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$CD8^+$ T Lymphocyte Apoptosis is Regulated by Protein O-Glycosylation

A dissertation submitted in partial satisfaction of the requirement for the degree

Doctor of Philosophy

in

Biomedical Sciences

by

Steven John Van Dyken

Committee in charge:

Professor Jamey D. Marth, Chair
Professor Steven F. Dowdy
Professor Ananda W. Goldrath
Professor Mitchell Kronenberg
Professor Ajit P. Varki

2006

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The dissertation of Steven John Van Dyken is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2006

DEDICATION

To my parents,

My role models,

John and Sandy Van Dyken,

For their unconditional love and support

And

To my wife, Jennifer

My heart and soul,

For her infinite love, endurance, and devotion,

I love you

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apoptosis," which has been submitted for publication in *Molecular and Cellular Biology*. I was the primary researcher and author of this work, and Dr. Jamey D. Marth directed and supervised the research that formed the basis for this chapter.

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3. Moody AM, North SJ, Reinhold B, **Van Dyken SJ**, Rogers ME, Panico M, Dell A, Morris HR, Marth JD, Reinherz EL. (2003). Sialic acid capping of CD8β core 1 O-glycans controls thymocyte-major histocompatibility complex class I interaction. *J. Biol. Chem.* 278(9): 7240-6.

4. Wagner JG, **Van Dyken SJ**, Wierenga JR, Hotchkiss JA, Harkema JR. (2003). Ozone exposure enhances endotoxin-induced mucous cell metaplasia in rat pulmonary airways. *Toxicol. Sci.* 74(2): 437-46.

5. Ubels JL, Aupperlee MD, Jackson II RS, **Van Dyken SJ**, McCartney MD. (2002). Topically applied 15-(S)-HETE stimulates mucin production by corneal epithelium. *Adv. Exp. Med. Biol.* 506 (A): 317-21.

6. Wagner JG, **Van Dyken SJ**, Hotchkiss JA, Harkema JR. (2001). Endotoxin enhancement of ozone-induced mucous cell metaplasia is neutrophil dependent in rat nasal epithelium. *Toxicol. Sci.* 60(2): 338-47.

7. Jackson RS II, **Van Dyken SJ**, McCartney MD, Ubels JL. (2001). The eicosanoid, 15-(S)-HETE, stimulates secretion of a mucin-like glycoprotein by the corneal epithelium. *Cornea* 20(5): 516-21.

Abstracts:

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2. **Van Dyken SJ**, Marth JD. (2005). Control of post-immune $CD8^+$ T cell apoptosis by O-glycan-dependent sialylation. *Glycobiology* 15(11): 1239.

3. **Van Dyken SJ**, Marth JD. (2003). Neither absence of CD43, nor deficiency of core 2 GlcNAcT-I, eliminates 1B11 antibody reactivity, the apoptotic phenotype, and the reduction of $CD8^+$ T lymphocyte levels in ST3Gal-I null mice. *Glycobiology* 13(11): 880-1.

4. **Van Dyken SJ**, Marth JD. (2003). Control of $CD8^+$ T cell homeostasis and apoptosis by the ST3Gal-I sialyltransferase. *FASEB J* 17(5 pt. II): A990.

5. **Van Dyken SJ**, Marth JD. (2002). Homeostatic and apoptotic regulation of the peripheral $CD8^+$ T cell compartment by the ST3Gal-I sialyltransferase. *Glycobiology* 12(10): 671-2.

6. Moody AM, North S, Reinhold B, **Van Dyken SJ**, Rogers ME, Dell A, Morris HR, Marth JD, Reinherz EL. (2002). Sialic acid capping of CD8β core 1 O-glycans controls thymocyte-MHCI interaction. *Glycobiology* 12(10): 646-7.

7. Harkema JR, Bennett CB, **Van Dyken SJ**, Hotchkiss JA. (2000). Strain-related differences in ozone-induced rhinitis in F344 and Brown Norway rats. *Am. J. Respir. Crit. Care Med.* 159 (3 pt. 2): A492.

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ABSTRACT OF THE DISSERTATION

$CD8^+$ T Lymphocyte Apoptosis is Regulated by Protein O-Glycosylation

by

Steven John Van Dyken

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2006

Professor Jamey D. Marth, Chair

Homeostasis is maintained after an immune response by apoptotic death of the majority of clonally expanded T cells. Evidence for the involvement of specific extracellular O-glycan modifications occurring during immune activation has previously emerged from analysis of $CD8^+$ T cells deficient in ST3Gal-I, which

express the activated O-glycan phenotype and undergo apoptosis *in vivo* in the absence of activation stimuli. The studies described in this dissertation further explore the influence of ST3Gal-I-mediated sialylation on $CD8^+$ T cell apoptosis and the maintenance of homeostasis throughout immune responses produced *in vitro* and *in vivo*. To determine the effect of constitutive presence of ST3Gal-I on O-glycans production during immune activation, mice expressing an ST3Gal-I transgene in the T cell lineage were generated, characterized, and challenged with various immunologic stimuli. The specific involvement of potential mediators of $CD8^+$ T cell apoptosis induced by ST3Gal-I deficiency was tested by analyzing double mutant mice. Finally, the mechanism of apoptosis was addressed by determining the involvement of intracellular signaling proteins Bcl-2 and Bim, which have been previously implicated in mediating $CD8^+$ T cell apoptosis.

These studies reveal novel mechanistic and structural features of apoptosis regulated by protein O-glycosylation, and a clear relationship to the post-immune contraction of $CD8^+$ T cells. On wild-type $CD8^+$ T cells, increased presence of unsialylated Core 1 O-glycans correlates with apoptosis and sensitizes them to *in vitro* apoptotic death induced by O-glycan crosslinking. Immune activation of ST3Gal-I transgenic $CD8^+$ T cells reveals that unsialylated Core 1 O-glycans can be generated specifically on cells undergoing apoptotic death, by a post-transcriptional mechanism. This O-glycan-dependent CD8 T cell apoptosis does not act through CD43, Core 2 GlcNAcT-1-generated Core 2 O-glycan structures, or Galgt1-

generated complex gangliosides. Furthermore, $CD8^+$ T cell apoptosis mediated by O-glycans does not rely on Bcl-2 levels, and can attenuate the *in vivo* accumulation of $CD8^+$ T cells normally induced by the absence of Bcl-2 family member Bim. Thus, these studies reveal novel mechanistic features of an essential physiologic mechanism of $CD8^+$ T cell apoptosis that follows TCR stimulation and enables contraction upon immune signal attenuation.

CHAPTER 1

Introduction

Apoptosis is essential to the maintenance of T cell homeostasis

Cellular homeostasis within the adaptive immune system is maintained by balancing the life and death of cells that are in a constant state of flux. Apoptotic death is an essential component of T cell homeostasis, aiding in the normal maintenance of a stable population of heterogeneous T cells, while also serving to contain cell numbers during and after immune function (Marrack and Kappler, 2004). Death that occurs upon resolution of the immune response is particularly important, as it ensures the elimination of the vast majority of clonally expanded and potentially harmful activated T cells, leaving only a small percentage of surviving cells, which form the basis for immunologic memory. This post-immune contraction in $CD8^+$ T cell number is linked to cell death by apoptosis; however, the mechanism(s) controlling this process remain to be elucidated (Goldrath and Bevan, 1999; Haring et al., 2006). This essential death process appears to be regulated by multiple pathways and is observed in both $CD4^+$ and $CD8^+$ T cells, although there is reason to believe that different types of lymphocytes undergo apoptosis by distinct mechanisms, and recent studies have found that this includes T cell receptor-induced cell death among Th_1 and Th_2 $CD4^+$ T cell subtypes (Seder and Ahmed, 2003; Devadas et al., 2006; Sharma et al., 2006).

Extrinsic pathway of CD8$^+$ T cell apoptosis

Current concepts of CD8$^+$ T cell apoptosis include two discrete but overlapping mechanisms, the extrinsic or death-receptor-mediated pathway and the intrinsic or mitochondrial death pathway (Strasser, 2005). The extrinsic pathway is thought to depend on extracellular receptor-ligand binding to directly initiate a death signal, which has been described as mediated by various members of the tumor necrosis factor (TNF) receptor family. *In vitro* studies have implicated a role for Fas and TNF signaling following activation (Lenardo et al., 1999). These ligand-receptor systems have also been found to induce non-apoptotic signals such as co-stimulation depending on culture conditions involving the use of different lymphoid cell types or immortalized cell lines (Siegel et al., 2000). *In vivo*, mice deficient in Fas or Fas ligand (*lpr* and *gld* mice, respectively) exhibit lymphoproliferative disorders resulting from the accumulation of abnormal CD4$^-$CD8$^-$ T lymphocytes in lymph nodes and spleen, along with autoantibody formation (Cohen and Eisenberg, 1991); however, these effects are not observed in mice with conditional inactivation of Fas in T cells, which, paradoxically, display increased T cell apoptosis and severe lymphopenia over time (Hao et al., 2004). Importantly, mice deficient in these receptors and their ligands exhibit normal CD8$^+$ T cell apoptotic contraction following immune stimulation *in vivo* (Razvi et al., 1995; Lohman et al., 1996; Nguyen et al., 2000; Reich et al., 2000; Hildeman et al., 2002; Pellegrini et al., 2003), indicating that these particular death receptor interactions are not required for CD8$^+$ T cell apoptosis during *in vivo* immune responses. Also, mice with T cell-

specific mutations in downstream adaptors of TNF family signaling, such as FADD and caspase 8, also display increased apoptosis, lymphopenia, and defective proliferation responses, further highlighting non-apoptotic functions of death-receptor signaling pathways (Zhang et al., 1998; Newton et al., 1998; Walsh et al., 1998; Beisner et al., 2003; Salmena et al., 2003).

Intrinsic pathway of $CD8^+$ T cell apoptosis

In contrast to the extrinsic pathway, the intrinsic pathway of $CD8^+$ T cell apoptosis is thought to depend on signals arising in a cell-autonomous manner, which tip the balance of intracellular pro- and anti-apoptotic proteins that include the Bcl-2 family, resulting in altered mitochondrial membrane permeability (Marrack and Kappler, 2004; Green, 2005). Indeed, Bcl-2 transgenic T cells are more resistant to apoptotic death *in vitro* induced by various apoptotic stimuli such as cytokine withdrawal, dexamethasone treatment, γ-irradiation, ionomycin and PMA (Strasser et al., 1991). *In vivo*, T cells from these mice display prolonged survival in response to immunization with the superantigen *Staphylococcus aureus* enterotoxin B (SEB; Strasser et al., 1991; Hildeman et al., 2002), although the contraction phase in response to LCMV infection appears to be normal (Razvi et al., 1995; Petschner et al., 1998). Additionally, transgenic expression of Bcl-2 has been shown to rescue T cell lymphopoiesis and development in both γc- and IL-7Rα-deficient mice (Kondo et al., 1997; Akashi et al., 1997; Maraskovsky et al., 1997). The pro-apoptotic Bcl-2 family member Bim has also been identified as a key intracellular factor in

regulating the death of mature T cells in both the resting state and after immune stimulation. Interestingly, in contrast to Bcl-2 and in the absence of immunization, Bim-deficient mice accumulate peripheral T and B lymphocytes *in vivo*, and thymocytes from these mice are resistant to *in vitro* death stimuli, particularly cytokine withdrawal and treatment with ionomycin or taxol (Bouillet et al., 1999). In addition, peripheral $CD4^+$ and $CD8^+$ T cells lacking Bim display prolonged survival in response to SEB immunization as well as following infection with herpes simplex virus (Hildeman et al., 2002; Pellegrini et al., 2003). Mice deficient in Bim have also recently been shown to rescue T cell production and function in mice also lacking IL-7Rα (Pellegrini et al., 2004).

Other mediators of $CD8^+$ T cell apoptosis

Other factors have been suggested to mediate apoptosis of post-immune activated $CD8^+$ T cells, including perforin, interferon-γ, granzyme B, and reactive oxygen species (ROS). Nevertheless, perforin-deficient mice exhibit normal contraction of $CD8^+$ T cells in response to an attenuated strain of *L. monocytogenes*, while chronic LCMV infection in these mice leads to accumulation of viral-specific $CD8^+$ T cells (Matloubian et al., 1999; Badovinac et al., 2000; Zhou et al., 2002). Interferon-γ-deficient mice display delayed T cell contraction after bacterial and viral infection, which could be due to hyperproliferation with failure to effectively clear pathogen (Lohman and Welsh, 1998; Badovinac et al., 2000; Bartholdy et al., 2000; Ou et al., 2001). Interferon-γ-dependent inflammation early in the immune response

does appear to regulate the initial expansion phase that makes $CD8^+$ T cell contraction possible (Badovinac et al., 2004). Activated T cell death has also been suggested to be mediated by ROS, since *in vitro* inhibition of ROS generation by a superoxide dismutase mimetic (MnTBAP) protected T cells from death post-activation *in vivo* by SEB immunization (Hildeman et al., 1999). In addition, the role of serine protease inhibitor 6 has been described recently in protecting against cytotoxic T lymphocyte suicide caused by granzyme B-mediated breakdown of cytotoxic granules (Zhang et al., 2006).

Thus, it is evident that the apoptotic death of post-activated $CD8^+$ T cells may be modulated by multiple factors, although the potential remains for the existence of an apoptotic signaling network that is enabled and perhaps activated by one or few types of cell surface molecules. In this case, the attributes of such a cell surface molecule or modification might include its induction or activation at the cell surface following immune stimulation, and perhaps at later stages of activation. Moreover, recapitulating the induction of such a protein or modification among naïve $CD8^+$ T cells may induce caspase-dependent apoptotic signals that result in death and a decrease in peripheral $CD8^+$ T cell number in the absence of immune stimulation.

A novel role for O-glycosylation in regulating $CD8^+$ T cell apoptosis

A candidate cell surface alteration that fits the profile of an apoptotic regulator operating in post-immune $CD8^+$ T cell contraction has been identified as a change in protein O-glycosylation involving the ST3Gal-I sialyltransferase (Priatel et

al., 2000). A significant reduction in the sialic acid linkage on Core 1 O-glycans indicative ST3Gal-I deficiency marks activated and effector $CD8^+$ T cells that are destined either for apoptosis or in some cases differentiation into memory $CD8^+$ T cells, the latter of which appear with increased levels of sialylated Core 1 O-glycans indicative of ST3Gal-I activity (Galvan et al., 1998; Priatel et al., 2000). The unsialylated Core 1 O-glycan structure is a ligand for the peanut agglutinin (PNA) lectin, and the ST3Gal-I deficiency state is also permissive for an increase of 1B11 antibody-reactive Core 2 O-glycans (**Figure 1-1**). During T cell development, immature CD4/CD8 double-positive (DP) thymocytes express low levels of terminal sialic acid on Core 1 O-glycans linked to various cell surface glycoproteins, resulting in exposure of underlying galactose residues in a characteristic conformation of Galβ1-3GalNAcα-Ser/Thr. This specific glycan structure can be detected experimentally using PNA lectin, which binds to Galβ1-3GalNAcα-Ser/Thr (Pereira et al., 1976) and is thus a well-established marker for immature thymocytes as well as activated and leukemic cells (Reisner et al., 1976, 1979; Raedler et al., 1981; Rose and Malchiodi 1981; Chervenak and Cohen, 1982).

The preferential modification of this particular O-glycan structure by the sialyltranferase ST3Gal-I is observed *in vitro* (Kono et al., 1997) and *in vivo* (Priatel et al. 2000) upon thymic maturation, producing Siaα2-3Galβ1-3GalNAcα-Ser/Thr, which is no longer a substrate for PNA and is the predominant form detected on mature CD8 single-positive (SP) thymocytes and naive peripheral $CD8^+$ T cells. This change upon thymic maturation has been documented in several vertebrate

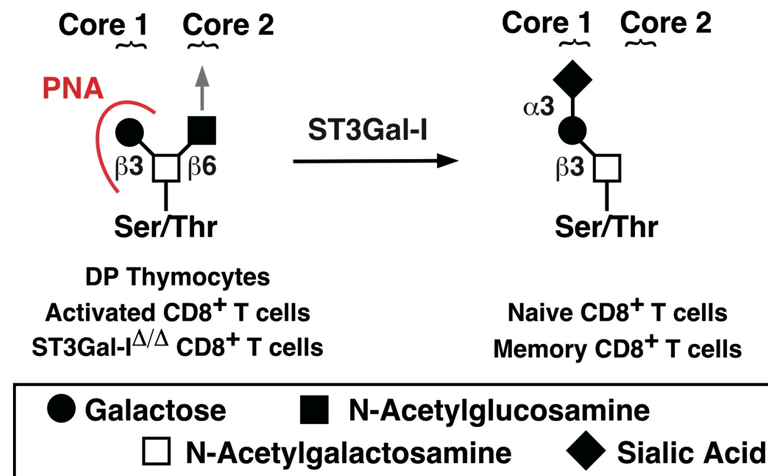

Figure 1-1. ST3Gal-I mediates O-glycan structural alterations during wild-type $CD8^+$ T cell development, immune function, and memory formation. ST3Gal-I-dependent sialylation of the Core 1 O-glycan Galβ1-3GalNAc-Ser/Thr can be monitored by differential PNA binding. Immature CD4/CD8 double-positive (DP) thymocytes and activated peripheral $CD8^+$ T cells, as well as ST3Gal-$I^{\Delta/\Delta}$ $CD8^+$ T cells, express the unsialylated Core 1 O-glycan Galβ1-3GalNAc-Ser/Thr, which is the ligand for PNA lectin, whereas this structure is predominantly sialylated among wild-type naive and memory cells. Position of Core 2 branch and possible extension is indicated by arrow.

species ranging from lizards to humans (Reisner et al., 1976, 1979; Rose and Malchiodi 1981; Kabir et al., 1983; Uni and Heller, 1991; Mansour et al., 1995). After immune stimulation or in the absence of ST3Gal-I, however, $CD8^+$ T cells display increased PNA ligands along with concurrent elevation of 1B11-reactive Core 2 O-glycans on cells that are destined to die by apoptosis after activation. In contrast, both of these O-glycan structures are comparatively reduced on the small proportion of viable memory $CD8^+$ T cells (**Figure 1-1**; Galvan et al., 1998; Priatel et al., 2000; Harrington et al., 2000).

The requirement of ST3Gal-I in regulating the appearance of both of these specific O-glycan structures suggests an interaction between glycosyltransferases within the Golgi apparatus that modify and perhaps compete with each other for specific glycoprotein substrates (Schachter and Brockhausen, 1989; Priatel et al., 2000). Twenty different sialyltransferases are encoded by the mammalian genome that can variably catalyze the addition of sialic acid to different acceptor substrates in various anomeric linkages (**Figure 1-2**). ST3Gal-I acts preferentially to transfer sialic acids in an α2-3 linkage to O-glycan substrates, which are initially generated by addition of the monosaccharide N-acetylgalactosamine (GalNAc) to serine and threonine residues by the family of polypeptide GalNac transferases, and subsequently branched by the activity of the Core 1 GalT and Core 2 GlcNAcTs enzymes to produce Core 1 and Core 2 O-glycan structures, which are then further modified by sialyltransferases and fucosyltransferases (Marth, 1999). It is at this point that certain glycoproteins transiting through the Golgi apparatus appear to be

Enzyme	ST3Gal-I ST3Gal-II ST3Gal-IV ST3Gal-IV ST3Gal-V ST3Gal-VI	ST6Gal-I ST6Gal-II	ST6GalNAc-I ST6GalNAc-II ST6GalNAc-III ST6GalNAc-IV ST6GalNAc-V ST6GalNAc-VI	ST8Sia-I ST8Sia-II ST8Sia-III ST8Sia-IV ST8Sia-V ST8Sia-VI
Acceptor	● Galactose	● Galactose	□ GalNAc	◆ Sialic acid
Linkage	α2-3	α2-6	α2-6	α2-8

Figure 1-2. Sialyltransferases in the mammalian genome. ST3Gal-I (highlighted) is one of 20 different sialyltransferases in the mammalian genome that can catalyze the addition of sialic acid to different acceptor substrates in various anomeric linkages.

subject to competition for modification by either ST3Gal-I or Core 2 GlcNAcT enzymes, a phenomenon possibly related to the specific location of these enzymes within particular Golgi compartments (Priatel et al., 2000).

