 292: C1690-1700.

Kabra NH, Kang C, Hsing LC, Zhang J and Winoto A. (2001). T cell-specific FADD-deficient mice: FADD is required for early T cell development. Proc Natl Acad Sci USA. 98: 6307-6312.

Kaech SM, Wherry EJ and Ahmed R. (2002). Effector and memory T-cell

differentiation: implications for vaccine development. Nat Rev Immunol. 2: 251-262.

Kägi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM and Hengartner H. (1994a). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature. 369: 31-37.

Kägi D, Vignaux F, Ledermann B, Bürki K, Depraetere V, Nagata S, Hengartner H and Golstein P. (1994b). Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science. 265: 528-530.

Kane KP, Champoux P and Mescher MF. (1989). Solid-phase binding of class I and II MHC proteins: immunoassay and T cell recognition. Mol Immunol. 26: 759-768.

Kane KP and Mescher MF. (1993). Activation of CD8-dependent cytotoxic T lymphocyte adhesion and degranulation by peptide class I antigen complexes. J Immunol. 150: 4788-4797.

Kane KP, Sherman LA and Mescher MF. (1989). Molecular interactions required for triggering alloantigen-specific cytolytic T lymphocytes. J Immunol. 142: 4153-4160.

Kang SM, Schneider DB, Lin Z, Hanahan D, Dichek DA, Stock PG and Baekkeskov S. (1997). Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. Nature Med. 3: 738-743.

Kassahn D, Nachbur U, Conus S, Micheau O, Schneider P, Simon HU and Brunner T. (2009). Distinct requirements for activation-induced cell surface expression of preformed Fas/CD95 ligand and cytolytic granule markers in T cells. Cell Death Differ. 16: 115-124.

Kavurma MM and Khachigian LM. (2003). Signaling and transcriptional control of Fas ligand gene expression. Cell Death Differ. 10: 36-44.

Kayagaki N, Kawasaki A, Ebata T, Ohmoto H, Ikeda S, Inoue S, Yoshino K, Okumura K and Yagita H. (1995). Metalloproteinase-mediated release of human Fas ligand. J Exp Med. 182: 1777-1783.

Keane MM, Ettenberg SA, Lowrey GA, Russell EK and Lipkowitz S. (1996). Fas expression and function in normal and malignant breast cell lines. Cancer Res. 56: 4791-4798.

Kessler B, Hudrisier D, Schroeter M, Tschopp J, Cerottini JC and Luescher IF. (1998). Peptide modification or blocking of CD8, resulting in weak TCR signaling, can activate CTL for Fas- but not perforin-dependent cytotoxicity or cytokine production. J Immunol. 161: 6939-6946.

Kidoya H, Umemura M, Kawabe T, Matsuzaki G, Yahagi A, Imamura R and Suda T. (2005). Fas ligand induces cell-autonomous IL-23 production in dendritic cells, a mechanism for Fas ligand-induced IL-17 production. J Immunol. 175: 8024-8031.

Kiener PA, Davis PM, Rankin BM, Klebanoff SJ, Ledbetter JA, Starling GC and Liles WC. (1997). Human monocytic cells contain high levels of intracellular Fas ligand: rapid release following cellular activation. J Immunol. 159: 1594-1598.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH and Peter ME. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. 14: 5579-5588.

Kischkel FC, Lawrence DA, Tinel A, LeBlanc H, Virmani A, Schow P, Gazdar A, Blenis J, Arnott D and Ashkenazi A. (2001). Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. J Biol Chem. 276: 46639-46646.

Kojima H, Shinohara N, Hanaoka S, Someya-Shirota Y, Takagaki Y, Ohno H, Saito T, Katayama T, Yagita H, Okumura K, et al. (1994). Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. Immunity. 1: 357-364.

Kojima Y, Kawasaki-Koyanagi A, Sueyoshi N, Kanai A, Yagita H and Okumura K. (2002). Localization of Fas ligand in cytoplasmic granules of $CD8^+$ cytotoxic T lymphocytes and natural killer cells: participation of Fas

ligand in granule exocytosis model of cytotoxicity. Biochem Biophys Res Commun. 296: 328-336.

Krammer PH, Arnold R and Lavrik IN. (2007). Life and death in peripheral T cells. Nat Rev Immunol. 7: 532-542.

Krammer PH, Galle PR, Möller P and Debatin KM. (1998). CD95(APO-1/Fas)-mediated apoptosis in normal and malignant liver, colon, and hematopoietic cells. Adv Cancer Res. 75: 251-273.

Kupfer A and Dennert G. (1984). Reorientation of the microtubule-organizing center and the Golgi apparatus in cloned cytotoxic lymphocytes triggered by binding to lysable target cells. J Immunol. 133: 2762-2766.

Kupfer A, Singer SJ and Dennert G. (1986). On the mechanism of unidirectional killing in mixtures of two cytotoxic T lymphocytes. Unidirectional polarization of cytoplasmic organelles and the membrane-associated cytoskeleton in the effector cell. J Exp Med. 163: 489-498.

Kurts C, Heath WR, Kosaka H, Miller JF and Carbone FR. (1998). The peripheral deletion of autoreactive $CD8^+$ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). J Exp Med. 188: 415-420.

Kuwano K and Arai S. (1996). Involvement of two distinct killing mechanisms in bystander target cell lysis induced by a cytotoxic T lymphocyte clone. Cell Immunol. 169: 288-293.

Lafleur EA, Jia SF, Worth LL, Zhou Z, Owen-Schaub LB and Kleinerman ES. (2001). Interleukin (IL)-12 and IL-12 gene transfer up-regulate Fas expression in human osteosarcoma and breast cancer cells. Cancer Res. 61: 4066-4071.

Latinis KM, Carr LL, Peterson EJ, Norian LA, Eliason SL and Koretzky GA. (1997). Regulation of CD95 (Fas) ligand expression by TCR-mediated signaling events. J Immunol. 158: 4602-4611.