Thus, in the absence of ST3Gal-I, glycoproteins on the mature T cell surface display the post-activated O-glycotype that is comprised of both unsialylated Core 1 and 1B11-reactive Core 2 O-glycan structures, without prior immune stimulation. Remarkably, this alteration induces specific apoptosis of naïve peripheral $CD8^+$ T cells, manifesting in a 70-90% reduction in the number of $CD8^+$ T cells in the blood, lymph nodes, and spleen, as compared to wild-type littermates. Conditional mutagenesis experiments further demonstrated that the apoptotic phenotype of the ST3Gal-I-deficient $CD8^+$ T cells was T cell-intrinsic, and that these cells could be induced to undergo caspase-dependent apoptosis *in vitro* by crosslinking cell surface O-glycans (Priatel et al., 2000). The $CD8^+$ T cell apoptosis phenotype induced by ST3Gal-I deficiency, therefore, uniquely links distal extracellular O-glycan modifications with intracellular death signals in a cell-type-specific manner. This phenotype, coupled with observations in wild-type $CD8^+$ T cells during immune activation, led to the model proposed by Priatel et al. (2000), which suggests that the altered O-glycosylation in both cases leads to apoptotic death and a subsequent reduction in the pool of cytotoxic T cells (**Figure 1-3**).

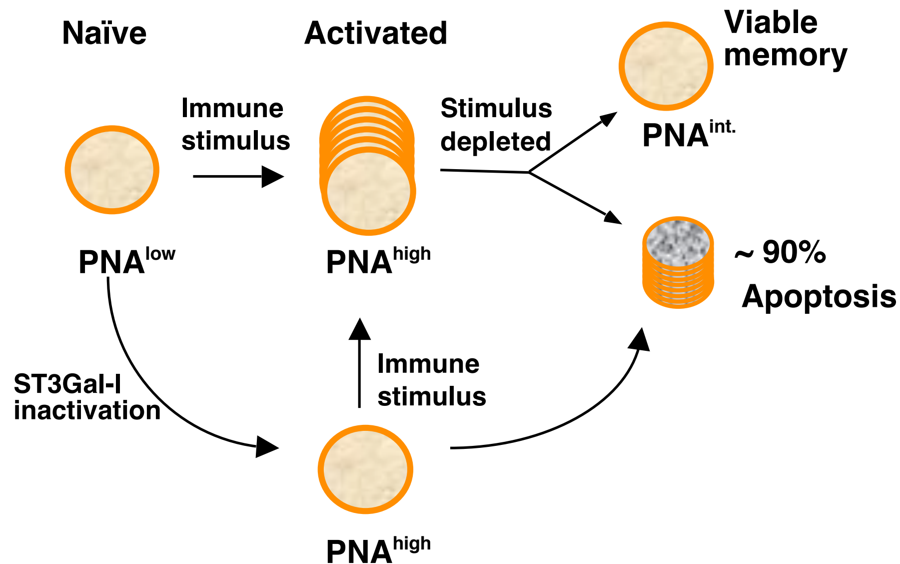

Figure 1-3. Model of peripheral CD8$^+$ T cell homeostasis: a default apoptotic pathway induced by activation. Production of the PNA^{high} phenotype occurs after immune stimulation of wild-type CD8$^+$ T cells, or in the naïve state upon genetic inactivation of ST3Gal-I. In both cases, 90% of these cells undergo apoptotic death *in vivo*, by a mechanism that may involve O-glycan crosslinking by an endogenous lectin. Model adapted from Priatel et al. (2000).

Summary and aims

This dissertation, therefore, addresses the influence of ST3Gal-I-mediated sialylation on $CD8^+$ T cell homeostasis and apoptosis in the naïve state and also during immune responses *in vitro* and *in vivo*. The previous generation and characterization of ST3Gal-I null mice revealed an essential role for this particular posttranslational modification in maintaining a viable peripheral $CD8^+$ T cell population. Thus, in an effort to investigate whether the constitutive presence of ST3Gal-I alters the apoptotic phenotype or O-glycans produced during immune activation, mice expressing an ST3Gal-I transgene specifically in the T cell lineage were generated, characterized, and challenged with various immunologic stimuli. In addition, the specific involvement of potential O-glycoproteins and candidate alterations occurring coincident with $CD8^+$ T cell apoptosis induced by ST3Gal-I deficiency was tested genetically by analyzing double mutant mice. Finally, the mechanism of apoptosis induced by ST3Gal-I deficiency was addressed by exploring the involvement of intracellular signaling proteins Bcl-2 and Bim, which have been implicated in mediating $CD8^+$ T cell apoptosis *in vivo*.

The results described in Chapter 2 indicate that on wild-type $CD8^+$ T cells, increased presence of unsialylated Core 1 O-glycans correlates with apoptosis and sensitizes these cells to *in vitro* apoptotic death induced by O-glycan crosslinking. Immune activation of ST3Gal-I transgenic $CD8^+$ T cells further reveals that unsialylated Core 1 O-glycans can be generated specifically on cells undergoing apoptotic death by a post-transcriptional mechanism. In addition, this O-glycan-

dependent $CD8^+$ T cell apoptosis is not dependent on either CD43, a major substrate of ST3Gal-I, Core 2 GlcNAcT-I-generated Core 2 O-glycan structures, or Galgt1-generated complex gangliosides. Finally, in an effort to clarify the intracellular machinery controlling this apoptotic pathway, Chapter 3 describes experiments demonstrating that $CD8^+$ T cell apoptosis mediated by O-glycans does not rely on Bcl-2 levels, and can attenuate the *in vivo* accumulation of $CD8^+$ T cells normally induced by the absence of Bcl-2 family member Bim. These effects appear to be influenced by the physiologic number and anatomic location of $CD8^+$ T cells, suggesting a model of $CD8^+$ T cell apoptosis that is summarized in Chapter 4, wherein loss of the sialic acid produced by ST3Gal-I on Core 1 O-glycans exposes a ligand for a limited number of endogenous lectins that crosslink one or more O-glycoproteins, inducing annexin V expression and subsequent apoptosis. Taken together, the studies described in this dissertation reveal novel structural and mechanistic features of an essential physiologic mechanism of $CD8^+$ T cell apoptosis mediated by unsialylated Core 1 O-glycans that is linked to TCR stimulation and enables contraction upon immune signal attenuation.

ACKNOWLEDGEMENTS

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apoptosis," which has been submitted for publication in *Molecular and Cellular Biology*. I was the primary researcher and author of this work, and Dr. Jamey D. Marth directed and supervised the research that formed the basis for this chapter.

CHAPTER 2

Regulation and Structural Features of ST3Gal-I-Mediated O-Glycosylation and Relationship to $CD8^+$ T Cell Apoptosis

INTRODUCTION

The experiments described in this chapter were designed to further investigate the regulation and relationship between ST3Gal-I-mediated O-glycan alterations and $CD8^+$ apoptosis during immune responses *in vitro* and *in vivo*. Since loss of sialic acid on Core 1 O-glycans due to genetic ST3Gal-I deficiency induced apoptotic $CD8^+$ T cell death *in vivo* (Priatel et al., 2000), the studies presented here test whether the increased presence of this structure among wild-type $CD8^+$ T cells also results in sensitivity to apoptotic death, and determine whether apoptosis or homeostasis is altered by constitutive expression of ST3Gal-I in T cells. Thus, transgenic mice in which human ST3Gal-I was expressed specifically in the T cell lineage through a CD2 promoter-containing minigene cassette were generated and characterized, and challenged with various *in vitro* and *in vivo* immunologic stimuli in an effort to inhibit the loss of sialic acid on Core 1 O-glycans normally observed among wild-type cells.

Also, the potential involvement of CD43, Core 2 O-glycans, and complex gangliosides is examined. The extracellular glycoprotein CD43 is one of the few glycoproteins on $CD8^+$ T cells that contains both Core 1 and Core 2 O-glycans that are increased in response to activation stimuli or ST3Gal-I deficiency (Piller et al, 1988; Priatel et al., 2000), so it represents a major candidate in mediating the apoptotic phenotype observed due to ST3Gal-I deficiency. Furthermore, the increase in both PNA ligands and 1B11-reactive Core 2 O-glycans observed in the genetic absence of ST3Gal-I indicates that ST3Gal-I is normally responsible for both

sialylating Core 1 O-glycans and inhibiting expression of 1B11-reactive Core 2 O-glycans. Therefore, in order to determine whether CD43 or Core 2 O-glycans that are generated by the Core 2 branching enzyme Core 2 GlcNAcT-1 are responsible for the apoptotic phenotype in post-activated or ST3Gal-I deficient CD8 T cells, mice deficient in either CD43 or Core 2 GlcNAcT-1, as well as ST3Gal-I^{Tg} mice, are examined as single mutants or also on an ST3Gal-I deficient background to determine the effect on post-activated apoptosis and peripheral CD8^{+} T cell numbers *in vivo*. Moreover, recent work demonstrating that the glycolipid asialo-GM1 can be recognized by PNA and is present at increased levels on activated CD8^{+} T cells (Amado et al., 2004) suggests the possible involvement of complex gangliosides in the O-glycan-mediated CD8^{+} T cell apoptosis. Thus, mice deficient in ST3Gal-I and the *Galgt1* gene, which lack the glycosyltransferase GM2/GD2 synthase and as a result do not express complex gangliosides including asialo-GM1 (Liu et al., 1999), were also generated and examined for the effect on peripheral CD8^{+} T cell numbers.

The results described in this chapter give new insights into the production of unsialylated Core 1 O-glycans on CD8^{+} T cells and their relationship to apoptosis during immune responses, and further clarify the involvement of candidate glycoproteins, glycolipids, and alternate O-glycan structures that are also modulated by ST3Gal-I and potentially mediate CD8^{+} T cell apoptosis. Furthermore, by utilizing an *in vitro* assay that tests the existence of this relationship in primary wild-type CD8^{+} T cells, important aspects of the apoptotic model are reinforced, along with a recapitulation of the *in vivo* phenotype.

MATERIALS AND METHODS

Mice

The ST3Gal-I transgene used in generating ST3Gal-I transgenic (ST3Gal-I^{Tg}) mice was constructed by inserting the 2 kb Eco RI fragment of human ST3Gal-I cDNA (Kitagawa and Paulson, 1994; GenBank accession #L29555) into the VA hCD2 minigene cassette (Zhumabekov et al., 1995). The resulting 13.2 kb transgene (liberated from plasmid with Kpn I and Xba I) was microinjected into the pronuclei of zygotes from hybrid strain CB6 (F1 generation of BALB/c x C57Bl/6 mating). Embryos were then transferred to the oviducts of pseudopregnant females and the genotype of pups was verified by PCR using transgene-specific primers. Of eight founder lines established with decreased PNA binding to T cell surfaces, two founder lines were backcrossed to wild-type C57Bl/6 mice for at least six generations before further analysis. GM2 / GD2 synthase null (Galgt$1^{\Delta/\Delta}$) mice have been described (Liu et al., 1999) and were provided by the Consortium for Functional Glycomics, grant number GM2116. CD43 null (CD$43^{\Delta/\Delta}$) mice have been described (Manjunath et al, 1995; Carlow et al., 2001) and were kindly provided by Dr. H. Ziltener (Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia, Canada). Mice lacking Core 2 GlcNAcT-1 (C2GNT$1^{\Delta/\Delta}$) and ST3Gal-I (ST3Gal-$I^{\Delta/\Delta}$) have also been previously reported (Ellies et al., 1998; Priatel et al., 2000). Animals were used between 8-12 weeks of age, and in compliance with standards

and procedures approved by the UCSD Institutional Animal Care and Use Committee.

Cell preparation

Single cell suspensions were prepared from isolated lymphoid tissues in phosphate-buffered saline (PBS) containing 2% heat-inactivated fetal bovine serum (FBS) after red blood cells lysis with ammonium chloride solution (BD PharM Lyse, BD Biosciences, San Jose, CA). For enrichment of $CD8^+$ T cells, negative depletion using magnetic beads was performed. Briefly, mixed cell suspensions from lymph nodes (pooled from axillary, brachial, cervical, inguinal, and mesenteric lymph nodes) and spleen were incubated with biotinylated antibodies to CD4, B220, Mac-1, NK1.1, and Gr-1 (BD Biosciences), washed with PBS, and labeled cells were depleted with Dynabeads M-280 streptavidin (Invitrogen Co., Carlsbad, CA) according to the manufacturer's recommendations. Resulting cell preparations had a purity of ≥90% $CD8^+$ T cells.

Antibodies, lectins, and flow cytometry

Cells were labeled for flow cytometry using annexin V-APC according to the manufacturer's recommendations (Caltag Laboratories, Burlingame, CA) and in combination with 7-AAD, PE-1B11, PE-CD25 (3C7), PE-CD122 (5H4), PE-CD127 (SB/199), PE- or FITC-anti-CD4 (RM4-5), PE- or APC-anti-CD8α (53-6.7) (BD Biosciences) or PNA-FITC (Vector Laboratories, Burlingame, CA). Mouse Vβ TCR

repertoire analysis was performed with FITC-conjugated antibodies to TCR Vβ 2, 3, 4, 5.1 and 5.2, 6, 7, 8.1 and 8.2, 8.3, 9, 10^b, 11, 12, 13, 14, and 17^a (BD Biosciences). All antibody incubations for flow cytometry were performed on ice for 10 minutes. Data were acquired with a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences).

PCR

ST3Gal-I^{Tg} $CD8^+$ T cells from lymph node and spleen (purified by negative depletion) were cultured at 37°C with 5% CO_2 and aliquots removed at 0, 24, 48, and 72 hours post-activation (for 24 hours) with immobilized anti-CD3 (145-2C11; 1 μg/ml; BD Biosciences) in RPMI 1640 media containing 10% heat-inactivated FBS, 1X penicillin / streptomycin / L-glutamine, and 2-mercaptoethanol (Invitrogen). Total RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNase I (DNA-free, Ambion, Austin, TX) to remove any genomic DNA contamination. Reverse transcription was performed using 100 ng total RNA with 40 U M-MLV reverse transcriptase containing either oligo$(dT)_{15}$ or random hexamer primers (Promega, Madison, WI) in a final volume of 20 μl. Control reactions lacking reverse transcriptase were performed in parallel. Resulting cDNA from reverse transcription (1μl) was amplified by PCR using primers specific for the ST3Gal-I transgene or 18s rRNA (QuantumRNA 18s Internal Standards, Ambion). PCR reaction products were separated on 2% agarose gels, stained with ethidium bromide, and quantified by densitometry.

In vitro T cell activation

For in vitro apoptosis analysis, cells from lymph nodes or spleen were prepared as described, resuspended in RPMI 1640 media containing 10% FBS, 1X penicillin / streptomycin / L-glutamine, 2-mercaptoethanol, and stimulated with immobilized anti-CD3 (145-2C11; 1 μg/ml; BD Biosciences) or ionomycin (0.5 μM) / PMA (10 ng/ml) (Sigma-Aldrich Co., St. Louis, MO) for 24 hours in culture at 37°C with 5% CO_2. The cells were then removed from activation stimuli, resuspended in fresh media, and further incubated without stimulus for the next 48 hours. At this time (72 hours post-activation), cells were removed from culture, labeled and analyzed by flow cytometry. Where indicated, interleukin-2 (50 U / ml; R & D Systems, Minneapolis, MN) was added to the cultures after removal of anti-CD3 at 24 hours.

Neuraminidase and lectin application

Lymph node cells (5 x 10^6) from C57Bl/6 mice were isolated as described and treated with or without 3 mU protease-free neuraminidase (sialidase) from *Vibrio cholerae* (EC 3.2.1.18, Roche Diagnostics, Indianapolis, IN) in RPMI 1640 media for 25 minutes at 37° C. After washing with PBS, cells were resuspended at 1 x 10^6 cells/ml in RPMI 1640 media containing PNA, ECL (Vector Laboratories), or media alone. Where indicated, lectin addition was preceded by a 6 hour incubation with immobilized anti-CD3 (145-2C11; 1 μg/ml) or accompanied by D-galactose (25

mM; Sigma-Aldrich). After 24 hours in culture at 37°C with 5% CO_2, cells were counted using a hemacytometer and trypan blue exclusion to identify live cells. The proportion of $CD8^+$ T cells in culture was determined by flow cytometry and used in calculating the total $CD8^+$ T cell count.

Lymphocytic choriomeningitis virus (LCMV) infection

ST3Gal-I^{Tg}, ST3Gal-$I^{\Delta/\Delta}$, and wild-type littermate mice were inoculated with an intraperitoneal injection of 2 x 10^5 plaque-forming units of LCMV-Armstrong in 0.2 ml sterile PBS. On days 0, 8, 15, and 30 post-inoculation, mice were euthanized, spleen and lymph nodes harvested, and cell suspensions were prepared for counting and flow cytometry as described. LCMV-specific T cells were defined by labeling with PE-conjugated D^b gp33-41 MHC class I tetramers (Beckman Coulter, Fullerton, CA), and in combination with other markers as described above.

Staphylococcal enterotoxin B (SEB) immunization

Mice were injected intravenously on day 0 with 150 μg staphylococcal enterotoxin B from *Staphylococcus aureus* (SEB; Sigma-Aldrich) in 0.2 ml sterile PBS. On days 0, 2, and 10 post-injection, mice were euthanized, spleen and lymph nodes harvested, and cell suspensions were prepared for counting and flow cytometry, as well as used to enrich $CD8^+$ T cells by negative depletion followed by culture in RPMI 1640 as described above. $CD8^+$ T cells expressing the Vβ8 TCR

were identified using the PE-conjugated anti-Vβ8 antibody (F23.1; BD Biosciences) and in combination with other antibody markers, as described.

RESULTS

Desialylation of Core 1 O-glycans sensitizes wild-type $CD8^+$ T cells to apoptotic death by O-glycan crosslinking *in vitro*.

As genetic loss of sialic acid formation on Core 1 O-glycans induces $CD8^+$ T cell apoptosis *in vivo*, due to ST3Gal-I deficiency, we investigated whether exogenous enzymatic removal of sialic acid from the normal $CD8^+$ T cell surface increases sensitivity to apoptotic death. Sialidase treatment of wild-type $CD8^+$ T cells removed all cell surface sialic acid linkages without reducing cell viability *in vitro*, and resulted in the maximal exposure of underlying galactose residues among various glycan structures, thereby increasing cell surface expression of ligands for both PNA and ECA lectins (**Figure 2-1A**). The level of PNA ligands induced on wild-type $CD8^+$ T cells treated with sialidase was similar to the level observed among ST3Gal-I deficient $CD8^+$ T cells, indicating that ST3Gal-I function fully accounts for the sialylation of the Galβ1-3GalNAc Core 1 O-glycan. Remarkably, sialidase-treated but not untreated wild-type $CD8^+$ T cells were susceptible to PNA-induced cell death that occurred within 24 hours of PNA addition *in vitro*, matching results obtained using non-treated ST3Gal-I deficient $CD8^+$ T cells (**Figure 2-1B**). Moreover, cell death was PNA dose-dependent and could be inhibited by the addition of free galactose or by T cell receptor (TCR) activation using anti-CD3 for 6

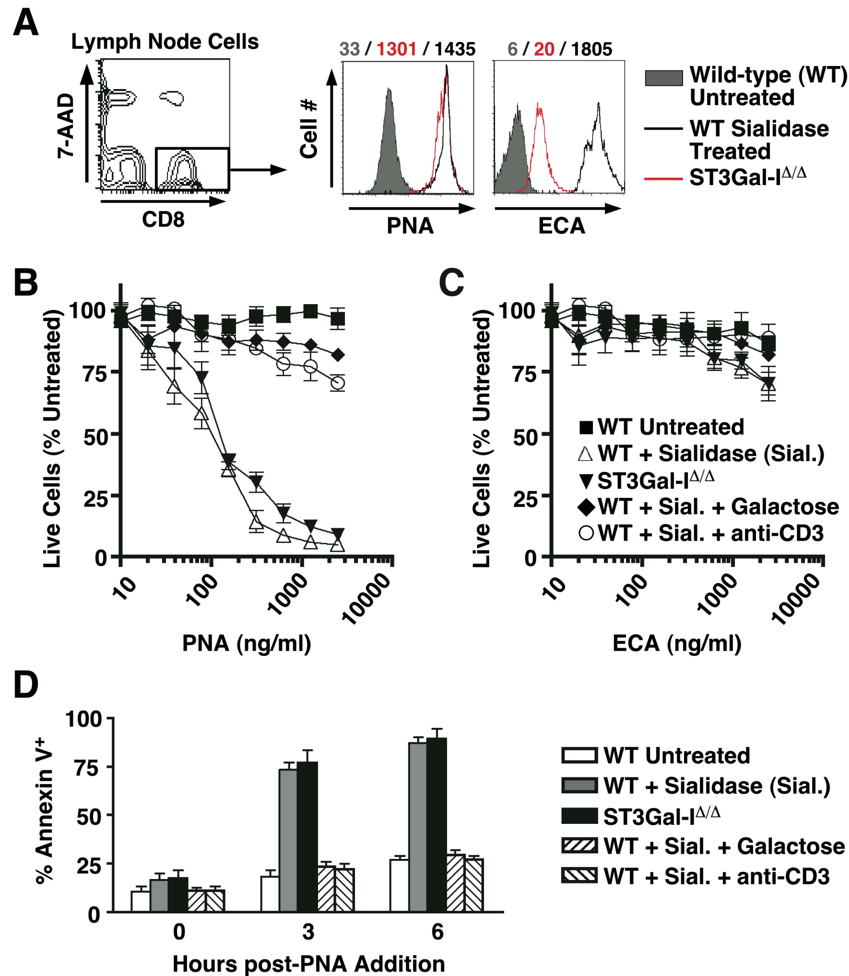

Figure 2-1. O-glycan structures and sialidase treatment sensitize wild-type $CD8^+$ T cells to apoptotic death *in vitro*. **(A)** Flow cytometric analysis of PNA and ECA ligand levels among live (7-AAD$^-$) wild-type $CD8^+$ T cells treated with *Vibrio cholerae* sialidase, along with non-treated ST3Gal-I$^{\Delta/\Delta}$ $CD8^+$ T cells. Mean fluorescence intensity values are indicated by the corresponding colored numbers. These cells were subsequently cultured for 24 hours with PNA **(B)** or ECL **(C)** at the indicated concentrations. Cell counts are presented as a percentage of live $CD8^+$ T cells compared to control cultures not treated with sialidase or lectin. **(D)** Percentage of annexin V^+ cells, among the live (7-AAD$^-$) cell population, in culture 0, 3, and 6 hours after addition of 1000 ng/ml PNA. Data are presented as mean ± SEM; n = 4.

hours prior to sialidase treatment. In contrast, treatment with ECA, which binds to Galβ1-4GlcNAc, failed to induce cell death among sialidase-treated or ST3Gal-I deficient $CD8^+$ T cells (**Figure 2-1C**), indicating a high degree of specificity for apoptotic signaling involving the O-glycan structure modified by ST3Gal-I. This response is preceded by the activation of caspases and the increased externalization of phosphatidylserine, detected by annexin V binding (**Figure 2-1D**; Priatel et al., 2000). In contrast, $CD4^+$ T cells were not as sensitive to PNA-induced apoptosis, consistent with previous results among ST3Gal-I deficient mice (data not shown; Priatel et al., 2000). These results recapitulate in wild-type $CD8^+$ T cells the acquired sensitivity to apoptosis observed among ST3Gal-$I^{\Delta/\Delta}$ $CD8^+$ T cells, and support the view that $CD8^+$ T cell apoptosis may normally occur among post-activated $CD8^+$ T cells bearing altered O-glycosylation.

Constitutive ST3Gal-I transgene expression in T cell ontogeny and peripheral homeostasis.

The model of post-immune $CD8^+$ T cell apoptosis previously reported by Priatel et al. (2000) predicts that the apoptosis of $CD8^+$ T cells due to absence of sialic acid on Core 1 O-glycan structures would be inhibited by constitutive expression of ST3Gal-I. We therefore generated transgenic mice using the human ST3Gal-I cDNA, which is over 90% identical to the mouse sequence, expressed in the T cell lineage by the human CD2 promoter (**Figure 2-2A**). ST3Gal-I transgenic (ST3Gal-I^{Tg}) mice were viable, fertile, and normal upon examination. Histologic

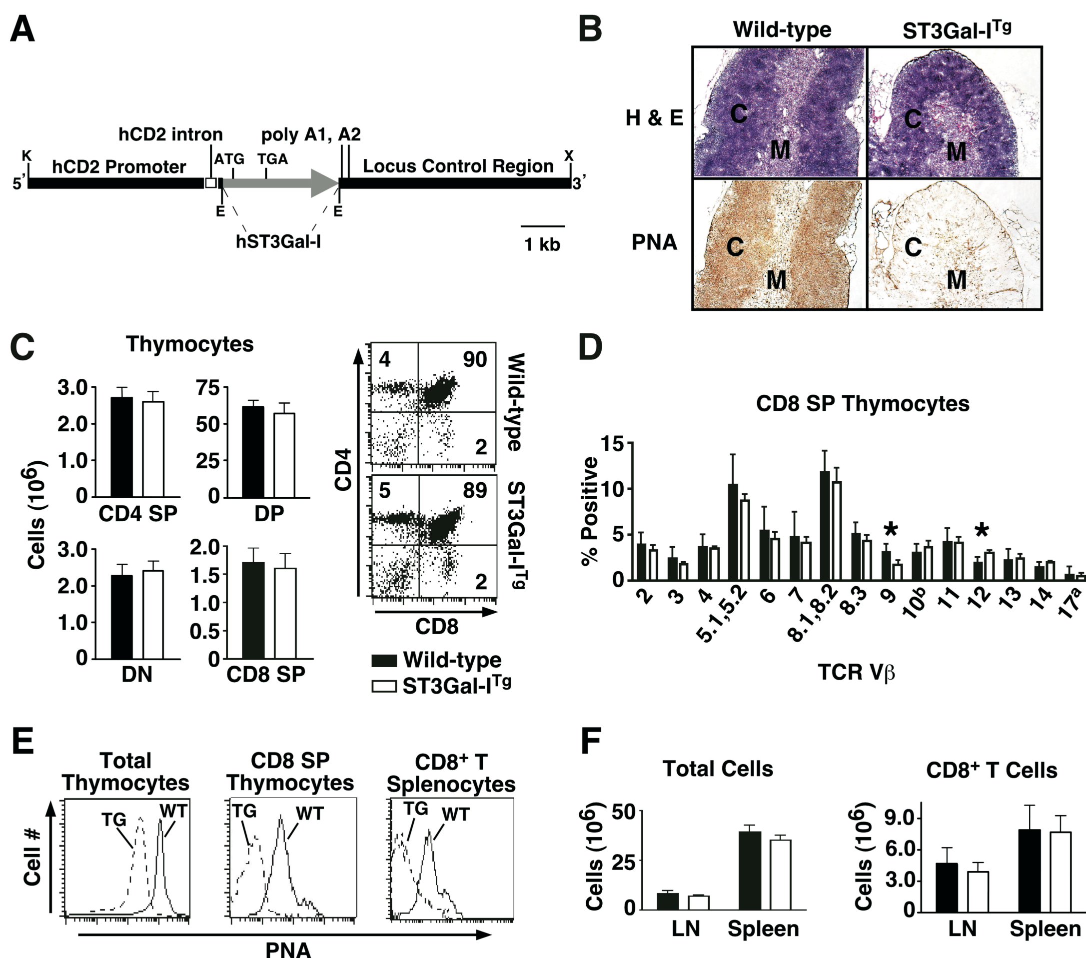

Figure 2-2. Constitutive ST3Gal-I transgene expression in T cell ontogeny and peripheral T lymphocyte homeostasis. (A) ST3Gal-I transgene construct. The Eco RI fragment of human ST3Gal-I cDNA was inserted into the VA-hCD2 minigene cassette, which contains the human CD2 promoter sequence. E=EcoR I; K=Kpn I; X=Xba I. **(B)** Thymic tissue sections from ST3Gal-I^{Tg} animals and wild-type littermates reveal a loss of PNA ligands in both thymic cortex (C) and medulla (M) as a result of constitutive ST3Gal-I expression in T cells. **(C)** Total thymocyte subset numbers and frequencies among CD4/CD8 double-positive (DP), double-negative (DN), and single-positive (SP) T cells in ST3Gal-I^{Tg} and wild-type littermate mice. **(D)** TCR Vβ repertoire expression in ST3Gal-I^{Tg} and wild-type littermate CD8 SP thymocyte populations. **(E)** Analysis by flow cytometry indicates decreased PNA ligands on ST3Gal-I^{Tg} cells as compared to littermate control cells among total thymocytes, CD8 SP thymocytes, and CD8^{+} T cells from spleen. **(F)** Total cell and CD8^{+} T cell numbers in spleen and lymph nodes in ST3Gal-I^{Tg} and wild-type littermate animals. Data are presented as mean ± SEM; n = 6. * = statistically significant difference from wild-type littermates ($p < 0.05$, paired t-test).

examination of the thymus revealed an overall normal organization and appearance in sections stained with hematoxylin and eosin, but a profound reduction in PNA ligands in both the cortex and medulla, indicative of high level ST3Gal-I function in thymocytes (**Figure 2-2B**). Elevated ST3Gal-I expression did not significantly affect thymocyte cellularity, or ontogeny judged by CD4/CD8 subset frequencies (**Figure 2-2C**). The CD8 single-positive (SP) thymocyte population, however, displayed specific alterations in TCR Vβ repertoire expression (**Figure 2-2D**). Notably, frequencies of Vβ 9 and Vβ 12 were significantly reduced and induced, respectively. These findings are opposite of, and consistent with, those observed in ST3Gal-I deficient CD8 SP thymocytes and likely reflect proposed alterations in some MHC Class I interactions that require CD8 (Moody et al., 2001). The increase in Core 1 O-glycan sialylation was measured as a 3-5-fold decrease on average in PNA binding to ST3Gal-I^{Tg} thymic and peripheral $CD8^+$ T cells among the spleen, lymph nodes, and blood (**Figure 2-2E** and data not shown). This increase in ST3Gal-I function, however, did not significantly alter peripheral lymphoid tissue cellularity or total $CD8^+$ T cell numbers in the lymph nodes or spleen of littermate mice at 8-10 weeks of age, or those aged for 7-12 months (**Figure 2-2F** and data not shown).

Annexin V induction in $CD8^+$ T cell apoptosis is linked to loss of Core 1 O-glycan sialylation by a post-transcriptional mechanism.

The appearance of unsialylated Core 1 O-glycans on the cell surface of post-activated $CD8^+$ T cells may be inhibited in ST3Gal-I^{Tg} $CD8^+$ T cells bearing constitutive ST3Gal-I expression. Consistent with this expectation, PNA binding was virtually absent prior to immune activation, reflecting the enhanced level of ST3Gal-I activity. Unexpectedly, however, *in vitro* immune stimulation with anti-CD3 resulted in a significant increase in unsialylated Core 1 O-glycans by 72 hours in culture on ST3Gal-I^{Tg} $CD8^+$ T cells, and comparable with the increase observed on wild-type $CD8^+$ T cells (**Figure 2-3A, B**). The appearance of the annexin V marker of cell surface phosphatidylserine exposure was always found in association with the induction of PNA ligands that mark the unsialylated Core 1 O-glycan. Moreover, the percentage of annexin V^+ PNA^+ cells could be reduced by either continuous anti-CD3 stimulation or by the addition of exogenous IL-2 among wild-type and transgenic $CD8^+$ T cells. Interestingly, stimulation with ionomycin and phorbol ester PMA also greatly reduced the appearance of annexin V^+ PNA^+ $CD8^+$ T cells, further revealing the close link of the annexin V apoptotic marker with the appearance of unsialylated Core 1 O-glycans. The induction of PNA^+ cells that are at least initially annexin V^- is consistent with the role of TCR activation and treatment with some cytokines in blocking annexin V expression and cell death by apoptosis (**Figure 2-3A, B**; Priatel et al., 2000). Annexin V^+ cells were no different from annexin V^- cells in the expression of various receptors for cytokines including IL-2, IL-15, IL-7, and the common γ chain CD132 (**Figure 2-4**).

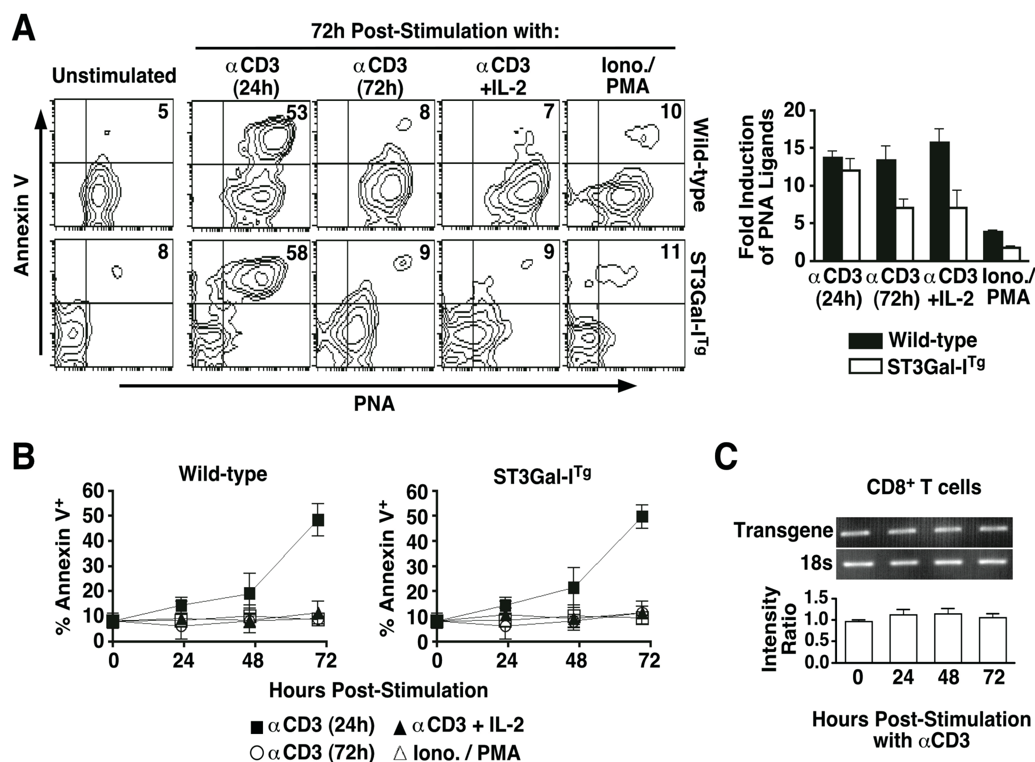

Figure 2-3. Annexin V induction in CD8$^+$ T cell apoptosis is linked to loss of Core 1 O-glycan sialylation by a post-transcriptional mechanism. (A) CD8$^+$ T cells from ST3Gal-I^{Tg} and littermate wild-type mice were activated *in vitro* with the indicated stimuli and analyzed for PNA ligand levels. Flow cytometry plots depict live (7-AAD$^-$) CD8$^+$ T cells; numbers indicate percentage of cells in upper right quadrant, and are representative of three separate experiments. PNA ligand levels were assessed by flow cytometry among stimulated cells (at 72 hours) and compared to unstimulated CD8$^+$ T cells to calculate fold induction. **(B)** Percentage of live (7-AAD$^-$) annexin V$^+$ CD8$^+$ T cells at the indicated time points after activation with the indicated stimuli. **(C)** RT-PCR analysis of ST3Gal-I transgene expression (normalized to 18s ribosomal RNA expression) was performed on purified CD8$^+$ T cells isolated at 0, 24, 48, and 72 hours post-anti-CD3-activation. Intensity ratio represents the ratio of ST3Gal-I transgene to 18s bands as assessed by densitometry.

No significant change was observed in ST3Gal-I transgene mRNA expression throughout the *in vitro* activation time course (**Figure 2-3C**), indicating that the appearance of unsialylated Core 1 O-glycans on $CD8^+$ T cells is likely due to a post-transcriptional mechanism that inactivates ST3Gal-I function. This finding is consistent with recent studies reporting no significant difference in endogenous ST3Gal-I mRNA levels among resting and activated wild-type $CD8^+$ T cells (Amado et al., 2004).

Relationship of Core 1 O-glycan structure to $CD8^+$ T cell apoptosis during an anti-viral immune response *in vivo*.

We sought to further investigate the relationship between unsialylated Core 1 O-glycans and apoptosis during *in vivo* immune responses. Acute infection of mice with lymphocytic choriomeningitis virus (LCMV) induces a dramatic clonal expansion of virus-specific $CD8^+$ T cells which peaks 8 days post-infection then undergoes a contraction phase, leaving a stable population of viable memory cells by 30 days post-infection (Lau et al., 1994; Murali-Krishna et al., 1998). We tested the influence of differential Core 1 O-glycan sialylation on this *in vivo* response by infecting wild-type, ST3Gal-I^{Tg}, and ST3Gal-$I^{\Delta/\Delta}$ mice with LCMV and further measuring the level of annexin V binding, which has been used to identify virus-specific $CD8^+$ T cells undergoing apoptosis (Wang et al., 2003).

We observed a close relationship between unsialylated Core 1 O-glycans detected by PNA and annexin V binding on gp33-41 tetramer-positive cells after

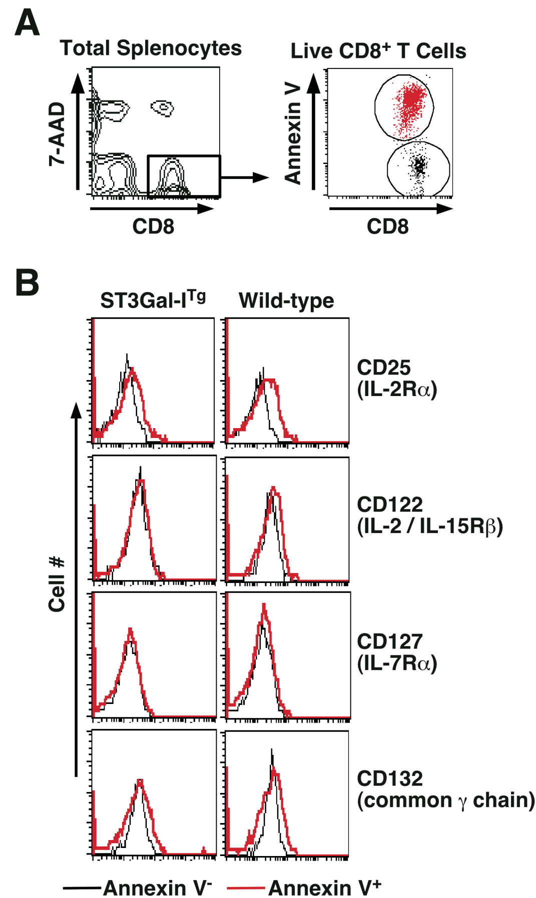

Figure 2-4. Cytokine receptor expression on annexin V$^-$ and annexin V$^+$ subsets from activated wild-type and ST3Gal-I^{Tg} CD8$^+$ T cells. **(A)** Flow cytometric analysis of live (7-AAD$^-$) splenic wild-type and ST3Gal-I^{Tg} CD8$^+$ T cells 72 hours post-activation with anti-CD3 (as in **Fig. 2-3**). Comparative expression of CD25, CD122, CD127, and CD132 on annexin V$^-$ and annexin V$^+$ subsets is shown in **(B)**. Results are representative of 3 independent experiments.