Lavrik IN, Golks A, Riess D, Bentele M, Eils R and Krammer PH. (2007).

Analysis of CD95 threshold signaling: triggering of CD95 (FAS/APO-1) at low concentrations primarily results in survival signaling. J Biol Chem. 282: 13664-13671.

Lee KH, Feig C, Tchikov V, Schickel R, Hallas C, Schütze S, Peter ME and Chan AC. (2006). The role of receptor internalization in CD95 signaling. EMBO J. 25: 1009-1023.

Leithauser F, Dhein J, Mechtersheimer G, Koretz K, Bruderlein S, Henne C, Schmidt A, Debatin KM, Krammer PH and Moller P. (1993). Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells. Lab Invest. 69: 415-429.

Lenardo M, Chan KM, Hornung F, McFarland H, Siegel R, Wang J and Zheng L. (1999). Mature T lymphocyte apoptosis: immune regulation in a dynamic and unpredictable antigenic environment. Annu Rev Immunol. 17: 221-253.

Lettau M, Qian J and Janssen O. (2004). Activation-dependent FasL expression in T lymphocytes and natural killer cells. Signal Transduction. 5-6: 206-211.

Lettau M, Qian J, Linkermann A, Latreille M, Larose L, Kabelitz D and Janssen O. (2006). The adaptor protein Nck interacts with Fas ligand: Guiding the death factor to the cytotoxic immunological synapse. Proc Natl Acad Sci USA. 103: 5911-5916.

Li JH, Rosen D, Ronen D, Behrens CK, Krammer PH, Clark WR and Berke G. (1998). The regulation of CD95 ligand expression and function in CTL. J Immunol. 161: 3943-3949.

Liblau RS, Wong FS, Mars LT and Santamaria P. (2002). Autoreactive CD8 T cells in organ-specific autoimmunity: emerging targets for therapeutic intervention. Immunity. 17: 1-6.

Lieberman J and Fan Z. (2003). Nuclear war: the granzyme A-bomb. Curr Opin Immunol. 15: 553-559.

Locksley RM, Killeen N and Lenardo MJ. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell. 104: 487-501.

Lord SJ, Rajotte RV, Korbutt GS and Bleackley RC. (2003). Granzyme B: a natural born killer. Immunol Rev. 193: 31-38.

Lowin B, Hahne M, Mattmann C and Tschopp J. (1994). Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature. 370: 650-652.

Lozza L, Rivino L, Guarda G, Jarrossay D, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A and Geginat J. (2008). The strength of T cell stimulation determines IL-7 responsiveness, secondary expansion, and lineage commitment of primed human $CD4^{+}$IL-7Rhi T cells. Eur J Immunol. 38: 30-39.

Luik RM, Wang B, Prakriya M, Wu MM and Lewis RS. (2008). Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. Nature. 454: 538-542.

Luik RM, Wu MM, Buchanan J, Lewis RS. Luik RM, Wu MM, Buchanan J and Lewis RS. (2006). The elementary unit of store-operated Ca^{2+} entry: local activation of CRAC channels by STIM1 at ER-plasma membrane junctions. J Cell Biol. 174: 815-825.

Lyubchenko TA, Wurth GA and Zweifach A. (2001). Role of calcium influx in cytotoxic T lymphocyte lytic granule exocytosis during target cell killing. Immunity. 15: 847-859.

Ma Y, Liu H, Tu-Rapp H, Thiesen HJ, Ibrahim SM, Cole SM and Pope RM. (2004). Fas ligation on macrophages enhances IL-1R1-Toll-like receptor 4 signaling and promotes chronic inflammation. Nat Immunol. 5: 380-387.

Mabrouk I, Buart S, Hasmim M, Michiels C, Connault E, Opolon P, Chiocchia G, Lévi-Strauss M, Chouaib S and Karray S. (2008). Prevention of autoimmunity and control of recall response to exogenous antigen by Fas death receptor ligand expression on T cells. Immunity. 29: 922-933.

Maecker HL, Yun Z, Maecker HT and Giaccia AJ. (2002). Epigenetic changes in tumor Fas levels determine immune escape and response to therapy. Cancer Cell. 2: 139-148.

Manderson AP, Kay JG, Hammond LA, Brown DL and Stow JL. (2007).

Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFalpha. J Cell Biol. 178: 57-69.

Martínez-Lorenzo MJ, Anel A, Gamen S, Monle n I, Lasierra P, Larrad L, Piñeiro A, Alava MA and Naval J. (1999). Activated human T cells release bioactive Fas ligand and APO2 ligand in microvesicles. J Immunol. 163: 1274-1281.

Matsui M, Machida S, Itani-Yohda T, Akatsuka T. (2002). Downregulation of the proteasome subunits, transporter, and antigen presentation in hepatocellular carcinoma, and their restoration by interferon-γ. J Gastroenterol Hepatol. 17: 897-907.

Maxfield FR and McGraw TE. (2004). Endocytic recycling. Nat Rev Mol Cell Biol. 5: 121-132.

Melo RC, Spencer LA, Dvorak AM and Weller PF. (2008). Mechanisms of eosinophil secretion: large vesiculotubular carriers mediate transport and release of granule-derived cytokines and other proteins. J Leukoc Biol. 83: 229-236.

Melo RC, Spencer LA, Perez SA, Ghiran I, Dvorak AM and Weller PF. (2005). Human eosinophils secrete preformed, granule-stored interleukin-4 through distinct vesicular compartments. Traffic. 6: 1047-1057.

Ménager MM, Ménasché G, Romao M, Knapnougel P, Ho CH, Garfa M, Raposo G, Feldmann J, Fischer A and de Saint Basile G. (2007). Secretory cytotoxic granule maturation and exocytosis require the effector protein hMunc13-4. Nat Immunol. 8: 257-267.