LCMV infection (**Figure 2-5A**). Moreover, among ST3Gal-I^{Tg} tetramer-positive cells, only those that expressed unsialylated Core 1 O-glycans were positive for annexin V binding, as was observed *in vitro* upon anti-CD3 activation (**Figure 2-3A**). The induction of unsialylated Core 1 O-glycans among gp33-41 tetramer-positive T cells peaked at day 8 in both ST3Gal-I^{Tg} and wild-type mice with a 6- to 10-fold increase in PNA ligands, respectively, that subsided by day 30, in agreement with previous findings (**Figure 2-5A**; Galvan et al., 1998; Priatel et al., 2000). Both wild-type and ST3Gal-I^{Tg} mice showed similar expansion and contraction responses involving total CD8^{+} T cells and gp33-41 tetramer-positive CD8^{+} T cells in the lymph nodes (**Figure 2-5B**) and the spleen (**Figure 2-5C**). Notably, ST3Gal-I$^{\Delta/\Delta}$ cells, which constitutively displayed unsialylated Core 1 O-glycans, also maintained the highest percentage of annexin V^{+} cells throughout the infection time course, which corresponded with low numbers of viable cells in the lymph nodes and spleen at every time tested. ST3Gal-I$^{\Delta/\Delta}$ mice also displayed reduced numbers of both total and gp33-41 tetramer-positive CD8^{+} T cells at all time points examined, although distinct expansion and contraction phases involving these cells were observed. These observations are consistent with previous results implicating the normal response of ST3Gal-I deficient naïve CD8^{+} T cells to TCR stimuli in the presence of attenuated cytotoxic T cell responses *in vivo* to MHC-mismatched tumor cell inoculation due to reduced CD8^{+} T cell numbers (Priatel et al., 2000).

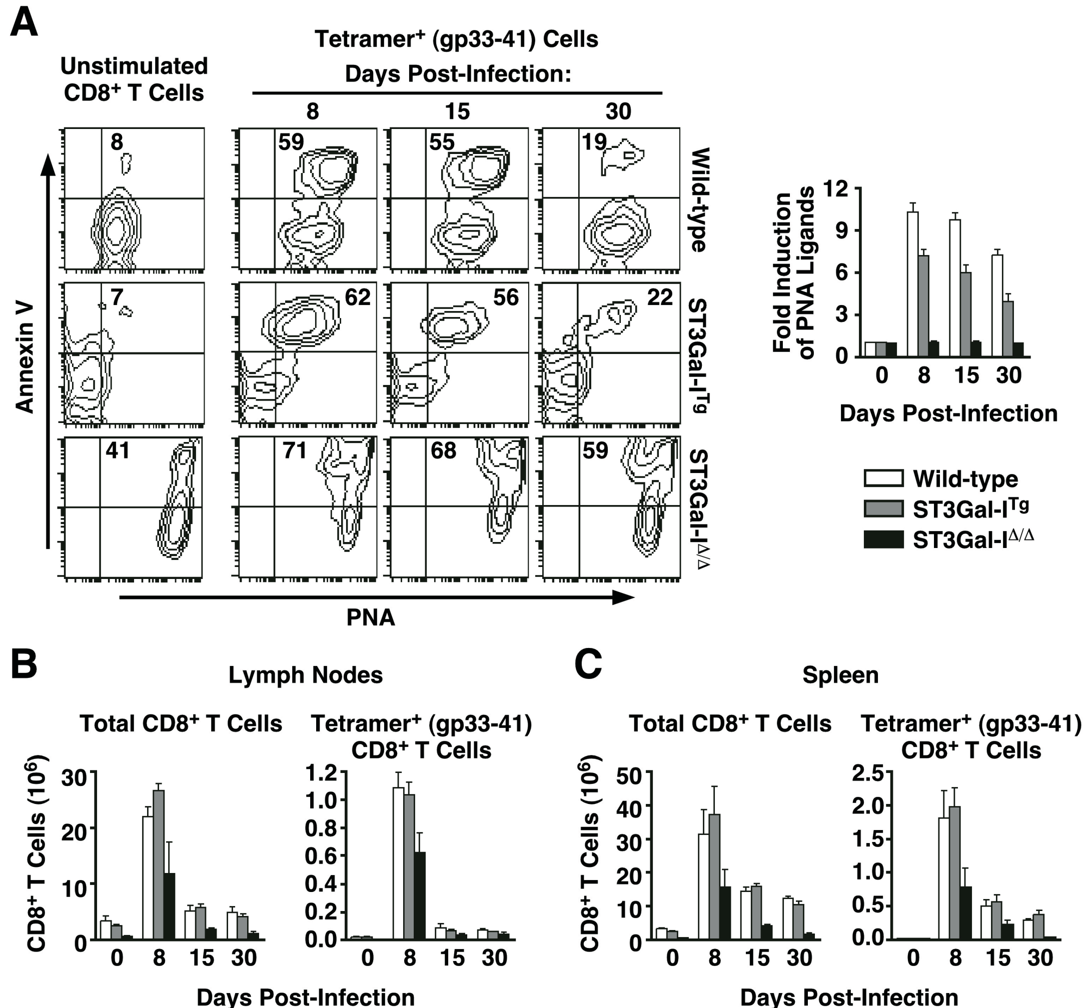

Figure 2-5. Relationship of Core 1 O-glycans structure to CD8$^+$ T cell apoptosis following an anti-viral immune response *in vivo*. (A) Live (7-AAD$^-$) unstimulated CD8$^+$ T cells (day 0) or gp33-41 tetramer-positive cells (days 8, 15, and 30) labeled with PNA and annexin V. Numbers indicate percentage of cells in upper right quadrant, and are representative of three separate experiments. PNA ligand levels were assessed by flow cytometry among gp33-41 tetramer-positive cells (days 8, 15, and 30 post-infection) and compared to unstimulated CD8$^+$ T cells (day 0) to calculate fold induction. Total numbers of CD8$^+$ T cells (left) and CD8$^+$ T cells specific for D^b gp33-41 MHC class I tetramers (right) in lymph nodes (**B**) and spleens (**C**) isolated from wild-type, ST3Gal-I^{Tg}, and ST3Gal-I$^{\Delta/\Delta}$ mice during LCMV infection. Cells were enumerated by hemacytometer counts in combination with percentages obtained by flow cytometric analysis. Data are presented as mean ± SEM; n = 3 - 6.

Relationship of Core 1 O-glycan structure to CD8$^+$ T cell apoptosis during a bacterial antigen-driven immune response *in vivo*.

We then investigated the immune response *in vivo* upon challenge with staphylococcal enterotoxin B (SEB) superantigen, which recruits CD8$^+$ T cells including the TCR Vβ8$^+$ population (Hildeman et al., 2002). Using wild-type, ST3Gal-I^{Tg}, and ST3Gal-I$^{\Delta/\Delta}$ mice, we found a similar pattern in the SEB response to that observed with LCMV challenge, specifically, all CD8$^+$ T cells that became annexin V$^+$ also expressed unsialylated Core 1 O-glycans (**Figure 2-6A**). In addition, the expansion and contraction of Vβ8$^+$ CD8$^+$ T cells displayed similar kinetics among all genotypes (**Figure 2-6B**). When compared to anti-CD3 stimulation or LCMV inoculation, many fewer cells became annexin V$^+$ measured at the indicated times. However, we observed that a significant percentage of Vβ8$^+$ CD8$^+$ T cells from both wild-type and ST3Gal-I^{Tg} mice (44 and 41%, respectively) become annexin V$^+$ PNA$^+$ during a subsequent 24-48 hour culture period *in vitro* (**Figure 2-6C**). As with LCMV infection, the corresponding ST3Gal-I$^{\Delta/\Delta}$ CD8$^+$ T cells constitutively expressed unsialylated Core 1 O-glycans, coinciding with a high percentage of annexin V$^+$ PNA$^+$ cells and fewer cell number throughout the time course and subsequent culture, while decreased viable cell number upon *in vitro* culture was comparable among wild-type, ST3Gal-I^{Tg}, and ST3Gal-I$^{\Delta/\Delta}$ Vβ8$^+$ CD8$^+$ T cells (**Figure 2-6D**).

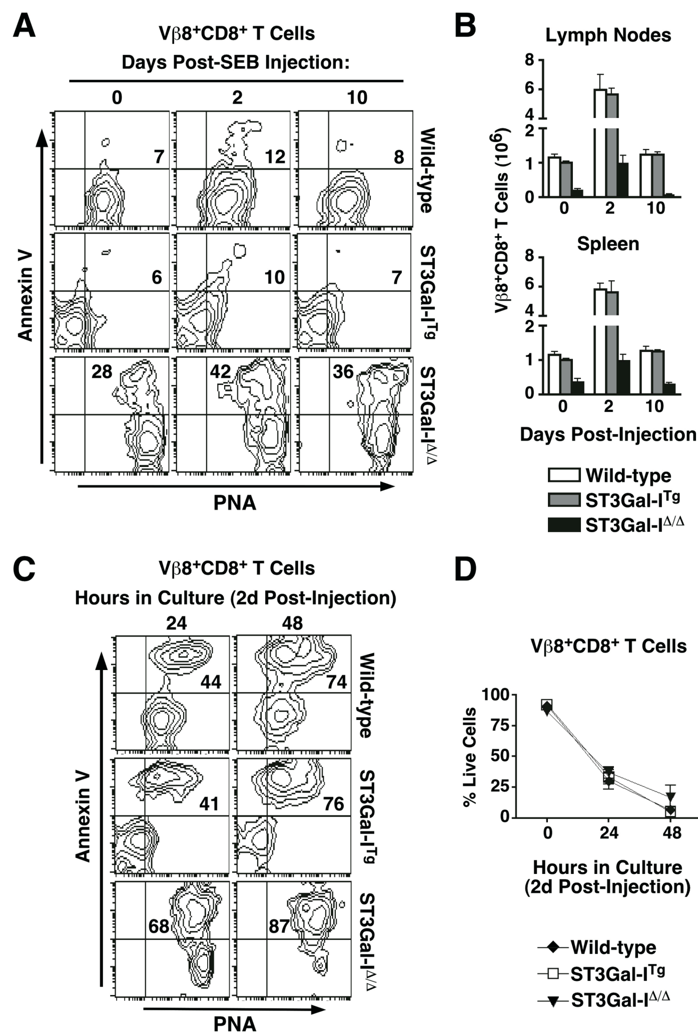

Figure 2-6. Relationship of Core 1 O-glycan structure to CD8$^+$ T cell apoptosis following a bacterial antigen-driven immune response *in vivo*. **(A)** PNA ligands and annexin V reactivity were assessed by flow cytometry among live (7-AAD$^-$) Vβ8$^+$CD8$^+$ T cells isolated from wild-type, ST3Gal-I^{Tg}, and ST3Gal-I$^{\Delta/\Delta}$ mice 0, 2, and 10 days post-SEB injection. **(B)** Total number of Vβ8$^+$CD8$^+$ T cells from lymph nodes and spleen of wild-type, ST3Gal-I^{Tg}, and ST3Gal-I$^{\Delta/\Delta}$ mice at 0, 2, and 10 days post-injection with SEB. Cells were enumerated by hemacytometer counts in combination with percentages obtained by flow cytometric analysis. **(C)** PNA ligands and annexin V reactivity were assessed by flow cytometry among live (7-AAD$^-$) Vβ8$^+$CD8$^+$ T cells removed from wild-type, ST3Gal-I^{Tg}, and ST3Gal-I$^{\Delta/\Delta}$ mice 2 days post-SEB injection, and analyzed after 24 or 48 hours in culture. Numbers in **(A)** and **(C)** indicate percentage of cells in upper right quadrant, and are representative of three separate experiments. **(D)** Percentage of live (7-AAD$^-$) Vβ8$^+$CD8$^+$ T cells during the 48 hours in culture following isolation at 2 days post-SEB injection. Data in **(B)** and **(D)** are presented as mean ± SEM; n = 3.

Eliminating roles for the induced Core 2 O-glycan branch, the CD43 glycoprotein, and Galgt1-generated complex gangliosides.

Concurrent with the induction of unsialylated Core 1 O-glycans following immune activation, $CD8^+$ T cells induce Core 2 O-glycans on the cell surface, which is enabled by elevated Core 2 GlcNAcT-1 expression and the loss of ST3Gal-I function (**Figure 1-1**; Piller et al., 1988; Priatel et al., 2000). Thus, the specific O-glycan structure that induces apoptosis and which may be a ligand for an endogenous lectin, might include both Core 1 and Core 2 branch modifications. To further resolve the glycan structure required for inducing $CD8^+$ T cell apoptosis, we analyzed $CD8^+$ T cell numbers and apoptotic markers among Core 2 GlcNAcT-1-deficient mice and those further lacking ST3Gal-I. Similar studies with multiple gene deficiency states were performed in mice lacking CD43 and Galgt1-dependent glycolipids. CD43 is a major carrier of Core 1 O-glycans along with induced levels of Core 2 O-glycan branching (Piller et al., 1988). In addition, Galgt1-dependent glycolipid structures have been found to bind to the PNA lectin (Amado et al., 2004) and therefore mice lacking this glycosyltransferase were also examined.

Absence of Core 2 GlcNAcT-1 failed to restore normal $CD8^+$ T cell levels in mice also lacking ST3Gal-I. Identical results were observed among mice lacking either CD43 or Galgt1 in the context of ST3Gal-I deficiency (**Figure 2-7**). Restoration of normal $CD8^+$ T cell homeostasis was achieved, however, in ST3Gal-I deficient mice bearing the human ST3Gal-I transgene. Levels of Core 2 O-glycosylation, unsialylated Core 1 O-glycans, and annexin V binding were also

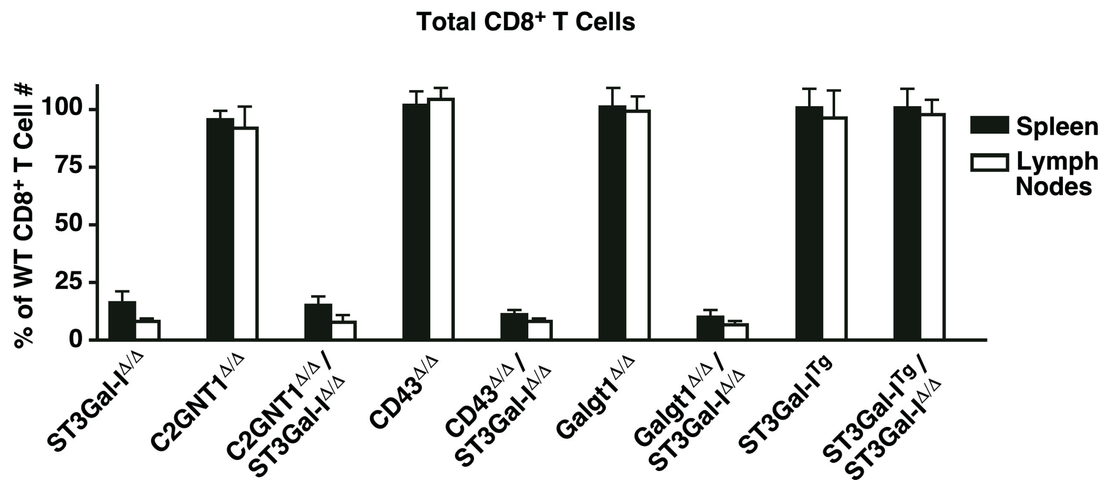

Figure 2-7. Eliminating potential apoptotic roles for the Core 2 O-glycan branch, the CD43 glycoprotein, and Galgt1-generated complex gangliosides. Peripheral deficiency of $CD8^+$ T cells observed in ST3Gal-I deficiency is rescued by transgenic expression of ST3Gal-I, but is not altered in the absence of CD43, Core 2 GlcNAcT-1 (C2GNT1), or Galgt1. Total $CD8^+$ T cell numbers in lymph nodes and spleen are represented as a percentage of the number obtained from wild-type littermate mice. Data are presented as mean ± SEM; n = 6.

measured in resting and activated $CD8^+$ T cells. Remarkably, transgenic ST3Gal-I expression blocked the induction of Core 2 O-glycans upon immune activation, further highlighting the competition that occurs in the Golgi for the identical substrates of Core 2 GlcNAcT-1 and ST3Gal-I (**Figure 2-8**; Priatel et al., 2000). As increased PNA ligand formation and annexin V binding still occurs in ST3Gal-I^{Tg} mice, we can conclude that Core 2 O-glycan induction is not a central determinant in the glycan structure contributing to the expression of the annexin V apoptotic marker and peripheral homeostasis in post-activated $CD8^+$ T cells. Identical results were obtained among ST3Gal-I^{Tg} $CD8^+$ T cells during the *in vivo* immune response to LCMV (data not shown). CD43 deficiency also failed to diminish the induction of unsialylated Core 1 O-glycans and annexin V binding, while the level of Core 2 O-glycans normally measured was reduced by more than 60%. In addition, there was no contribution to $CD8^+$ T cell homeostasis evident among mice lacking glycolipids produced by Galgt1 or in collaboration with ST3Gal-I.

DISCUSSION

Expression of unsialylated Core 1 and 1B11-reactive Core 2 O-glycan structures on the $CD8^+$ T cell surface is controlled by the sialyltransferase ST3Gal-I. The production of this O-glycotype, normally only observed on mature $CD8^+$ T cells after immune stimulation, is invariably linked to the onset of mature $CD8^+$ T cell apoptosis, and thus this particular glycan alteration fits the criteria expected for a regulator of post-immune contraction. Mice with a null mutation in the *ST3Gal-I*

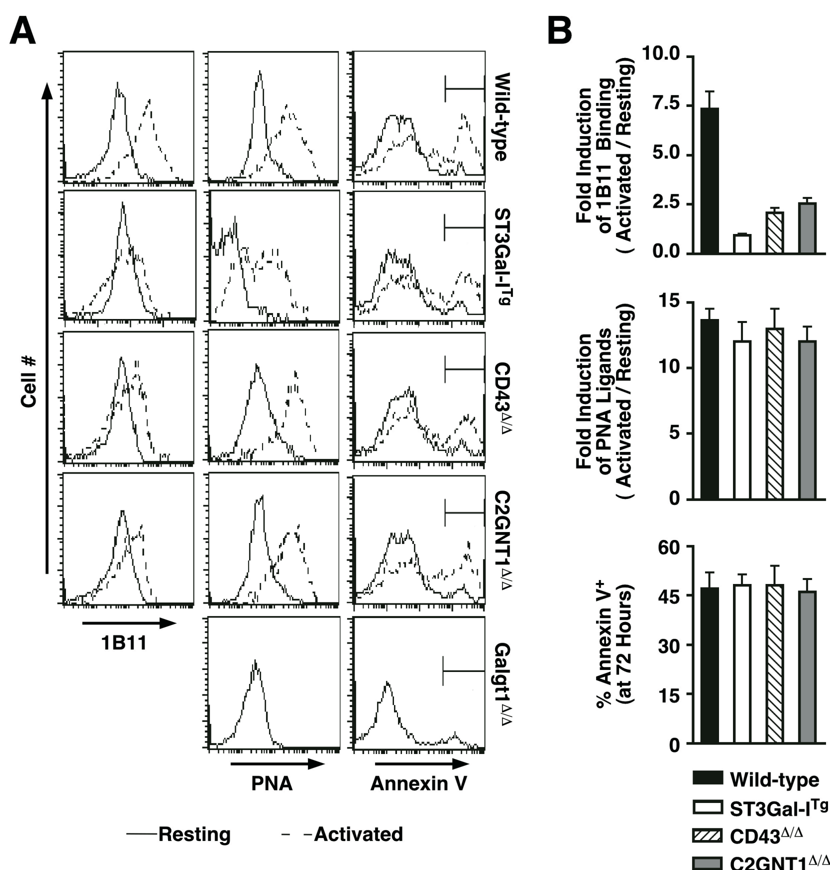

Figure 2-8. Induction of 1B11-reactive Core 2 O-glycans during activation is inhibited by transgenic ST3Gal-I expression, Core 2 GlcNAcT-1 deficiency, or absence of CD43. Activation-induced formation of 1B11-reactive Core 2 O-glycans is inhibited in ST3Gal-I^{Tg} CD8^{+} T cells, CD43$^{\Delta/\Delta}$ and C2GNT1$^{\Delta/\Delta}$ CD8^{+} T cells; however, PNA ligands continue to appear coincident with the normal induction of apoptotic annexin V-positive cells. Histograms in **(A)** show live (7-AAD^{-}) CD8^{+} T cells in a resting state (solid lines) and 72 hours post-anti-CD3 activation (dotted line; as described in **Figure 2-3**). **(B)** Fold induction of 1B11 antibody binding and PNA ligands between resting and activated cells, as well as the percentage of annexin V^{+} cells is presented as mean ± SEM; n = 3.

gene display a dramatic reduction in the number of peripheral $CD8^+$ T cells due to apoptosis that is associated with increased cell surface expression of unsialylated Core 1 and 1B11-reactive Core 2 O-glycan structures (Priatel et al., 2000). The aim of the experiments described in this chapter was to further clarify the identity of the O-glycoprotein responsible for apoptosis, determine the contribution of Core 1 or Core 2 O-glycan structures, and further test the influence of ST3Gal-I-mediated sialylation on the survival of $CD8^+$ T cells both in the naïve state and during immune stimulation by generating mice that expressed a ST3Gal-I transgene specifically in the T cell lineage.

Constitutive expression of the ST3Gal-I in T cells was able to abolish PNA ligand expression on naïve $CD8^+$ T cells in comparison to wild-type cells, indicating that the enzyme produced by the transgene was capable of sialylating Core 1 O-glycan structures that were normally unsialylated. This activity, however, had no observable effect on the survival of these cells in the thymus or peripheral lymphoid tissues, establishing that the production of additional post-translational sialic acid modifications on Core 1 O-glycans does not provide $CD8^+$ T cells with a survival advantage over wild-type cells, which can sufficiently sialylate the appropriate O-glycoprotein that is involved in apoptotic death. Importantly, the relevant sialylated O-glycoprotein on wild-type $CD8^+$ T cells could be desialylated via enzymatic treatment and further crosslinked to induce apoptotic death *in vitro*. This confirmed previous findings among ST3Gal-$I^{\Delta/\Delta}$ cells (Priatel et al., 2000) and recapitulated the null phenotype in wild-type cells, demonstrating that the relevant O-glycoprotein is

present and can operate in normal wild-type cells when a decrease in sialylation is induced and the O-glycans are crosslinked.

Importantly, this particular reduction in Core 1 O-glycan sialylation also occurs coincident with the onset of post-activation $CD8^+$ T cell apoptosis during the contraction phase of the immune response. Increased ST3Gal-I function in transgenic mice eliminated PNA ligand expression in naïve $CD8^+$ T cells, but was unable to completely prevent the generation of unsialylated Core 1 O-glycans after *in vitro* and *in vivo* immune stimulation. Thus, it remains to be seen whether complete inhibition of PNA induction can be achieved by other methods and if this indeed prevents apoptosis. Interestingly, however, the unsialylated Core 1 O-glycans were found only among cells that were apoptotic, allowing a clear demarcation between apoptotic cell populations with reduced ST3Gal-I function and those that retained sialylation and did not externalize phosphatidylserine. Further, the apoptotic phenotype could be inhibited by continued TCR stimulation or addition of exogenous IL-2. This finding casts new light on how the increase in PNA ligands normally observed in response to immune activation is linked to the apoptosis of $CD8^+$ T cells, and indicates the existence of a highly regulated process that occurs upon activation to generate unsialylated Core 1 O-glycan structures on the $CD8^+$ T cell surface independent of ST3Gal-I mRNA expression.

Three mechanistic possibilities seem likely as to how this could occur: (1) cleavage of sialic acid linkages by an endogenous sialidase, (2) regulation of ST3Gal-I protein levels in the Golgi, or (3) differential localization of ST3Gal-I or

other enzymes involved in glycan synthesis or modification. The possibility that an endogenous sialidase is responsible for generating unsialylated Core 1 structures is supported by reports of increased sialidase activity upon T cell activation (Landolfi et al., 1985; Taira and Nariuchi, 1988; Galvan et al., 1998), and has been tested recently (Amado et al., 2004). In this study, the authors investigated the involvement of *Neu1* and *Neu3*, two genes encoding sialidases with possible participation in producing unsialylated Core 1 structures on $CD8^+$ T cells. No significant difference was observed, though, in the PNA ligand induction among activated $CD8^+$ T cells from C57Bl/6 mice and *Neu1* sialidase-deficient SM/J mice. Furthermore, the authors reported that even though a significant increase in *Neu3* gene expression was observed in activated $CD8^+$ T cells, PNA ligand binding was not altered among cells cultured in the presence of the sialidase inhibitor Neu5Ac2en (Amado et al., 2004). In support of this, no effects of Neu5Ac2en on PNA induction were observed in either wild-type or ST3Gal-I^{Tg} $CD8^+$ T cells (data not shown), indicating that cleavage of sialic acid linkages to Core 1 O-glycans by an extracellular sialidase induced during $CD8^+$ T cell activation is unlikely, and thus does not account for the increase in PNA ligands observed in activated $CD8^+$ T cells.

In contrast, the possibility that the ST3Gal-I sialyltransferase itself is cleaved and secreted has several lines of evidence. First, other examples of sialyltransferases, such as ST6Gal-I, have been reported to contain a signal sequence and can be secreted from the cell in soluble form (Weinstein et al., 1987). Soluble sialyltransferases have also been detected in body fluids such as breast milk and

serum, and are increased in disease states and during inflammation (Paulson and Colley, 1989). In addition, Amado et al. (2004) reported that although no significant alterations were observed in ST3Gal-I gene expression, the sialyltransferase activity in cell lysates from activated $CD8^+$ T cells was decreased in comparison to resting cells. Whether this is due to cleavage and secretion of the enzyme remains to be investigated, and definitive experiments may require the development of a specific antibody to ST3Gal-I. Related to this is the last possibility, that activation results in altered localization of ST3Gal-I or other enzymes involved in glycan modification, which could subsequently lead to differential activity on glycan substrates. Again, the availibility of specific antibodies to the enzymes involved in glycan biosynthesis will allow for a better understanding of the regulation of this key event in $CD8^+$ T cell post-immune apoptosis.

The apoptotic death of peripheral $CD8^+$ T cells due to ST3Gal-I deficiency was previously suggested to occur by the aggregation of specific ST3Gal-I substrates on $CD8^+$ T cell glycoproteins (Priatel et al., 2000). A major candidate substrate was CD43, an O-glycoprotein which differs in expression level between $CD4^+$ and $CD8^+$ T cells, induces both unsialylated Core 1 and 1B11-reactive Core 2 O-glycans in the absence of ST3Gal-I, and has further been reported to participate in T cell apoptosis (Brown et al., 1996; Priatel et al., 2000; Onami et al., 2002). In addition, since Core 2 O-glycan branching is regulated in concert with the activity of ST3Gal-I, the possibility existed that Core 2 GlcNAcT-1-generated O-glycan structural elements were responsible for the apoptotic phenotype induced by ST3Gal-I deficiency. In

both cases, however, the lack of rescue in double-deficient animals clearly demonstrated that neither CD43 itself, nor Core 2 O-glycans generated by Core 2 GlcNAcT-1, are involved in the apoptotic signaling initiated in the absence of ST3Gal-I. Further, these molecules do not appear to play a role during the apoptotic response induced during activation, as $CD8^+$ T cells deficient in CD43 or Core 2 GlcNAcT-1, as well as $ST3Gal\text{-}I^{Tg}$ cells, displayed PNA induction and normal apoptosis, even while formation of 1B11-reactive Core 2 O-glycan structures was inhibited. Intriguingly, transgenic ST3Gal-I expression completely blocked the induction of these Core 2 O-glycan structures after immune activation *in vitro* and *in vivo*, lending further support to the notion that the ST3Gal-I and Core 2 GlcNAcT-1 glycosyltransferases compete in the Golgi for the same O-glycan substrates (Schachter and Brockhausen, 1989; Priatel et al., 2000). These observations clarify that the alteration in O-glycosylation induced by activation and ST3Gal-I deficiency apoptosis that is relevant to apoptosis is most likely the PNA-reactive unsialylated Core 1 branch rather than Core 2 O-glycans.

In addition to CD43, CD8 and CD45 were also identified as other candidate glycoproteins that contain PNA ligands (Casabo et al., 1994; Wu et al., 1996; Priatel et al., 2000; Moody et al., 2001, 2003; Amado et al., 2004). Recently, crosslinking of CD8 with antibodies to CD8 or MHC class I ligands has been described to induce apoptosis in immature DP thymocytes but not in mature $CD8^+$ T cells (Grebe et al., 2004); however, this activity does not appear to depend on ST3Gal-I-mediated sialylation (Kao et al., 2006). CD45 has also been suggested to participate in T cell

apoptosis (Ong et al., 1994; Perillo et al., 1995), but antibodies to CD45 were unable to either induce apoptosis or block PNA-induced apoptosis in ST3Gal-I-deficient T cells (data not shown; Priatel et al., 2000). Interestingly, *de novo* synthesis of CD45 occurs after $CD8^+$ T cell activation and has been reported to account for the majority of increased PNA ligands (Amado et al., 2004). This may involve the generation of different glycoforms of CD45, which are produced by alternative splicing of the ABC exons (Hermiston et al., 2003), but it remains to be seen how this process might contribute to CD45 activity or its potential relationship to post-immune apoptosis among $CD8^+$ T cells.

In summary, the experiments described in this chapter have uncovered novel aspects of the relationship between unsialylated Core 1 O-glycans and $CD8^+$ T cell apoptosis *in vitro* and *in vivo*, as well as given insights as to how this structure might be regulated during immune responses. The involvement of candidate molecules that potentially mediate $CD8^+$ T cell apoptosis due to ST3Gal-I deficiency has also been clarified, allowing a determination of the minimal O-glycan structural element relevant to $CD8^+$ T cell apoptosis. The data presented here indicate that CD43 can been ruled out as the operative glycoprotein on the $CD8^+$ T cell surface; however, future work might utilize a glycoproteomics approach to definitively identify the O-glycoprotein contributing to $CD8^+$ T cell apoptosis in the absence of ST3Gal-I-mediated sialylation.

ACKNOWLEDGEMENTS

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CHAPTER 3

Involvement of Intracellular Apoptotic Signaling Proteins in ST3Gal-I-Mediated $CD8^+$ T Cell Apoptosis

INTRODUCTION

The findings described in chapter 2 prompted further investigation of the apoptotic signaling pathway that is initiated *in vivo* among $CD8^+$ T cells in the absence of sialic acid on Core 1 O-glycans. Thus, in this chapter, an inside-out approach is utilized to identify intracellular signaling components that participate in the apoptotic pathway induced in ST3Gal-I deficient $CD8^+$ T cells, based on several recent studies that have highlighted the central role of Bcl-2 family members in regulating T cell apoptosis and homeostasis (Marrack and Kappler 2004; Strasser, 2005). As such, the involvement of these intracellular proteins in the apoptotic pathway induced by ST3Gal-I deficiency in $CD8^+$ T cells is specifically tested by breeding ST3Gal-I deficiency into mice bearing anti-apoptotic phenotypes afforded by altered Bcl-2 and Bim expression.

Previous work has demonstrated that transgenic expression of Bcl-2 in T cells protects them from *in vitro* death stimuli and after immune stimulation *in vivo* (Strasser et al., 1991; Hildeman et al., 2002), and can also correct T cell deficiency phenotypes associated with γc deficiency (Kondo et al., 1997) and IL-7Rα deficiency (Maraskovsky et al., 1997; Akashi et al., 1997). In addition, since the Bcl-2 transgene normally protects $CD8^+$ T cells from death following *in vivo* SEB challenge (Strasser et al., 1991; Hildeman et al., 2002), this protection in response to SEB is also tested in $Bcl\text{-}2^{Tg}$ / $ST3Gal\text{-}I^{\Delta/\Delta}$ $CD8^+$ T cells. In contrast to Bcl-2 transgenic animals, Bim deficient mice display accumulation of peripheral lymphocytes *in vivo* (Bouillet et al., 1999), and $CD4^+$ and $CD8^+$ T cells from these

mice display prolonged survival in response to both SEB administration (Hildeman et al., 2002) and after infection with herpes simplex virus (Pellegrini et al., 2003), in a lymphoid tissue-dependent manner. Mice deficient in Bim have also recently been shown to rescue T cell production and function in IL-7Rα-deficiency (Pellegrini et al., 2004). Therefore, the involvement of Bim, a pro-apoptotic Bcl-2 family member, is also investigated by generating mice deficient in both ST3Gal-I and Bim and additionally challenging them with SEB.

These studies uncover unexpected effects of ST3Gal-I deficiency on $CD8^+$ T cell phenotypes observed in Bcl-2 transgenic and Bim deficient mice, and further reveal new roles for these mediators in physiologic versus non-physiologic contexts. Accordingly, this allows the discernment of novel mechanistic attributes that point to an *in vivo* model regarding how altered protein O-glycosylation enables $CD8^+$ T cell apoptosis in the contraction phase of the immune response.

MATERIALS AND METHODS

Mice

Eμ-Bcl-2 transgenic [Bcl-2^{Tg}; strain B6.Cg-Tg(BCL2)25Wehi/J; (Strasser et al., 1991)] and Bim null [$Bim^{\Delta/\Delta}$; strain B6.129-$Bcl2l11^{tm1.1Ast}$/J; (Bouillet et al., 1999)] mice were obtained from The Jackson Laboratory (Bar Harbor, ME). ST3Gal-I (ST3Gal-I $^{\Delta/\Delta}$) mice have been previously described (Priatel et al., 2000). Animals were used between 8-12 weeks of age and in compliance with standards and procedures approved by the UCSD Institutional Animal Care and Use Committee.

Cell preparation

Single cell suspensions were prepared from isolated lymphoid tissues in phosphate-buffered saline (PBS) containing 2% heat-inactivated fetal bovine serum (FBS) after red blood cells lysis with ammonium chloride solution (BD PharM Lyse, BD Biosciences, San Jose, CA). For enrichment of $CD8^+$ T cells, negative depletion using magnetic beads was performed. Briefly, mixed cell suspensions from lymph nodes (pooled from axillary, brachial, cervical, inguinal, and mesenteric lymph nodes) and spleen were incubated with biotinylated antibodies to CD4, B220, Mac-1, NK1.1, and Gr-1 (BD Biosciences), washed with PBS, and labeled cells were depleted with Dynabeads M-280 streptavidin (Invitrogen Co., Carlsbad, CA) according to the manufacturer's recommendations. Resulting cell preparations had a purity of ≥90% $CD8^+$ T cells.

Antibodies, lectins, and flow cytometry

Cells were labeled for flow cytometry using annexin V-APC according to the manufacturer's recommendations (Caltag Laboratories, Burlingame, CA) and in combination with 7-AAD, PE- or FITC-anti-CD4 (RM4-5), PE- or APC-anti-CD8α (53-6.7) (BD Biosciences) or PNA-FITC (Vector Laboratories, Burlingame, CA). All antibody incubations for flow cytometry were performed on ice for 10 minutes. In some experiments, cell surface analyses were supplemented by intracellular labeling with PE-conjugated antibodies to either human Bcl-2 (6C8) or Armenian

hamster IgG isotype control (Ha4/8) using Cytofix / Cytoperm buffer (BD Biosciences) to permeabilize and fix the cells. Data were acquired with a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences). Polyclonal rabbit anti-Bim antibody (BD Biosciences) was used in western blotting of total thymocyte lysates.

Neuraminidase and lectin application

Lymph node cells (5 x 10^6) from indicated mice were isolated as described and resuspended at 1 x 10^6 cells/ml in RPMI 1640 media containing PNA or media alone. After 24 hours in culture at 37°C with 5% CO_2, cells were counted using a hemacytometer and trypan blue exclusion to identify live cells. The proportion of $CD8^+$ T cells in culture was determined by flow cytometry and used in calculating the total $CD8^+$ T cell count.

Staphylococcal enterotoxin B (SEB) immunization

Mice were injected intravenously on day 0 with 150 μg staphylococcal enterotoxin B from *Staphylococcus aureus* (SEB; Sigma-Aldrich) in 0.2 ml sterile PBS. On days 0, 2, and 10 post-injection, mice were euthanized, spleen and lymph nodes harvested, and cell suspensions were prepared for counting and flow cytometry, as well as used to enrich $CD8^+$ T cells by negative depletion followed by culture in RPMI 1640 as described above. $CD8^+$ T cells expressing the Vβ8 TCR

were identified using the PE-conjugated anti-Vβ8 antibody (F23.1; BD Biosciences) and in combination with other antibody markers, as described.

Adoptive transfer of T cells

Five million thymocytes, isolated from Bim$^{\Delta/\Delta}$, ST3Gal-I$^{\Delta/\Delta}$, and Bim$^{\Delta/\Delta}$ / ST3Gal-I$^{\Delta/\Delta}$ mice, enriched for double negative and CD8 SP thymocytes (present in identical proportions) as described previously, were suspended in 0.2 ml sterile PBS and adoptively transferred into *Rag1*$^{\Delta/\Delta}$ recipients by intravenous injection. After 2 weeks, mice were euthanized, and lymphocte suspensions were prepared from the peripheral blood, lymph nodes, or spleen. CD8 SP cells were identified by antibody lableling, and the percentage of annexin V^{+} cells was assessed by flow cytometry as described.

RESULTS

Loss of ST3Gal-I overcomes Bcl-2 expression to induce CD8^{+} T cell apoptosis *in vivo*, but not *in vitro*, coincident with expression of unsialylated Core 1 O-glycans.

Previous studies have demonstrated that transgenic expression of Bcl-2 in T cells protects them from apoptosis *in vitro* and after immune stimulation *in vivo* (Strasser et al., 1991; Hildeman et al., 2002). In order to further identify the molecular circuitry that explains how unsialylated Core 1 O-glycans induce CD8^{+} T cell apoptosis, we generated ST3Gal-I-deficient mice that also expressed a T-cell-

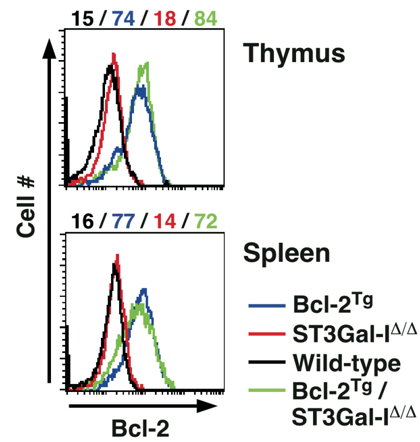

Figure 3-1. Bcl-2 expression in T cells is unaltered by ST3Gal-I deficiency. Flow cytometric analysis of intracellular Bcl-2 levels among live (7-AAD^{-}) thymic and splenic CD8 SP cells from wild-type, Bcl-2^{Tg}, ST3Gal-I$^{\Delta/\Delta}$, and Bcl-2^{Tg} / ST3Gal-I$^{\Delta/\Delta}$ mice. Mean fluorescence intensity values are indicated by the corresponding colored numbers, and are representative of 3 independent experiments.

specific Bcl-2 transgene (Strasser et al., 1991) and examined peripheral $CD8^+$ T cell numbers. We confirmed that the hBcl-2 transgene was expressed similarly in both Bcl-2^{Tg} and Bcl-2^{Tg} / ST3Gal-$I^{\Delta/\Delta}$ $CD8^+$ T cells (**Figure 3-1**). Bcl-2 transgene expression did not reduce the high level of PNA ligands or decrease the percentage of annexin V^+ $CD8^+$ T cells in Bcl-2^{Tg} / ST3Gal-$I^{\Delta/\Delta}$ mice, similar to results seen in ST3Gal-I deficiency alone (**Figure 3-2A**). A significant decrease in peripheral $CD8^+$ T cells was observed in ST3Gal-I deficient mice regardless of the presence of the Bcl-2 transgene, while no differences were observed in the $CD4^+$ T cell population (**Figure 3-2B, C**).