Ménasché G, Pastural E, Feldmann J, Certain S, Ersoy F, Dupuis S, Wulffraat N, Bianchi D, Fischer A, Le Deist F and de Saint Basile G. (2000). Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. Nat Genet. 25: 173-176.

Metkar SS, Menaa C, Pardo J, Wang B, Wallich R, Freudenberg M, Kim S, Raja

SM, Shi L, Simon MM and Froelich CJ. (2008). Human and mouse granzyme A induce a proinflammatory cytokine response. Immunity. 29: 720-733.

Migueles SA, Osborne CM, Royce C, et al. (2008). Lytic granule loading of $CD8^+$ T cells is required for HIV-infected cell elimination associated with immune control. Immunity. 29: 1009-1021.

Montel AH, Bochan MR, Hobbs JA, Lynch DH and Brahmi Z. (1995). Fas involvement in cytotoxicity mediated by human NK cells. Cell Immunol. 166: 236-246.

Moqbel R and Coughlin JJ. (2006). Differential secretion of cytokines. Sci STKE. 338: 26.

Murphy KM and Reiner SL. (2002). The lineage decisionsof helper T cells. Nature Rev Immunol. 2: 933-944.

Murray RZ, Kay JG, Sangermani DG and Stow JL. (2005). A role for the phagosome in cytokine secretion. Science. 310: 1492-1495.

Nagata S and Golstein P. (1995). The Fas death factor. Science. 267: 1449-1456.

Nguyen LT, McKall-Faienza K, Zakarian A, Speiser DE, Mak TW and Ohashi PS. (2000). TNF receptor 1 (TNFR1) and CD95 are not required for T cell deletion after virus infection but contribute to peptide-induced deletion under limited conditions. Eur J Immunol. 30: 683-688.

Nguyen T and Russell J. (2001). The regulation of FasL expression during activation-induced cell death (AICD). Immunology. 103: 426-434.

O'Connell J, Bennett MW, O'Sullivan GC, Collins JK and Shanahan F. (1999). The Fas counterattack: cancer as a site of immune privilege. Immunol Today. 20: 46-52.

O'Connell J, Houston A, Bennett MW, O'Sullivan GC and Shanahan F. (2001). Immune privilege or inflammation? Insights into the Fas ligand enigma. Nat Med. 7: 271-274.

O'Connell J, O'Sullivan GC, Collins JK and Shanahan F. (1996). The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med. 184: 1075-1082.

Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T and Nagata S. (1993). Lethal effect of the anti-Fas antibody in mice. Nature. 364: 806-809.

Oprins A, Duden R, Kreis TE, Geuze HJ and Slot JW. (1993). Beta-COP localizes mainly to the cis-Golgi side in exocrine pancreas. J Cell Biol. 121: 49-59.

Orlinick JR, Elkon KB and Chao MV. (1997a). Separate domains of the human fas ligand dictate self-association and receptor binding. J Biol Chem. 272: 32221-32229.

Orlinick JR, Vaishnaw A, Elkon KB and Chao MV. (1997b). Requirement of cysteine-rich repeats of the Fas receptor for binding by the Fas ligand. J Biol Chem. 272: 28889-28894.

O'Rourke AM, Apgar JR, Kane KP, Martz E and Mescher MF. (1991). Cytoskeletal function in CD8- and T cell receptor-mediated interaction of cytotoxic T lymphocytes with class I protein. J Exp Med. 173: 241-249.

Ostergaard H and Clark WR. (1987). The role of Ca^{2+} in activation of mature cytotoxic T lymphocytes for lysis. J Immunol. 139: 3573-3579.

Ostergaard HL and Clark WR. (1989). Evidence for multiple lytic pathways used by cytotoxic T lymphocytes. J Immunol. 143: 2120-2126.

Ostergaard HL, Kane KP, Mescher MF and Clark WR. (1987). Cytotoxic T lymphocyte mediated lysis without release of serine esterase. Nature. 330: 71-72.

Ostler T, Davidson W and Ehl S. (2002). Virus clearance and immunopathology by CD8(+) T cells during infection with respiratory syncytial virus are mediated by IFN-gamma. Eur J Immunol. 32: 2117-2123.

Ouyang W, Kolls J and Zheng Y. (2008). The Biological Functions of T Helper 17 Cell Effector Cytokines in Inflammation. Immunity. 28: 454-467.

Owen-Schaub L, Chan H, Cusack JC, Roth J and Hill LL. (2000). Fas and Fas ligand interactions in malignant disease. Int J Oncol. 17: 5-12.

Park CY, Hoover PJ, Mullins FM, Bachhawat P, Covington ED, Raunser S, Walz

T, Garcia KC, Dolmetsch RE and Lewis RS. (2009). STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. Cell. 136: 876-890.

Parra, B., M. T. Lin, S. A. Stohlman, C. C. Bergmann, R. Atkinson, and D. R. Hinton. (2000). Contributions of Fas-Fas ligand interactions to the pathogenesis of mouse hepatitis virus in the central nervous system. J Virol. 74: 2447-2450.

Pearce EL, Mullen AC, Martins GA, Krawczyk CM, Hutchins AS, Zediak VP, Banica M, DiCioccio CB, Gross DA, Mao CA, Shen H, Cereb N, Yang SY, Lindsten T, Rossant J, Hunter CA and Reiner SL. (2003). Control of Effector $CD8^+$ T Cell Function by the Transcription Factor Eomesodermin. Science. 302: 1041-1043.

Peter ME, Budd RC, Desbarats J, Hedrick SM, Hueber A, Newell MK, Owen LB, Pope RM, Tschopp J, Wajant H, Wallach D, Wiltrout RH, Zornig M and Lynch DH. (2007). The CD95 receptor: apoptosis revisited. Cell. 129: 447-450.