Effect of ST3Gal-I deficiency on *in vivo* and *in vitro* modulation of $CD8^+$ T cell apoptosis in Bcl-2 transgenic mice following SEB immunization.

We further determined whether the presence of the Bcl-2 transgene would inhibit the post-immune contraction of $CD8^+$ T cells by apoptosis in response to SEB. In agreement with reported findings, the Bcl-2 transgene by itself reduced contraction of $V\beta8^+$ $CD8^+$ T cells in the post-immune phase of the response among the lymph nodes and spleen, as compared with wild-type littermates. Remarkably, $V\beta8^+$ $CD8^+$ T cell contraction was re-established in Bcl-2^{Tg} mice that lacked ST3Gal-I, similar to findings in ST3Gal-I deficient controls (**Figure 3-3A**). The degree of cell contraction was directly proportional to the frequency of cells bearing unsialylated Core 1 O-glycans concurrent with the induction of annexin V binding (**Figure 3-3B**). Nevertheless, when activated $V\beta8^+$ $CD8^+$ T cells were removed at 2

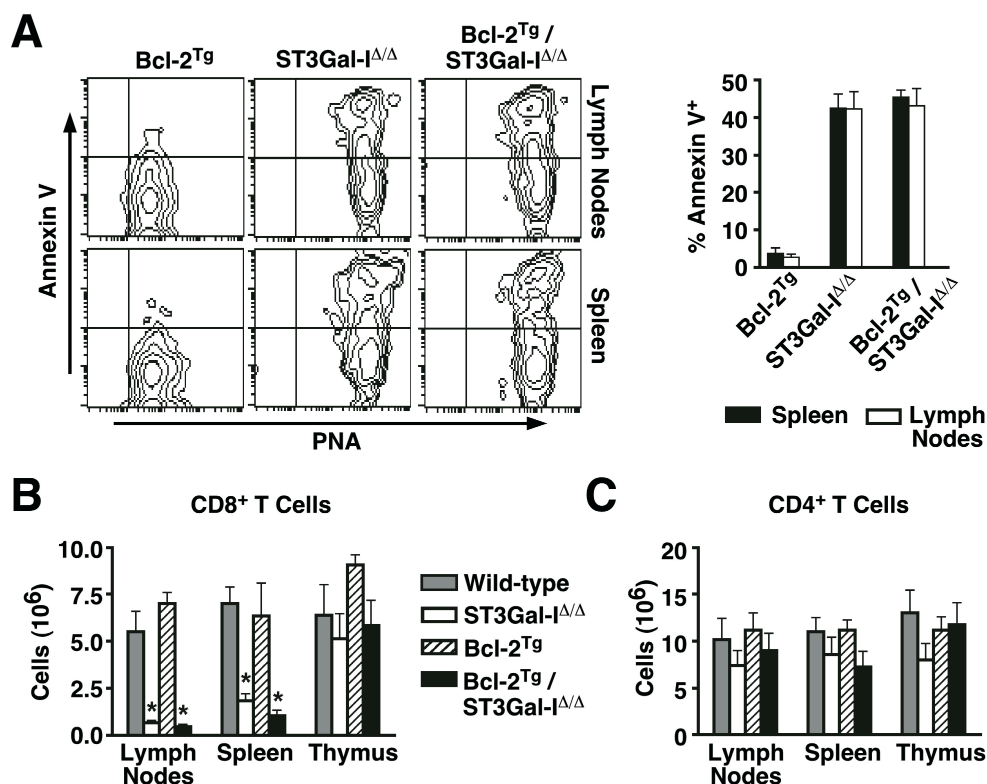

Figure 3-2. Loss of ST3Gal-I overcomes Bcl-2 expression to induce CD8$^+$ T cell apoptosis *in vivo*, coincident with the expression of unsialylated Core 1 O-glycans. (A) Flow cytometric analysis of live (7-AAD$^-$) CD8$^+$ cells from the lymph nodes and spleens of wild-type, Bcl-2^{Tg}, ST3Gal-I$^{\Delta/\Delta}$, and Bcl-2^{Tg} / ST3Gal-I$^{\Delta/\Delta}$ mice. The percentage of annexin V$^+$ cells is shown in the graph at right depicting mean ± SEM; n = 4. Total numbers of CD8 **(B)** and CD4 **(C)** SP cells from the lymphoid tissues of wild-type, Bcl-2^{Tg}, ST3Gal-I$^{\Delta/\Delta}$, and Bcl-2^{Tg} / ST3Gal-I$^{\Delta/\Delta}$ mice. Cells were enumerated by hemacytometer counts in combination with percentages obtained by flow cytometric analysis. Data are presented as mean ± SEM; n = 6; * = statistically significant difference from wild-type littermates ($p < 0.05$, unpaired t-test).

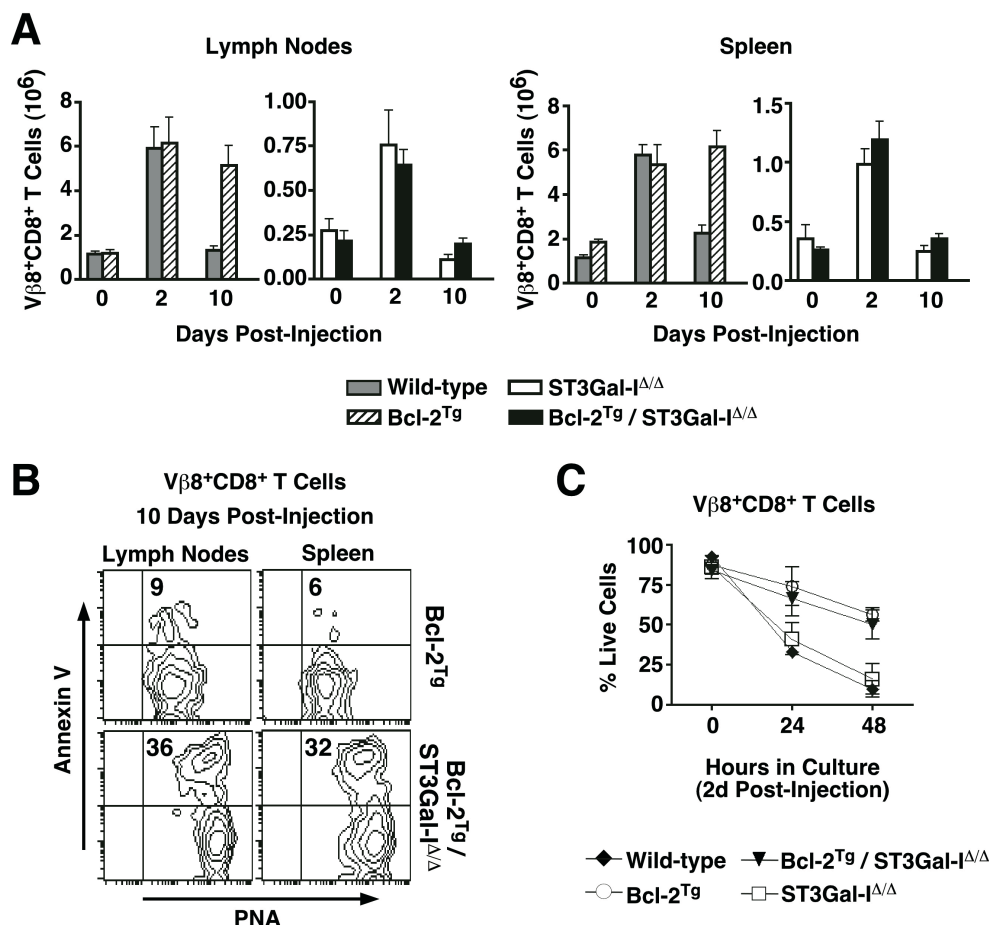

Figure 3-3. Effect of ST3Gal-I deficiency on *in vivo* and *in vitro* modulation of $CD8^+$ T cell apoptosis in Bcl-2 transgenic mice following SEB immunization. (A) Total numbers of $V\beta8^+$ $CD8^+$ T cells from lymph nodes and spleens of indicated mice at 0, 2, and 10 days post-injection with SEB. **(B)** PNA ligands and annexin V reactivity were assessed by flow cytometry among live (7-AAD^-) $V\beta8^+$ $CD8^+$ T cells removed from Bcl-2^{Tg} and Bcl-2^{Tg} / ST3Gal-$I^{\Delta/\Delta}$ mice 10 days post-SEB injection. **(C)** Percentage of live (7-AAD^-) $V\beta8^+$ $CD8^+$ T cells during the 48 hours in culture following isolation at 2 days post-SEB injection. Cells were enumerated by hemacytometer counts in combination with percentages obtained by flow cytometric analysis. Data are presented as mean ± SEM; n = 3; * = statistically significant difference from wild-type littermates ($p < 0.05$, unpaired t-test).

days post-SEB injection and placed in culture for 2 days, viable cell number was highest among both Bcl-2^{Tg} and Bcl-2^{Tg} / ST3Gal-$I^{\Delta/\Delta}$ V$\beta 8^+$ $CD8^+$ T cells, indirectly proportional to apoptotic marker expression, and as compared to V$\beta 8^+$ $CD8^+$ T cells from wild-type and ST3Gal-I deficient mice (**Figure 3-3C** and data not shown).

ST3Gal-I deficiency attenuates the accumulation of $CD8^+$ T cells in the absence of Bim.

Unlike Bcl-2 transgenic mice, Bim deficient mice accumulate lymphocytes even among experimentally and immunologically naïve animals (Bouillet et al., 1999). The presence and absence of Bim was verified among all genotypes (**Figure 3-4**). We further examined mice deficient in both ST3Gal-I and Bim and compared results with littermates that were either wild-type or deficient in either ST3Gal-I or Bim. Peripheral $CD8^+$ T cells from Bim deficient mice expressed low levels of the apoptotic annexin V marker, similar to Bcl-2 transgenic cells. In contrast to results obtained with the Bcl-2 transgene, Bim deficiency significantly reduced the frequency of annexin V^+ $CD8^+$ T cells in the absence of ST3Gal-I, as compared with ST3Gal-I deficiency alone (**Figure 3-5A**). This coincided with a significant increase in splenic $CD8^+$ T cell numbers to levels that were not significantly different from wild type mice, while lymph node $CD8^+$ T cell cellularity was increased by more than 2-fold to about 50% of normal levels (**Figure 3-5B**). This finding was specific to the $CD8^+$ T cell population, as $CD4^+$ T cells continued to accumulate abnormally in the absence of Bim, regardless of whether ST3Gal-I was present (**Figure 3-5C**).

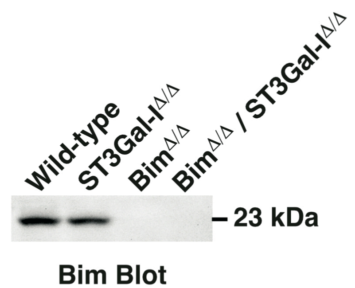

Figure 3-4. Bim expression in T cells is unaltered by ST3Gal-I deficiency. Western blot analysis of Bim levels in thymocyte lysates from wild-type, Bim$^{\Delta/\Delta}$, ST3Gal-I$^{\Delta/\Delta}$, and Bim$^{\Delta/\Delta}$ / ST3Gal-I$^{\Delta/\Delta}$. mice.

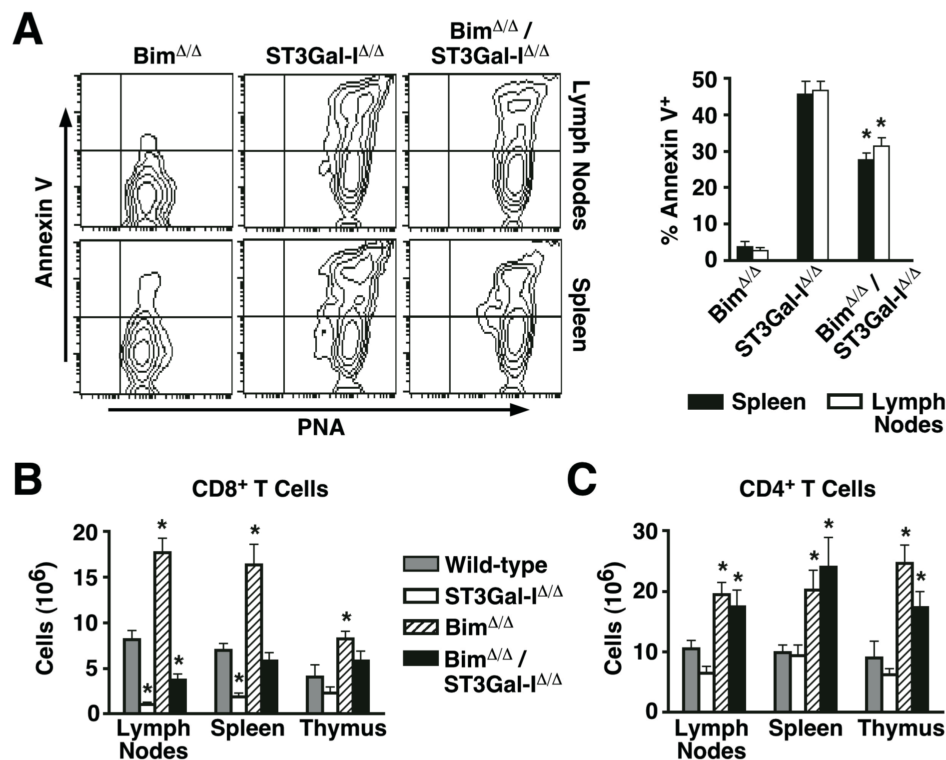

Figure 3-5. ST3Gal-I deficiency attenuates the accumulation of CD8^{+} T cells in the absence of Bim. (A) Flow cytometric analysis of live (7-AAD^{-}) CD8^{+} cells from the lymph nodes and spleens of wild-type, Bim$^{\Delta/\Delta}$, ST3Gal-I$^{\Delta/\Delta}$, and Bim$^{\Delta/\Delta}$ / ST3Gal-I$^{\Delta/\Delta}$ mice. The percentage of annexin V^{+} cells is shown in the graph at right depicting mean ± SEM; n = 4. Total numbers of CD8 **(B)** and CD4 **(C)** SP cells from lymphoid tissues of wild-type, Bim$^{\Delta/\Delta}$, ST3Gal-I$^{\Delta/\Delta}$, and Bim$^{\Delta/\Delta}$ / ST3Gal-I$^{\Delta/\Delta}$ mice. Cells were enumerated by hemacytometer counts in combination with percentages obtained by flow cytometric analysis. Data are presented as mean ± SEM; n = 6; * = statistically significant difference from ST3Gal-I$^{\Delta/\Delta}$ (in **A**) or wild-type littermates (in **B** and **C**; $p < 0.05$, unpaired t-test).

Effect of ST3Gal-I deficiency on *in vivo* and *in vitro* modulation of $CD8^+$ T cell apoptosis in Bim deficient mice following SEB immunization.

Bim deficiency was previously shown to protect $CD8^+$ T cells from death following *in vivo* SEB challenge (Hildeman et al., 2002). We therefore next examined whether ST3Gal-I deficiency altered this phenotype among mice also lacking Bim. As expected, Bim deficiency prevented the apoptotic post-immune contraction of $V\beta8^+$ $CD8^+$ T cells as compared with wild-type littermates, with a more pronounced accumulation occurring in the spleen than in the lymph nodes. Remarkably, the contraction of post-immune $V\beta8^+$ $CD8^+$ T cells residing in the spleen was inhibited among ST3Gal-I deficient mice that also lacked Bim, while the contraction response in the lymph nodes was reduced but remained significant (**Figure 3-6A**). These differing degrees of $CD8^+$ T cell contraction apportioned to specific tissue types nevertheless directly correlated with the frequency of $CD8^+$ T cells that expressed high levels of both unsialylated Core 1 O-glycans and the annexin V apoptotic marker (**Figure 3-6B**). Interestingly, and similar to results obtained in culture with Bcl-2 transgenic $V\beta8^+$ $CD8^+$ T cells, Bim deficiency equally reduced cell death by apoptosis *in vitro,* regardless of the presence of ST3Gal-I, and with cell survival again indirectly proportional to the frequency of cells expressing both unsialylated Core 1 O-glycans and annexin V (**Figure 3-6C** and data not shown).

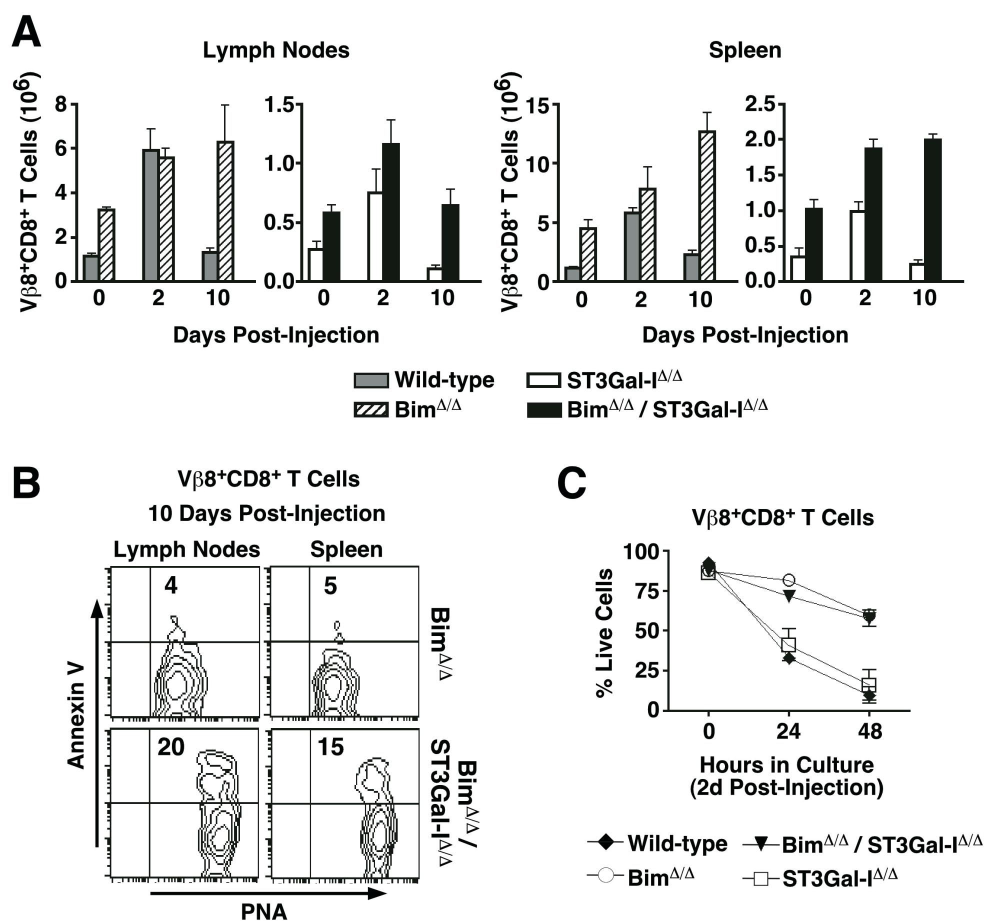

Figure 3-6. Effect of ST3Gal-I deficiency on *in vivo* and *in vitro* modulation of $CD8^+$ T cell apoptosis in Bim deficient mice following SEB immunization. (A) Total numbers of $V\beta8^+$ $CD8^+$ T cells from lymph nodes and spleens of indicated mice at 0, 2, and 10 days post-injection with SEB. **(B)** PNA ligands and annexin V reactivity were assessed by flow cytometry among live (7-AAD^-) $V\beta8^+$ $CD8^+$ T cells removed from $Bim^{\Delta/\Delta}$ and $Bim^{\Delta/\Delta}$ / ST3Gal-$I^{\Delta/\Delta}$ mice 10 days post-SEB injection. **(C)** Percentage of live (7-AAD^-) $V\beta8^+$ $CD8^+$ T cells during the 48 hours in culture following isolation at 2 days post-SEB injection. Cells were enumerated by hemacytometer counts in combination with percentages obtained by flow cytometric analysis. Data are presented as mean ± SEM; n = 3.