Pfeffer S. (2003). Membrane domains in the secretory and endocytic pathways. Cell. 112: 507-517.

Pfeffer S and Aivazian D. (2004). Targeting Rab GTPases to distinct membrane compartments. Nat Rev Mol Cell Biol. 5: 886-896.

Pipkin ME and Lieberman J. (2007). Delivering the kiss of death: progress on understanding how perforin works. Curr Opin Immunol. 19: 301-308.

Price GE, Huang L, Ou R, Zhang M and Moskophidis D. (2005). Perforin and Fas cytolytic pathways coordinately shape the selection and diversity of $CD8^+$-T-cell escape variants of influenza virus. J Virol. 79:8545-8559.

Purbhoo MA, Irvine DJ, Huppa JB and Davis MM. (2004). T cell killing does not require the formation of a stable mature immunological synapse. Nat Immunol. 5: 524-530.

Qin H, Trudeau JD, Reid GS, Lee IF, Dutz JP, Santamaria P, Verchere CB and

Tan R. (2004). Progression of spontaneous autoimmune diabetes is associated with a switch in the killing mechanism used by autoreactive CTL. Int Immunol. 16: 1657-1662.

Refaeli Y, Vanparijs L, London CA, Tschopp J and Abbas AK. (1998). Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. Immunity. 8: 615-623.

Rehermann B and Nascimbeni M. (2005). Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol. 5: 215-229.

Reich A, Körner H, Sedgwick JD and Pircher H. (2000). Immune down-regulation and peripheral deletion of CD8 T cells does not require TNF receptor-ligand interactions nor CD95 (Fas, APO-1). Eur J Immunol. 30: 678-682.

Restifo NP. (2000). Not so Fas: re-evaluating the mechanisms of immune privilege and tumor escape. Nature Med. 6: 493–495.

Restifo NP, Esquivel F, Kawakami Y, Yewdell JW, Mulé JJ, Rosenberg SA and Bennink JR. (1993). Identification of human cancers deficient in antigen processing. J Exp Med. 177: 265–272.

Rieux-Laucat F, Le Deist F and Fischer A. (2003). Autoimmune lymphoproliferative syndromes: genetic defects of apoptosis pathways. Cell Death Differ. 10: 124-133.

Rizzuto R, Marchi S, Bonora M, Aguiari P, Bononi A, De Stefani D, Giorgi C, Leo S, Rimessi A, Siviero R, Zecchini E and Pinton P. (2009). Ca(2+) transfer from the ER to mitochondria: When, how and why. Biochim Biophys Acta. 2009 Mar 30.

Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA and Pozzan T. (1998). Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. Science. 280: 1763-1766.

Roberts AI, Devadas S, Zhang X, Zhang L, Keegan A, Greeneltch K, Solomon J, Wei L, Das J, Sun E, Liu C, Yuan Z, Zhou JN and Shi Y. (2003). The role of activation-induced cell death in the differentiation of T-helper-cell subsets. Immunol Res. 28: 285-293.

Rode M, Balkow S, Sobek V, Brehm R, Martin P, Kersten A, Dumrese T, Stehle T, Müllbacher A, Wallich R and Simon MM. (2004). Perforin and Fas act together in the induction of apoptosis, and both are critical in the clearance of lymphocytic choriomeningitis virus infection. J Virol. 78: 12395-12405.

Rogers PR and Croft M. (1999). Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. J Immunol. 163: 1205-1213.

Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Veliçelebi G and Stauderman KA. (2005). STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J Cell Biol. 169: 435-445.

Rosette C, Werlen G, Daniels MA, Holman PO, Alam SM, Travers PJ, Gascoigne NR, Palmer E and Jameson SC. (2001). The impact of duration versus extent of TCR occupancy on T cell activation: a revision of the kinetic proofreading model. Immunity. 15: 59-70.

Rouvier E, Luciani MF and Golstein P. (1993). Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. J Exp Med. 177: 195-200.

Russell JH and Ley TJ. (2002). Lymphocyte-mediated cytotoxicity. Annu Rev Immunol. 20: 323-370.

Russell MR, Nickerson DP and Odorizzi G. (2006). Molecular mechanisms of late endosome morphology, identity and sorting. Curr Opin Cell Biol. 18: 422-428.

Rutigliano JA and Graham BS. (2004). Prolonged production of TNF-alpha exacerbates illness during respiratory syncytial virus infection. J Immunol. 173: 3408-3417.

Sakaguchi S. (2005). Naturally arising Foxp3-expressing $CD25^{+}CD4^{+}$ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol. 6: 345-352.

Sancho D, Vicente-Manzanares M, Mittelbrunn M, Montoya MC, Gordón-Alonso

M, Serrador JM and Sánchez-Madrid F. (2002). Regulation of microtubule-organizing center orientation and actomyosin cytoskeleton rearrangement during immune interactions. Immunol Rev. 189: 84-97.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH and Peter ME. (1998). Two CD95 (APO-1/Fas) signaling pathways. EMBO J. 17: 1675-1687.

Schneider P, Holler N, Bodmer JL, Hahne M, Frei K, Fontana A and Tschopp J. (1998). Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. J Exp Med. 187: 1205-1213.

Seki N, Brooks AD, Carter CR, Back TC, Parsoneault EM, Smyth MJ, Wiltrout RH and Sayers TJ. (2002). Tumor-specific CTL kill murine renal cancer cells using both perforin and Fas ligand-mediated lysis *in vitro*, but cause tumor regression *in vivo* in the absence of perforin. J Immunol. 168: 3484-3492.

Shen A, Puente LG and Ostergaard HL. (2005). Tyrosine kinase activity and remodelling of the actin cytoskeleton are co-temporally required for degranulation by cytotoxic T lymphocytes. Immunology. 116: 276-286.