Induction of apoptotic CD8$^+$ T cells in dose-response O-glycan crosslinking, among cell compartments, and in the leukopenic state.

The phenotypic modulation of CD8$^+$ T cell apoptosis in ST3Gal-I deficiency by Bcl-2 and Bim may be explained by the expression of an endogenous stimulus that may be more abundant in the lymph nodes than in the spleen but is absent from *in vitro* cell cultures. When present, such a stimulus appears to be a lectin, similar to PNA, that crosslinks one or more key O-glycoproteins bearing unsialylated Core 1 O-glycans and thereby induces apoptosis initially by the induction of phosphatidylserine as measured by the apoptotic marker annexin V. Alternatively, Bim may reside in an epistatic relationship with ST3Gal-I within an apoptotic signaling pathway that at least partially contributes to the post-immune CD8$^+$ T cell contraction phase. To further discern between these two possibilities, we explored the dose-response relationship of CD8$^+$ T cells from Bcl-2 transgenic and Bim deficient mice in the presence or absence of ST3Gal-I. No reduction was observed in the sensitivity to PNA-induced annexin V induction or cell death *in vitro*, implying that Bim deficiency does not alter the efficacy of apoptotic signaling invoked by crosslinking glycoproteins bearing unsialylated Core 1 O-glycans (**Figure 3-7A**). Nevertheless, there remained significant differences in the frequency of apoptotic cells among different peripheral compartments of wild-type and ST3Gal-I deficient mice, similar to those seen in the absence of Bim, with the highest frequencies of annexin V$^+$ cells in the spleen and lymph nodes as compared to thymus and blood (**Figure 3-7B**).

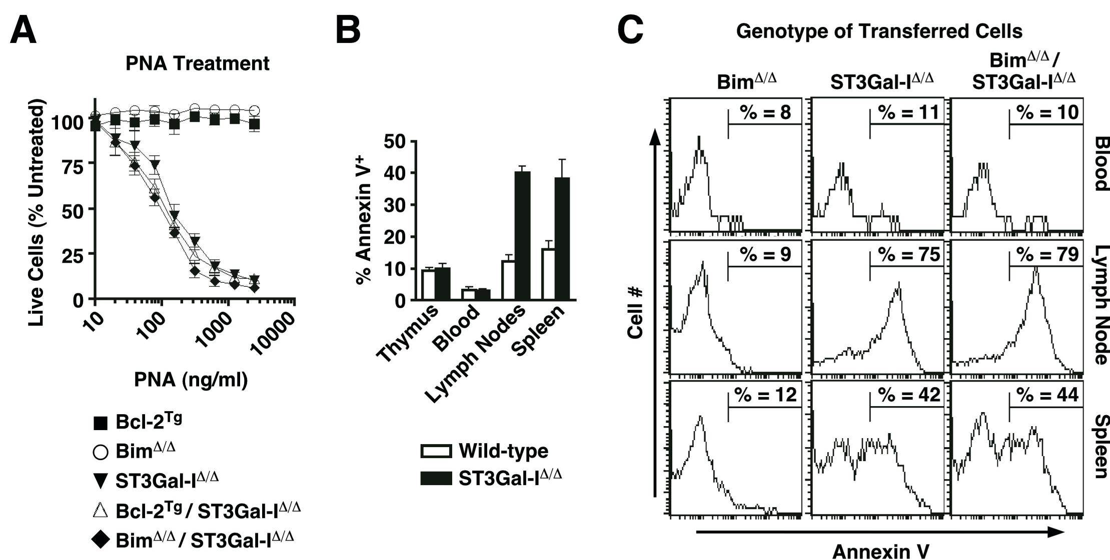

Figure 3-7. Dose-response induction of apoptosis and apoptotic CD8$^+$ T cell compartmentalization. (A) Crosslinking of the unsialylated Core 1 O-glycan Galβ1-3GalNAcα-Ser/Thr induces death in ST3Gal-I$^{\Delta/\Delta}$, Bcl-2^{Tg} / ST3Gal-I$^{\Delta/\Delta}$, or Bim$^{\Delta/\Delta}$ / ST3Gal-I$^{\Delta/\Delta}$, but not in Bcl-2^{Tg} or Bim$^{\Delta/\Delta}$ CD8$^+$ T cells (incubated with PNA, as in **Figure 2-1**). Cell counts are presented as a percentage (mean ± SEM; n = 3) of live CD8$^+$ T cells compared to control cultures not treated with lectin. **(B)** Percentage of CD8 SP T cells positive for annexin V upon isolation from thymus, peripheral blood, lymph nodes, or spleen of ST3Gal-I$^{\Delta/\Delta}$ or wild-type littermate mice (mean ± SEM; n = 3). **(C)** CD8 SP thymocytes isolated from the indicated mice were adoptively transferred into *Rag1*$^{\Delta/\Delta}$ recipients, recovered after 2 weeks from the peripheral blood, lymph nodes, or spleen, and the percentage of annexin V$^+$ cells (gating shown) was assessed by flow cytometry.

These findings further implied that the apoptotic stimulus *in vivo* may be restrictively expressed and in limiting supply. We therefore transplanted equal numbers of viable T cells of different genotypes into RAG-1 deficient mice, which lack lymphocytes, and measured the frequency of annexin V^+ $CD8^+$ T cells during the early phases of lymphocyte expansion, when peripheral lymphoid tissues were colonized by fewer T cells than in normal wild-type mice. Remarkably, the reduced frequency of annexin V^+ $CD8^+$ T cells detected in the absence of both Bim and ST3Gal-I, as compared to ST3Gal-I deficiency alone, failed to occur and equally high levels of annexin V^+ $CD8^+$ T cell numbers were observed among both genotypes (**Figure 3-7C**). Moreover, and similar to results evident in Bim deficiency, the frequency of $CD8^+$ T cells expressing the annexin V apoptotic marker was highest in the lymph nodes, followed by the spleen, whereas a very low percentage of annexin V^+ $CD8^+$ T cells were observed in peripheral blood. These results support a model of $CD8^+$ T cell apoptosis in the contraction phase of the immune response wherein loss of the sialic acid produced by ST3Gal-I on Core 1 O-glycans exposes a ligand for a limited number of endogenous lectins that crosslink one or more O-glycoproteins in inducing annexin V expression and caspase activation.

DISCUSSION

Apoptosis mediated by Bim and other Bcl-2 family members is thought to be initiated by a loss of survival signals such as cytokine withdrawal (Strasser, 2005),

and rescue of T cell production and function in IL-7Rα-deficiency by a Bcl-2 transgene or Bim deficiency supports this concept (Maraskovsky et al., 1997; Akashi et al., 1997; Pellegrini et al., 2004). Our data indicate that the loss of sialic acid on specific $CD8^+$ T cell glycoproteins makes these cells susceptible to ligand-induced death, rather than making them incapable of receiving a survival signal (**Figure 3-7A**). As such, both sialidase-treated and ST3Gal-I-deficient $CD8^+$ T cells are responsive to cytokines such as IL-2, -7, and -15 *in vitro* (data not shown) and can proliferate in response to immune stimulation, as shown during LCMV and SEB challenge *in vivo* (described in Chapter 2). In addition, IL-7 signaling is required for thymic development of T cells (Peschon et al., 1994; von Freeden-Jeffry et al., 1995) and no such thymic defect is observed in the ST3Gal-I deficient mice (Priatel et al., 2000). Thus, the apoptotic signal arising as a result of ST3Gal-I deficiency evidently acts through direct death induction by the interaction of an unknown endogenous lectin with a $CD8^+$ T cell surface glycoprotein. Our data further indicate that this death is induced in a tissue-restricted manner and may result from differential anatomic expression of this putative lectin.

This concept is supported in light of the *in vivo* results obtained from animals deficient in both ST3Gal-I and Bim or lacking ST3Gal-I with a Bcl-2 transgene. The peripheral $CD8^+$ T cell deficiency caused by lack of ST3Gal-I is restored to wild-type levels in the spleen by the additional loss of Bim, but not in the lymph nodes or by transgenic expression of Bcl-2. Further, the accumulation of $CD8^+$ T cells normally observed in Bim-deficient mice (Bouillet et al., 1999) was clearly

attenuated in mice deficient in both Bim and ST3Gal-I, but remained elevated with respect to $CD4^+$ T cells (and B cells; data not shown). This highlights the impact of ST3Gal-I loss among $CD8^+$ T cells specifically, and also demonstrates a differential susceptibility to $CD8^+$ T cell death among different lymphoid tissues in a physiologic setting. Previous work demonstrated that the contraction of Bim deficient $CD8^+$ T cells proceeded normally in the lymph nodes after herpes simplex virus infection, and occurred to a greater degree in the lymph nodes than in the spleen in the case of SEB (Hildeman et al., 2002; Pellegrini et al., 2003), consistent with our observations in the lymph nodes and spleen on a ST3Gal-I-deficient background. Interestingly, however, both Bim deficiency and the Bcl-2 transgene had a protective effect on ST3Gal-I-deficient $CD8^+$ T cells that were activated *in vivo*, removed, and placed in culture.

These results can be explained by the existence of an tissue-restricted endogenous ligand that is present in limiting abundance and induces caspase activation, phosphatidylserine externalization, and death of the ST3Gal-I-deficient $CD8^+$ T cells *in vivo*, but is not present *in vitro*. In support of this, both Bcl-2^{Tg} / ST3Gal-$I^{\Delta/\Delta}$ and $Bim^{\Delta/\Delta}$ / ST3Gal-$I^{\Delta/\Delta}$ $CD8^+$ T cells die *in vitro* due to PNA at doses identical to those that induce death in ST3Gal-$I^{\Delta/\Delta}$ $CD8^+$ T cells. In the case of Bim deficiency, the restoration of $CD8^+$ T cells to wild-type levels observed in the spleen may be a result of the balance between the dramatic accumulation of $CD8^+$ T cells produced in the absence of Bim and the depleting effects of ST3Gal-I deficiency alone. The increased number of cells, therefore, inundates the death signal provided

by the endogenous lectin and therefore is unable to deplete the cells to the same extent as normally observed. In support of this, adoptive transfer of an equal number of CD8 SP thymocytes from $Bim^{\Delta/\Delta}$, ST3Gal-$I^{\Delta/\Delta}$, and $Bim^{\Delta/\Delta}$ / ST3Gal-$I^{\Delta/\Delta}$ into lymphopenic RAG-1 null mice resulted in similar apoptosis of both ST3Gal-$I^{\Delta/\Delta}$ and $Bim^{\Delta/\Delta}$ / ST3Gal-$I^{\Delta/\Delta}$ cells, which was most pronounced in the lymph nodes and spleen compared to peripheral blood. This suggests that expression of the endogenous lectin may be restricted to particular lymphoid tissues or compartments. As such, among ST3Gal-I-deficient lymphoid tissues, only lymph nodes and spleen harbor a high percentage of annexin V^+ $CD8^+$ T cells compared to wild-type tissues, in contrast to those isolated from the thymus or peripheral blood.

Thus, the protective effect of increased Bcl-2 levels or Bim deficiency in $CD8^+$ T cells varies according to the *in vivo* or *in vitro* context of the cells and the presence or absence of ST3Gal-I-mediated sialylation of Core 1 O-glycans. Further studies are warranted to determine the exact anatomic location of the putative endogenous lectin, along with the identity of the $CD8^+$ T cell glycoprotein containing the PNA ligand relevant to apoptosis, as well as the possible involvement of other Bcl-2 family members such as Bax and Bak in the O-glycan-induced apoptotic pathway. It is clear that, *in vivo*, both Bim and ST3Gal-I play a role in regulating the total numbers of $CD8^+$ T cells in the lymphoid tissues; however, the results presented here indicate that these signals are not directly related, and are further influenced by the physiologic number and anatomic location of $CD8^+$ T cells, which act in concert to maintain a balance between life and death.

ACKNOWLEDGEMENTS

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CHAPTER 4

Conclusions and Future Perspectives

Summary

A specific modification to protein O-glycosylation expressed on the post-activated T cell surface is closely linked to peripheral $CD8^+$ T cell apoptosis and appears to play a major role in the contraction phase of the immune response. The experiments described in this dissertation extend the initial report by Priatel et al. (2000) that identified the ST3Gal-I sialyltransferase as regulating this O-glycan alteration, providing evidence that apoptosis among wild-type $CD8^+$ T cells is also linked with this same O-glycan change following both *in vitro* and *in vivo* immune stimulation regimes. Unexpectedly, this regulation of protein O-glycosylation appears to be controlled by a novel post-transcriptional mechanism that mediates ST3Gal-I function, and these studies further identify the minimal O-glycan structure that is sufficient to induce apoptosis as an unsialylated Core 1 O-glycan, lacking a Core 2 O-glycan branch. Its relationship to mechanisms inducing apoptosis suggests that this O-glycan is a ligand for an endogenous lectin that may be expressed differentially among specific tissue compartments *in vivo*. The molecular constituents in this apoptotic pathway remain to be fully established, but operate by caspase activation, and do not appear to involve most if not all of the presently known glycoproteins that bear unsialylated Core 1 O-glycans. The mechanism of action *in vivo* overrides Bcl-2 transgene expression, and to a significant extent Bim deficiency also, thereby reducing $CD8^+$ T cell accumulation, and suggesting a mechanism of apoptosis linked to a saturable compartmentalized apoptotic stimulus.

Relationship of CD8$^+$ T cell apoptosis to altered protein O-glycosylation

Following immune activation *in vitro* or *in vivo*, both CD4$^+$ and CD8$^+$ T cells produce a measurable change in protein O-glycoslyation detected by a 10-20-fold increase in PNA lectin binding to the intact cell surface. Nevertheless, this induction of unsialylated Core 1 O-glycans only induces apoptosis among the CD8$^+$ T cell population when constitutively induced in both cell types among mice lacking ST3Gal-I (Priatel et al., 2000). The experiments described in Chapter 2 demonstrate that inducing this O-glycan change by cell surface desialylation of wild-type CD8$^+$ T cells sensitizes them to PNA-induced apoptosis, which is preceded by the induction of caspase activation and phosphatidylserine externalization on the outer plasma membrane leaflet as measured by the annexin V apoptotic marker. The specificity of this response to unsialylated Core 1 O-glycans was evident as increased ECA lectin binding to other galactose-bearing glycan branch termini also occurred upon sialidase treatment but failed to alter cell viability, eliminating the possibility of a non-specific effect of global desialylation.

In all genotypes of CD8$^+$ T cells studied, including wild-type, ST3Gal-I deficient, ST3Gal-I transgenic, Bcl-2 transgenic, Bim deficient, and combinations of these, the acquisition of a PNA$^+$ annexin V$^+$ cell surface phenotype invariably tracked with subsequent apoptotic death and reduced peripheral CD8$^+$ T cell numbers. *In vitro*, this measure of apoptotic fate among wild-type CD8$^+$ T cells correlated precisely with removal of TCR stimuli, such as anti-CD3 antibody, that had induced immune activation 48-72 hours earlier. Moreover, the appearance of

PNA$^+$ annexin V$^+$ CD8$^+$ T cells following TCR stimulation can be blocked by the addition of IL-2. Interestingly, the low apoptotic response normally seen following ionomycin and PMA treatment, which maximally activates T cells but bypasses direct TCR stimulation, coincided with a low frequency of CD8$^+$ T cells that became PNA$^+$ annexin V$^+$. The linkage of annexin V binding on CD8$^+$ T cells with the presence of unsialylated Core 1 O-glycans was further evident upon *in vivo* immune activation experiments that included a viral challenge (LCMV) as well as immunization with a bacterial superantigen (SEB) among mice of the genotypes described above. In all studies, a clonal expansion phase was observed, which was followed by a measurable contraction phase, the latter being proportional to the appearance and frequency of PNA$^+$ annexin V$^+$ CD8$^+$ T cells among the relevant responding populations. These results indicate that the activation response initiated at the level of TCR stimulation invariably induces unsialylated Core 1 O-glycans followed by cell surface phosphatidylserine externalization once immune stimulation begins to wane. These findings closely link the induced unsialylated Core 1 O-glycan structure recognized by the PNA lectin and annexin V$^+$ CD8$^+$ T cells as those destined for apoptosis. Moreover, unlike CD4$^+$ T cells which can become annexin V$^+$ in the absence of significant PNA binding, all annexin V$^+$ CD8$^+$ T cells are also PNA$^+$ having undergone an induction of unsialylated Core 1 O-glycans, whereas memory CD8$^+$ T cells are more similar to resting CD8$^+$ T cells in having increased levels of sialic acid on cell surface Core 1 O-glycans (Priatel et al., 2000; Galvan et al., 1998).

Structure and regulation of protein O-glycosylation in CD8$^+$ T cell apoptosis

The appearance of unsialylated Core 1 O-glycans following TCR stimulation is normally accompanied by an induction of Core 2 O-glycan branching, due to loss of ST3Gal-I substrate competition with endogenous Core 2 GlcNAcT activity (Piller et al., 1988; Priatel et al., 2000). Nevertheless, observations described in Chapter 2 indicate that Core 2 O-glycan branching is not involved in CD8$^+$ T cell apoptosis induced by the absence of ST3Gal-I. Core 2 GlcNAcT-1 deficiency substantially reduces activation-induced 1B11 antibody binding, a marker of Core 2 O-glycans, but does not affect CD8$^+$ T cell homeostasis. Importantly, no rescue of peripheral CD8$^+$ T cell numbers could be achieved in mice that were deficient in both ST3Gal-I and Core 2 GlcNAcT-1, and the frequency of CD8$^+$ T cells that were PNA$^+$ annexin V$^+$ remained elevated, similar to ST3Gal-I deficiency alone. Therefore, the change in O-glycans that occurs following CD8$^+$ T cell activation, which includes induction of Core 2 O-glycan branching, may reflect increased production of selectin ligands involved in lymphocyte trafficking (Snapp et al., 2001). Nevertheless, there is no involvement of the Core 2 O-glycan branch structure in CD8$^+$ T cell apoptosis, leaving the unsialylated Core 1 O-glycan disaccharide Galβ1-3GalNAc-Ser/Thr as the minimal structural determinant in apoptotic signaling.

Efforts to block the loss of sialic acid on Core 1 O-glycans following CD8$^+$ T cell activation involved the successful production of ST3Gal-I transgenic mice bearing constitutive ST3Gal-I function during T cell ontogeny and continuing

throughout T cell immune stimulation. Clear evidence of human ST3Gal-I function in the thymus was observed and the ability of the transgene to rescue $CD8^+$ T cell numbers in ST3Gal-I deficient mice was demonstrated. The loss of PNA binding in cortical thymoyctes correlated with changes in the same TCR Vβ chains affected in ST3Gal-I deficiency, and in an opposing manner, consistent with a role for ST3Gal-I activity in altering CD8-MHC interactions (Moody et al., 2001, 2003). These relatively minor changes in the TCR Vβ repertoire did not alter mature T cell activation responses, which were identical when compared with wild-type and ST3Gal-I deficient $CD8^+$ T cells (data not shown). Elevated and constitutive ST3Gal-I expression blocked the appearance of 1B11 binding determinants upon $CD8^+$ T cell activation that require Core 2 GlcNAcT-1, further revealing that ST3Gal-I can reside in the medial Golgi compartment as does Core 2 GlcNAcT-1, and compete with Core 2 GlcNAcT-1 for some of the same glycoprotein substrates *in vivo* (Schachter and Brockhausen, 1989; Priatel et al., 2000).