Shen X, Xu KF, Fan Q, Pacheco-Rodriguez G, Moss J and Vaughan M. (2006). Association of brefeldin A-inhibited guanine nucleotide-exchange protein 2 (BIG2) with recycling endosomes during transferrin uptake. Proc Natl Acad Sci USA. 103: 2635-2640.

Shin H and Wherry EJ. (2007). CD8 T cell dysfunction during chronic viral infection. Curr Opin Immunol. 19: 408-415.

Shlomchik WD. (2007). Graft-versus-host disease. Nat Rev Immunol. 7: 340-352.

Shrestha B and Diamond MS. (2007). Fas ligand interactions contribute to $CD8^+$ T-cell-mediated control of West Nile virus infection in the central nervous system. J Virol. 81: 11749-11757.

Shresta S, Graubert TA, Thomas DA, Raptis SZ and Ley TJ. (1999). Granzyme A initiates an alternative pathway for granule-mediated apoptosis. Immunity. 10: 595-605.

Siegel RM, Chan FK, Chun HJ, Lenardo MJ. Siegel RM, Chan FK, Chun HJ and Lenardo MJ. (2000). The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. Nat Immunol. 1: 469-474.

Smyth MJ. (1997). Fas ligand-mediated bystander lysis of syngeneic cells in response to an allogeneic stimulus. J Immunol. 158: 5765-5772.

Smyth MJ, Krasovskis E and Johnstone RW. (1998). Fas ligand-mediated lysis of self bystander targets by human papillomavirus-specific $CD8^+$ cytotoxic T lymphocytes. J Virol. 72: 5948-5954.

Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI and Trapani JA. (2000). Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med. 192: 755-760.

Sönnichsen B, De Renzis S, Nielsen E, Rietdorf J and Zerial M. (2000). Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol. 149: 901-914.

Starling GC, Bajorath J, Emswiler J, Ledbetter JA, Aruffo A and Kiener PA. (1997). Identification of amino acid residues important for ligand binding to Fas. J Exp Med. 185: 1487-1492.

Stinchcombe JC, Bossi G, Booth S and Griffiths GM. (2001a). The immunological synapse of CTL contains a secretory domain and membrane bridges. Immunity. 15: 751-761.

Stinchcombe JC, Barral DC, Mules EH, Booth S, Hume AN, Machesky LM, Seabra MC and Griffiths GM. (2001b). Rab27a is required for regulated secretion in cytotoxic T lymphocytes. J Cell Biol. 152:825-834.

Stinchcombe JC, Bossi G and Griffiths GM. (2004). Linking albinism and immunity: the secrets of secretory lysosomes. Science. 305: 55-59.

Stinchcombe JC and Griffiths GM. (1999). Regulated secretion from hemopoietic cells. J Cell Biol. 147: 1-6.

Stow JL, Manderson AP and Murray RZ. (2006). SNAREing immunity: the role of SNAREs in the immune system. Nat Rev Immunol. 6: 919-929.

Strand S, Hofmann WJ, Hug H, Muller M, Otto G, Strand D, Mariani SM,

Stremmel W, Krammer PH and Galle PR. (1996). Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells—a mechanism of immune evasion? Nat Med. 2: 1361-1366.

Stranges PB, Watson J, Cooper CJ, Choisy-Rossi CM, Stonebraker AC, Beighton RA, Hartig H, Sundberg JP, Servick S, Kaufmann G, Fink PJ and Chervonsky AV. (2007). Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity. Immunity. 26: 629-641.

Strasser A, O'Connor L and Dixit VM. (2000). Apoptosis signaling. Annu Rev Biochem. 69: 217-245.

Su X, Hu Q, Kristan JM, Costa C, Shen Y, Gero D, Matis LA and Wang Y. (2000). Significant role for Fas in the pathogenesis of autoimmune diabetes. J Immunol. 164: 2523-2532.

Suda T, Hashimoto H, Tanaka M, Ochi T and Nagata S. (1997). Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. J Exp Med. 186: 2045-2050.

Suda T, Okazaki T, Naito Y, Yokota T, Arai N, Ozaki S, Nakao K and Nagata S. (1995). Expression of the Fas ligand in cells of T cell lineage. J Immunol. 154: 3806-3813.

Suda T, Takahashi T, Golstein P and Nagata S. (1993). Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell. 75: 1169-1178.

Sun M, Ames KT, Suzuki I and Fink PJ. (2006). The cytoplasmic domain of Fas ligand costimulates TCR signals. J Immunol. 177: 1481-1491.

Sun M and Fink PJ. (2007). A new class of reverse signaling costimulators belongs to the TNF family. J Immunol. 179: 4307-4312.

Sun M, Lee S, Karray S, Levi-Strauss M, Ames KT and Fink PJ. (2007). Cutting edge: two distinct motifs within the Fas ligand tail regulate Fas ligand-mediated costimulation. J Immunol. 179: 5639-5643.

Suzuki I and Fink PJ. (1998). Maximal proliferation of cytotoxic T lymphocytes requires reverse signaling through Fas ligand. J Exp Med. 187: 123-128.

Suzuki I and Fink PJ. (2000). The dual functions of fas ligand in the regulation of peripheral $CD8^+$ and $CD4^+$ T cells. Proc Natl Acad Sci USA. 97: 1707-1712.

Suzuki I, Martin S, Boursalian TE, Beers C and Fink PJ. (2000). Fas ligand costimulates the *in vivo* proliferation of $CD8^+$ T cells. J Immunol. 165: 5537-5543.

Svensson M, Johansson-Lindbom B, Zapata F, Jaensson E, Austenaa LM, Blomhoff R and Agace WW. (2008). Retinoic acid receptor signaling levels and antigen dose regulate gut homing receptor expression on $CD8^+$ T cells. Mucosal Immunol. 1: 38-48.

Takayama H and Sitkovsky MV. (1987). Antigen receptor-regulated exocytosis in cytotoxic T lymphocytes. J Exp Med. 166: 725-743.

Tanaka M, Itai T, Adachi M and Nagata S. (1998). Downregulation of Fas ligand by shedding. Nat Med. 4: 31-36.

Teter K, Chandy G, Quiñones B, Pereyra K, Machen T and Moore HP. (1998). Cellubrevin-targeted fluorescence uncovers heterogeneity in the recycling endosomes. J Biol Chem. 273: 19625-19633.

Thastrup O, Cullen PJ, Drøbak BK, Hanley MR and Dawson AP. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. Proc Natl Acad Sci USA. 87: 2466-2470.

Togawa A, Morinaga N, Ogasawara M, Moss J and Vaughan M. (1999). Purification and cloning of a brefeldin A-inhibited guanine nucleotide-exchange protein for ADP-ribosylation factors. J Biol Chem. 274: 12308-12315.

Trambas CM and Griffiths GM. (2003). Delivering the kiss of death. Nat Immunol. 4: 399-403.

Trenn G, Takayama H and Sitkovsky MV. (1987). Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T-lymphocytes. Nature. 330: 72-74.

Trowbridge IS, Collawn JF and Hopkins CR. (1993). Signal-dependent membrane

protein trafficking in the endocytic pathway. Annu Rev Cell Biol. 9: 129-161.

Ullrich O, Reinsch S, Urbé S, Zerial M and Parton RG. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol. 135: 913-924.

Valitutti S, Müller S, Dessing M and Lanzavecchia A. (1996). Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. J Exp Med. 183: 1917-1921.

Vance JE. (1990). Phospholipid synthesis in a membrane fraction associated with mitochondria. J Biol Chem. 265: 7248-7256.

Vance JE. (1991). Newly made phosphatidylserine and phosphatidylethanolamine are preferentially translocated between rat liver mitochondria and endoplasmic reticulum. J Biol Chem. 266: 89-97.

van den Broek ME, Kägi D, Ossendorp F, Toes R, Vamvakas S, Lutz WK, Melief CJ, Zinkernagel RM and Hengartner H. (1996). Decreased tumor surveillance in perforin-deficient mice. J Exp Med. 184: 1781-1790.

van der Sluijs P, Hull M, Webster P, Goud B and Mellman I. (1992). The small GTP-binding protein rab4 controls an early sorting event on the endocytic pathway. Cell. 70: 729-740.

Vignaux F, Vivier E, Malissen B, Depraetere V, Nagata S and Golstein P. (1995). TCR/CD3 coupling to Fas-based cytotoxicity. J Exp Med. 181: 781-786.

Vizler C, Bercovici N, Cornet A, Cambouris C and Liblau RS. (1999). Role of autoreactive $CD8^+$ T cells in organ-specific autoimmune diseases: insight from transgenic mouse models. Immunol Rev. 169: 81-92.

Walsh CM, Matloubian M, Liu CC, Ueda R, Kurahara CG, Christensen JL, Huang MT, Young JD, Ahmed R and Clark WR. (1994a). Immune function in mice lacking the perforin gene. Proc Natl Acad Sci USA. 91: 10854-10858.

Walsh CM, Glass AA, Chiu V and Clark WR. (1994b). The role of the Fas lytic pathway in a perforin-less CTL hybridoma. J Immunol. 153: 2506-2514.

Walter U and Santamaria P. $CD8^+$ T cells in autoimmunity. (2005). Curr Opin

Immunol. 17: 624-631.

Wang J, Chun HJ, Wong W, Spencer DM and Lenardo MJ. (2001). Caspase-10 is an initiator caspase in death receptor signaling. Proc Natl Acad Sci USA. 98: 13884-13888.

Watanabe-Fukunaga R, Brannan CI, Itoh N, Yonehara S, Copeland NG, Jenkins NA and Nagata S. (1992). The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol. 148: 1274–1279.

Watts AD, Hunt NH, Wanigasekara Y, Bloomfield G, Wallach D, Roufogalis BD and Chaudhri G. (1999). A casein kinase I motif present in the cytoplasmic domain of members of the tumour necrosis factor ligand family is implicated in 'reverse signalling'. EMBO J. 18: 2119-2126.

Weant AE, Michalek RD, Khan IU, Holbrook BC, Willingham MC and Grayson JM. (2008). Apoptosis regulators Bim and Fas function concurrently to control autoimmunity and $CD8^+$ T cell contraction. Immunity. 28: 218-230.

Wenzel J, Sanzenbacher R, Ghadimi M, Lewitzky M, Zhou Q, Kaplan DR, Kabelitz D, Feller SM and Janssen O. (2001). Multiple interactions of the cytosolic polyproline region of the CD95 ligand: hints for the reverse signal transduction capacity of a death factor. FEBS Lett. 509: 255-262.

Wherry EJ and Ahmed R. (2004). Memory CD8 T-cell differentiation during viral infection. J Virol. 78: 5535-5545.

Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R and Ahmed R. (2003). Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol. 77: 4911-4927.

Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, Subramaniam S, Blattman JN, Barber DL and Ahmed R. (2007). Molecular signature of $CD8^+$ T cell exhaustion during chronic viral infection. Immunity. 27: 670-684.

Williams MA and Bevan MJ. (2007). Effector and memory CTL differentiation. Annu Rev Immunol. 25: 171-192.

Wolint P, Betts MR, Koup RA and Oxenius A. (2004). Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of $CD8^+$ T cells. J Exp Med. 199: 925-936.

Wu MM, Buchanan J, Luik RM and Lewis RS. (2006). Ca^{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. J Cell Biol. 174: 803-813.

Xiao S, Matsui K, Fine A, Zhu B, Marshak-Rothstein A, Widom RL and Ju ST. (1999). FasL promoter activation by IL-2 through SP1 and NFAT but not Egr-2 and Egr-3. Eur J Immunol. 29: 3456-3465.

Xiao S, Deshmukh US, Jodo S, Koike T, Sharma R, Furusaki A, Sung SS and Ju ST. (2004). Novel negative regulator of expression in Fas ligand (CD178) cytoplasmic tail: evidence for translational regulation and against Fas ligand retention in secretory lysosomes. J Immunol. 173: 5095-5102.

Zelinskyy G, Balkow S, Schimmer S, Schepers K, Simon MM and Dittmer U. (2004). Independent roles of perforin, granzymes, and Fas in the control of Friend retrovirus infection. Virology. 330: 365-374.

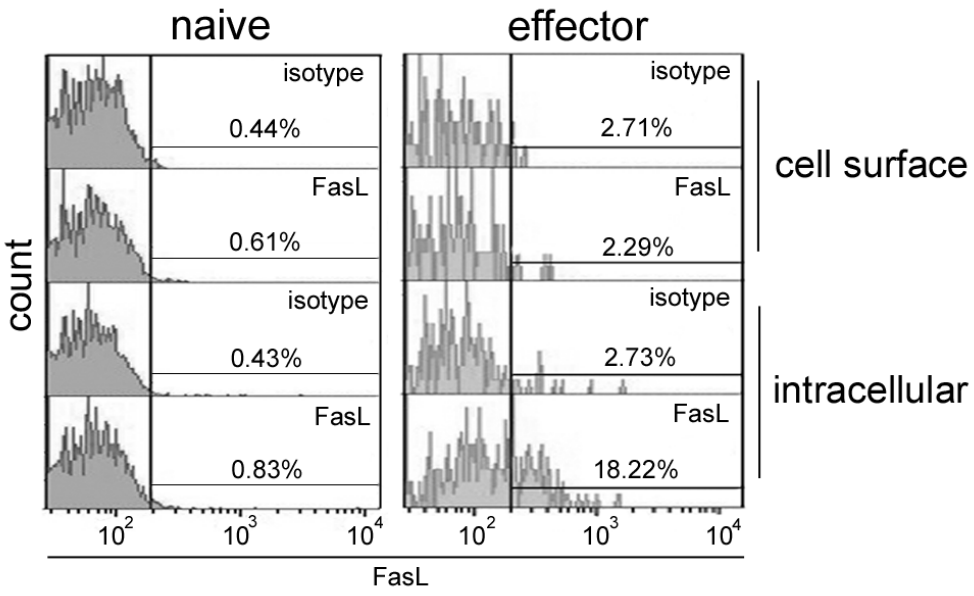
Zelinskyy G, Balkow S, Schimmer S, Werner T, Simon MM and Dittmer U. (2007). The level of friend retrovirus replication determines the cytolytic pathway of $CD8^+$ T-cell-mediated pathogen control. J Virol. 81: 11881-11890.

Zhang J, Cado D, Chen A, Kabra NH and Winoto A. (1998). Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature. 392: 296-300.

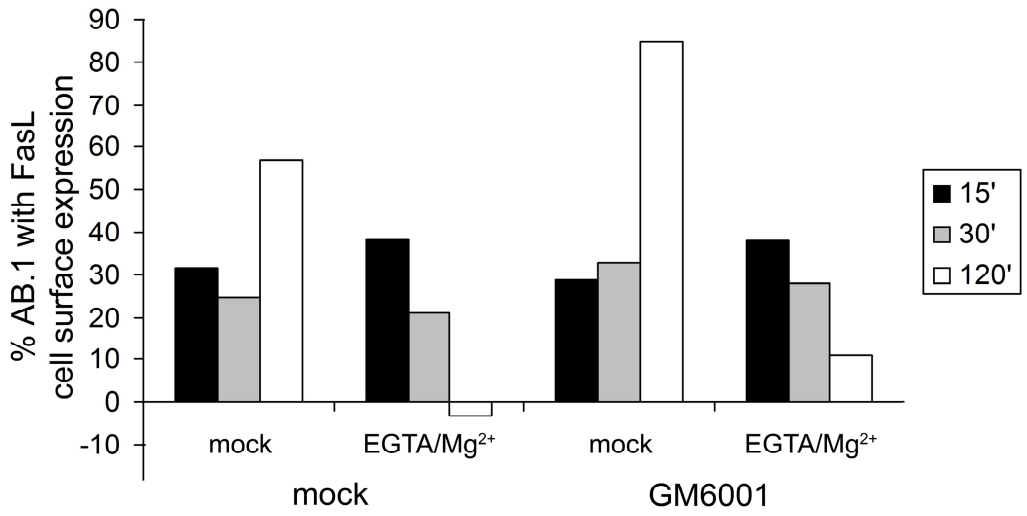
Zhang W and Samelson LE. (2000). The role of membrane-associated adaptors in T cell receptor signalling. Semin Immunol. 12: 35-41.

Zimmermann C, Rawiel M, Blaser C, Kaufmann M and Pircher H. (1996). Homeostatic regulation of $CD8^+$ T cells after antigen challenge in the absence of Fas (CD95). Eur J Immunol. 26: 2903-2910.

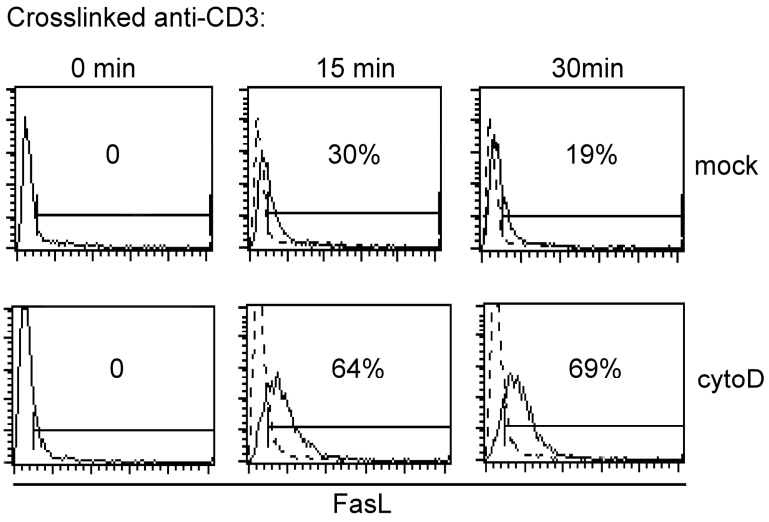
APPENDIX



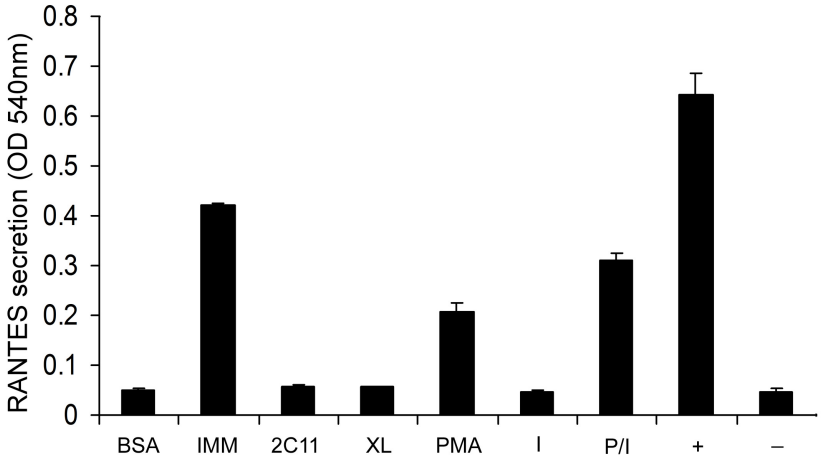
Appendix Figure 1. Intracellular pools of FasL in *ex vivo* effector $CD8^+$ T cells. Post 12 days of *in vivo* priming with allogenic cell EL4, splenocytes from Balb/C mice were isolated, subjected to Fc receptor blocking followed with multi-color staining and analyzed with FACS assay. Gated effector $CD8^+$ T cells ($CD3^+$ $CD8^+$ $CD11a^{hi}$ $CD62L^{lo}$) were examined with both surface and intracellular FasL expression. Data are from a single experiment.



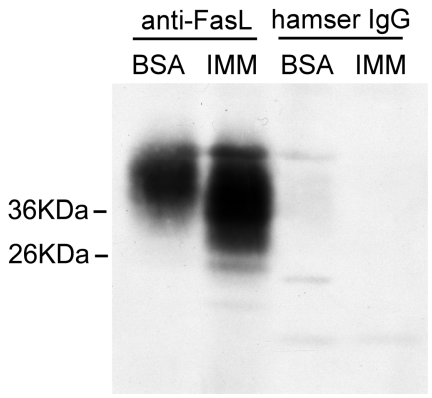
Appendix Figure 2. FasL from the stored pool and *de novo* synthesized pool have differential sensitivity to MMP. CTL clone AB.1 was preincubated with 5 μg/ml MMP inhibitor GM6001 for 30 min, then stimulated in the presence or absence of 4 mM EGTA/ 3 mM Mg^{2+} with cognate target cell EL4. After the indicated time, the specific conjugates were separated with 5 mM EDTA, and surface FasL on gated AB.1 was determined by flow cytometry. Indicated values are the percentage of FasL-positive AB.1 upon stimulation subtracted by that of AB.1 mixed with EL4 and sit one ice for 15min as negative control. Data are representative of three independent experiments.



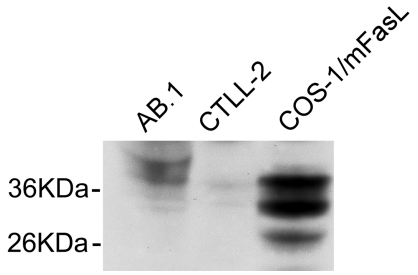
Appendix Figure 3. Significantly increased and prolonged early FasL cell surface expression by crosslinked anti-CD3 stimulation in the presence of cytochalasin D. CTL clone AB.1 was preincubated with 10 μM cytochalasin D (cytoD) for 20 min, then treated for the indicated time with soluble cross-linked anti-CD3 (145-2C11). Cell surface FasL expression was measured on stimulated cells (——) or unstimulated control cells (– – –). Indicated values are percentage of AB.1 with FasL cell surface translocation upon stimulation. Data are from a single experiment. Similar results were got from AB.1 pretreated with cytoD then stimulated with phorbol ester PMA.



Appendix Figure 4. Crosslinked anti-CD3 stimulation does not induce RANTES secretion. CTL clone AB.1 was stimulated for 4hr with plate-bound anti-CD3 (IMM) or BSA control, soluble anti-CD3 (2C11) or crosslinked anti-CD3 (XL), or 25 ng/ml PMA alone, or 3 μM ionomycin alone (I), or 10 ng/ml PMA plus 0.5 μM ionomycin (P/I). RANTES secretion was examined by ELISA. Positive control (+), RANTES standard. Negative control (-), blank well. Average of triplicated samples is shown; bars represent SD.



Appendix Figure 5. Different molecular weight of FasL from AB.1 before and after immobilized anti-CD3 stimulation. CTL clone AB.1 was treated with either plate-bound anti-CD3 (145-2C11) (IMM) or BSA negative control for 2 hr. FasL was immunoprecipitated from each sample. A negative control IP with isotype control Ab was also carried out. Immunoblot of FasL is shown.



Appendix Figure 6. Different molecular weight of FasL from AB.1, CTLL-2 and COS-1/mFasL. Samples of postnuclear lysate from CTL clone AB.1 and CTLL-2, as well as COS-1 transfected with mouse FasL (COS-1/mFasL) were examined. Immunoblot for FasL is shown.