Even while ST3Gal-I activity in the Golgi can block Core 2 O-glycan induction, constitutive ST3Gal-I expression cannot inhibit the appearance of unsialylated Core 1 O-glycans on post-activated $CD8^+$ T cells. This remarkable and unexpected finding implies that loss of ST3Gal-I function following $CD8^+$ T cell stimulation reflects a post-transcriptional regulatory mechanism, as elevated transgenic ST3Gal-I RNA expression and enzymatic function were maintained. The ability of the transgene to nevertheless compete with Core 2 GlcNAcT activity in the medial Golgi and abolish all unsialylated Core 1 O-glycans in resting $CD8^+$ T cells

reveals that this change in O-glycan structure likely results from an event that occurs in the late (trans) Golgi network or in post-Golgi processing. Therefore, the post-immune absence of the sialic acid normally produced by ST3Gal-I could reflect glycoprotein turnover that coincides with ST3Gal-I proteolysis or perhaps the activation of an endogenous sialidase. Although the possibility of proteolysis cannot be ruled out, there are reports of increased sialidase activity upon T cell activation (Landolfi et al., 1985; Taira and Nariuchi, 1988; Galvan et al., 1998). More recently, however, other studies have found that loss of potential sialidases, or inhibition of sialidase activity, has no effect on the appearance of unsialylated Core 1 O-glycans (Amado et al., 2004).

While this regulatory mechanism remains to be established, it is clear that not all glycoproteins normally bearing unsialylated Core 1 O-glycans participate in apoptotic signaling. For example, the high level of ST3Gal-I expression as a transgene greatly increases Core 1 O-glycan sialylation of all glycoproteins among resting peripheral $CD8^+$ T cells, as compared with wild-type peripheral $CD8^+$ T cells, and the only PNA^+ $CD8^+$ T cells among ST3Gal-I^{Tg} mice are those which are annexin V^+. Moreover, experiments described in Chapter 2 indicate that the CD43 glycoprotein, which bears unsialylated Core 1 O-glycans in addition to a significant amount of Core 2 O-glycans following $CD8^+$ T cell activation, and which was previously implicated in T cell apoptosis (Brown et al., 1996; Priatel et al., 2000; Onami et al., 2002), does not participate in $CD8^+$ T cell apoptosis provoked by ST3Gal-I deficiency.

Mechanism of apoptotic regulation by ST3Gal-I

Unsialylated Core 1 O-glycan crosslinking by PNA lectin treatment *in vitro* appears to replicate an *in vivo* stimulus that induces apoptosis among resting wild-type desialylated $CD8^+$ T cells, ST3Gal-I deficient $CD8^+$ T cells, or post-activated $CD8^+$ T cells of either genotype that lack ongoing immune signaling initiated by TCR stimulation. The susceptibility of $CD8^+$ T cells to PNA-induced apoptosis did not coincide with altered expression levels of various cytokine receptors, and was not altered by the addition of IL-7 (data not shown). Furthermore, not all O-glycoproteins bearing unsialylated Core 1 O-glycans are involved. Among known glycoprotein candidates other than CD43, which has been discounted in Chapter 2, both CD8 and CD45 are also modified with unsialylated Core 1 O-glycans (Casabo et al., 1994; Wu et al., 1996; Priatel et al., 2000; Moody et al., 2001, 2003; Amado et al., 2004). Recently, crosslinking of CD8 with antibodies to CD8 or MHC class I ligands has been found to induce apoptosis among immature $CD4^+CD8^+$ thymocytes but not mature $CD8^+$ T cells (Grebe et al., 2004). Moreover, this activity does not appear to be increased by loss of ST3Gal-I (Kao et al., 2006). Interestingly, *de novo* synthesis of CD45 after $CD8^+$ T cell activation has been reported to account for the majority of increased PNA ligands (Amado et al., 2004), and CD45 has been found to participate in apoptosis (Ong et al., 1994; Perillo et al., 1995). However, antibodies to CD45 are unable to induce or modulate apoptosis in ST3Gal-I-deficient T cells (data not shown; Priatel et al., 2000).

Using an inside-out approach to identify intracellular signaling components that instigate caspase activation and apoptotic cell death in ST3Gal-I deficiency, the studies described in Chapter 3 utilizing Bcl-2 transgenic and Bim-deficient model systems led to unexpected findings. The ability of Bcl-2 to reduce the $CD8^+$ T cell contraction phase of an immune response to SEB *in vivo* was completely eliminated by ST3Gal-I deficiency, resulting in few peripheral $CD8^+$ T cells that were mostly annexin V^+, while *in vitro* Bcl-2 transgene expression clearly increased cell viability, along with reduced frequencies of $CD8^+$ T cells that were PNA^+ and annexin V^+ at the cell surface. The implication is that an endogenous apoptotic stimulus is required and is typically lacking *in vitro*. The intermediate phenotype observed in Bim and ST3Gal-I deficient mice appears to reflect a balance between the hyperaccumulation of $CD8^+$ T cells due to Bim deficency, and the ongoing effect of ST3Gal-I deficiency to induce $CD8^+$ T cell apoptosis, as a significant increase in PNA^+ annexin V^+ $CD8^+$ T cells was evident in the absence of both Bim and ST3Gal-I, compared with normal wild type $CD8^+$ T cells. This is consistent with the finding that Bim deficiency failed to reduce the efficacy of PNA treatment as a means to induce apoptotic cell death in the absence of ST3Gal-I. In comparison, Bcl-2 transgene expression does not lead to abnormally high peripheral $CD8^+$ T cell numbers among experimentally naïve mice, and the ability of PNA to induce apoptosis *in vitro* was also unaffected by Bcl-2 overexpression in ST3Gal-I deficient $CD8^+$ T cells. Although the possibility remains that apoptotic regulation involving Bcl-2, Bim, and ST3Gal-I include epistatic relationships, it appears more likely that the death signal delivered

by crosslinking unsialylated Core 1 O-glycans operates independent of Bcl-2 and Bim function in physiologic contexts.

As such, these findings indicate that an endogenous multivalent lectin induces $CD8^+$ T cell apoptosis during the contraction phase of the immune response by crosslinking one or more cell surface O-glycoproteins that are modified by unsialylated Core 1 O-glycans following TCR activation. In this model, $CD8^+$ T cell immune activation thus induces a default apoptotic pathway to cell death that will become effective in the absence of immune signaling, inducing caspase activation and phosphatidylserine externalization that is independent of, and genetically dominant over, the apoptotic regulators Bcl-2 and Bim (**Figure 4-1**).

In Bim deficiency, the vast increase in $CD8^+$ T cell numbers saturates the number of lectin binding sites, and thereby partially reduces peripheral T cell numbers in some tissues to a normal homeostatic level. Sialylation by ST3Gal-I normally protects naïve and memory $CD8^+$ T cells from this apoptotic stimulus in compartments where the apoptotic lectin is present and capable of crosslinking unsialylated Core 1 O-glycans. The identity this putative apoptosis-inducing lectin is not yet known, although candidates include the β-galactoside-binding mammalian galectins, several of which have been reported to induce apoptosis in T cells (Perillo et al., 1995; Kuwabara et al., 2002; Fukumori et al., 2003; Kashio et al., 2003; Sturm et al., 2004). Although reconstitution of this stimulus *ex vivo* in cell culture systems has not been described here, indirect evidence implies that such a molecule exists among splenic and lymph node cell types, but appears absent from blood.

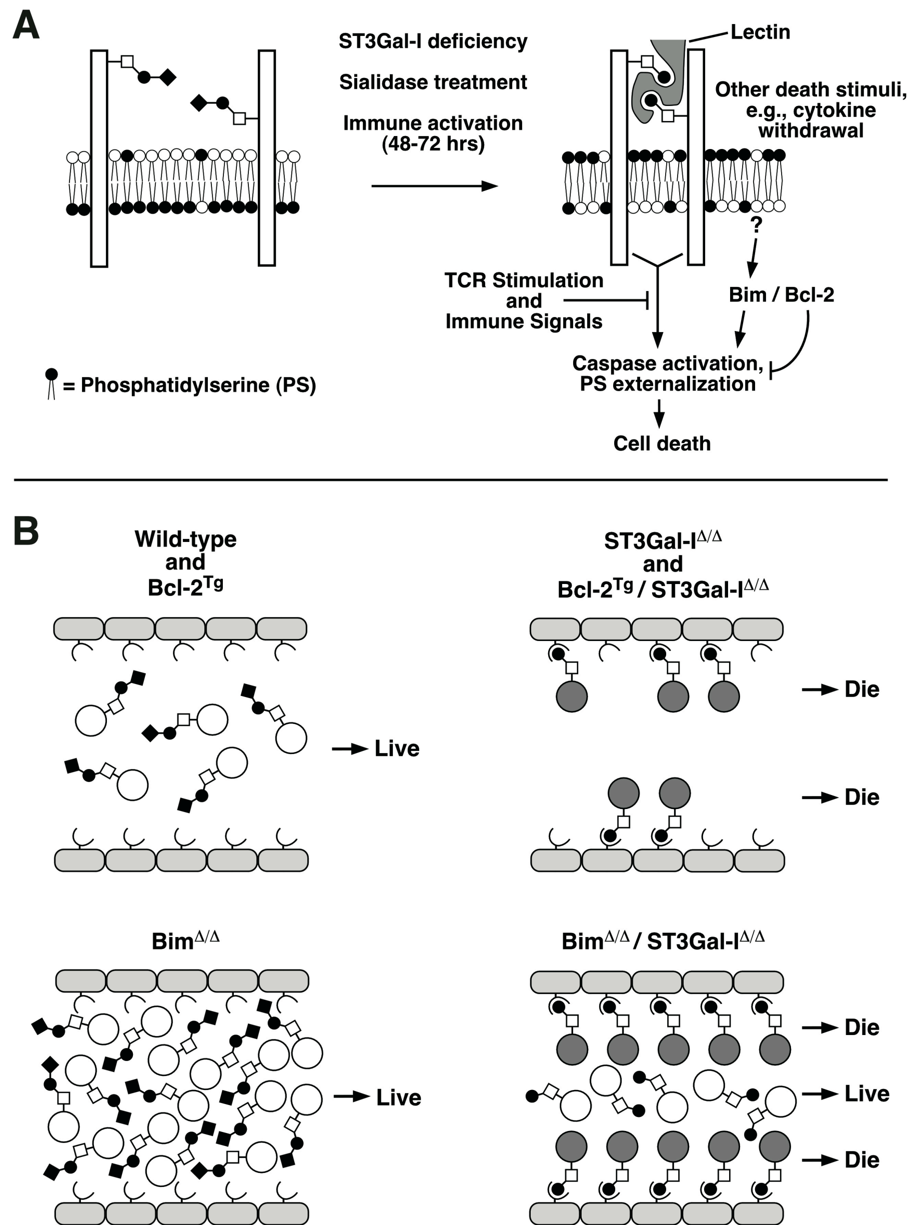

Figure 4-1. Model of ST3Gal-I function in CD8$^+$ T cell apoptosis. (A) Loss of ST3Gal-I-mediated Core 1 O-glycan sialylation induces sensitivity to apoptosis in CD8$^+$ T cells, which can be induced by glycan crosslinking, but is inhibited by TCR stimulation and immune signaling. **(B)** This effect is modulated *in vivo*, perhaps by anatomic restriction of the proposed endogenous lectin, resulting in reduced frequencies of apoptosis when numbers of CD8$^+$ T cells exceed normal levels, as in the case of Bim deficiency.

Reconstitution kinetics among lymphopenic RAG-1 deficient mice were consistent with an apoptosis-inducing lectin that is more highly expressed or active in lymph node compartments as compared with spleen, a finding consistent with the presence of a CD8^{+} T cell contraction phase following SEB immunization in lymph nodes, but not spleen, of Bim and ST3Gal-I deficient mice at the times studied. Previous studies found that the contraction of Bim-deficient CD8^{+} T cells proceeded normally in the lymph nodes after herpes simplex virus infection, and occurred to a greater degree in the lymph nodes than in the spleen in the case of SEB (Hildeman et al., 2002; Pellegrini et al., 2003). The precise anatomic locations and expression levels of the apoptotic stimulus remain to be resolved, as does the potential involvement of other apoptotic regulators, including Bax and Bak (Green, 2005).

Future perspectives

The homeostasis of CD8^{+} T cells may be critically dependent on the presence or absence of multiple factors; nevertheless, the studies described in this dissertation imply that a specific alteration in protein O-glycosylation manifested on the cell surface at late stages of TCR stimulation may play a dominant role in the CD8^{+} T cell contraction phase of the immune response, by linking extracellular glycoprotein structure with molecular interactions that lead to apoptotic signal formation once immune activation signals have subsided, and in the absence of the memory cell sialylation phenotype. The identification of the relevant glycoprotein(s) and apoptotic pathway(s) involved are important matters to resolve in understanding how

Core 1 O-glycan sialylation inhibits $CD8^+$ T cell apoptosis, and may require a glycoproteomics approach that is increasingly able to characterize biologically relevant glycan structures on glycoproteins (Dell and Morris, 2001; Chui et al., 2001; Moody et al., 2003). Moreover, the dysfunction of one or few glycoproteins can be responsible for the emergence of major phenotypes among glycosyltransferase deficient mice, and such studies have implied that protein and glycan determinants can collaborate in the formation of biologic specificity that encompass the production of endogenous ligands for mammalian lectins (Han et al., 2005; Ohtsubo et al., 2005; Ohtsubo and Marth, 2006). Although further studies are needed to determine the identity of the apoptotic stimulus, its precise anatomic distribution, and its molecular counter-receptor on the $CD8^+$ T cell surface, the ability to modulate $CD8^+$ T cell numbers and apoptotic signaling *in vivo* by altering ST3Gal-I activity may be useful in approaches to reduce cytotoxic T cell activity in transplant rejection and the pathogenesis of various diseases including glomerulonephritis and diabetes.

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REFERENCES

Amado, M., Q. Yan, E. M. Comelli, B. E. Collins, and J. C. Paulson. 2004. Peanut agglutinin high phenotype of activated $CD8^+$ T cells results from de novo synthesis of CD45 glycans. J. Biol. Chem. **279:**36689-36697.

Akashi, K., M. Kondo, U. von Freeden-Jeffry, R. Murray, and I. L. Weissman. 1997. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell **89:**1033-1041.

Badovinac, V. P., A. R. Tvinnereim, and J. T. Harty. 2000. Regulation of antigen-specific $CD8^+$ T cell homeostasis by perforin and interferon-γ. Science **290:**1354-1357.

Badovinac, V. P., B. B. Porter, and J. T. Harty. 2004. $CD8^+$ T cell contraction is controlled by early inflammation. Nat. Immunol. **5:**809-817.

Bartholdy, C., J. P. Christensen, D. Wodarz, and A. R. Thomsen. 2000. Persistent virus infection despite chronic cytotoxic T-lymphocyte activation in gamma interferon-deficient mice infected with lymphocytic choriomeningitis virus. J. Virol. **74:**10304-10311.

Beisner, D. R., I. H. Chu, A. F. Arechiga, S. M. Hedrick, and C. M. Walsh. 2003. The requirements for Fas-associated death domain signaling in mature T cell activation and survival. J. Immunol. **171:**247-256.

Bouillet, P., D. Metcalf, D. C. S. Huang, D. M. Tarlington, T. W. H. Kay, F. Köntgen, J. M. Adams, and A. Strasser. 1999. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science **286:**1735-1738

Brown, T. J., W. W. Shuford, W.-C. Wang, S. G. Nadler, T. S. Bailey, H. Marquhardt, and R. S. Mittler. 1996. Characterization of a CD43/leukosialin-mediated pathway for inducing apoptosis in human T-lymphoblastoid cells. J. Biol. Chem. **271:**27686-27695.

Carlow, D. A., S. Y. Corbel, and H. J. Ziltener. 2001. Absence of CD43 fails to alter T cell development and responsiveness. J Immunol. **166:**256-261.

Casabo, L. G., C. Mamalaki, D. Kioussis, and R. Zamoyska. 1994. T cell activation results in the physical modification of the mouse CD8b chain. J. Immunol. **152:**397-404.

Chervenak, R., and J. J. Cohen. 1982. Peanut lectin binding as a marker for activated T-lineage lymphocytes. Thymus **4:**61-67.

Chui, D., G. Sellakumar, R. S. Green, M. Sutton-Smith, T. McQuistan, K. W. Marek, H. R. Morris, A. Dell, and J. D. Marth. 2001. Genetic remodeling of protein glycosylation *in vivo* induces autoimmune disease. Proc. Natl. Acad. Sci. USA **98:**1142-1147.

Cohen, P. L., and R. A. Eisenberg. 1991. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. Ann. Rev. Immunol. **9:**243-269.

Dell, A., and H. R. Morris. 2001. Glycoprotein structure determination by mass spectrometry. Science **291:**2351-2356.

Devadas, S., J. Das, C. Liu, L. Zhang, A. I. Roberts, Z. Pan, P. A. Moore, G. Das, and Y. Shi. 2006. Granzyme B is critical for T cell receptor-induced cell death of Type 2 helper T cells. Immunity **25:**237-247.

Ellies, L. G., S. Tsuboi, B. Petryniak, J. B. Lowe, M. Fukuda, and J. D. Marth. 1998. Core 2 oligosaccharide biosythesis distinguishes between selectin ligands essential for leukocyte homing and inflammation. Immunity **9:**881-890.

Fukumori, T., Y. Takenaka, T. Yoshii, H. R. Kim, V. Hogan, H. Inohara, S. Kagawa, and A. Raz. 2003. CD29 and CD7 mediate galectin-3-induced type II T-cell apoptosis. Cancer Res. **63:**8302-8311.

Galvan, M., K. Murali-Krishna, L. L. Ming, L. Baum, and R. Ahmed. 1998. Alterations in cell surface carbohydrates on T cells from virally infected mice can distinguish effector / memory $CD8^+$ T cells from naive cells. J. Immunol. **161:**641-648.

Goldrath, A. W., and M. J. Bevan. 1999. Selecting and maintaining a diverse T-cell repertoire. Nature **402:**255-262.

Grebe, K. M., R. L. Clarke, and T. A. Potter. 2004. Ligation of CD8 leads to apoptosis of thymocytes that have not undergone positive selection. Proc. Natl Acad. Sci. USA **101:**10410-10415.

Green, D. R. 2005. Apoptotic pathways: ten minutes to dead. Cell **121:**671-674.

Han, S., B. E. Collins, P. Bengston, and J. C. Paulson. 2005. Homomultimeric complexes of CD22 in B cells revealed by protein-glycan cross-linking. Nat. Chem. Biol. **1:**93-97.

Haring, J. S., V. P. Badovinac, and J. T. Harty. 2006. Inflaming the $CD8^+$ T cell response. Immunity **25:**19-29.

Harrington, L. E., M. Galvan, L. G. Baum, J. D. Altman, and R. Ahmed. 2000. Differentiating between memory and effector CD8 T cells by altered expression of cell surface O-glycans. J. Exp. Med. **7:**1241-1246.

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

Hermiston, M. L., Z. Xu, and A. Weiss. 2003. CD45: a critical regulator of signaling thresholds in immune cells. Annu. Rev. Immunol. **21:**107-137.

Hildeman, D. A., T. Mitchell, T. K. Teague, P. Henson, B. J. Day, J. Kappler, and P. C. Marrack. 1999. Reactive oxygen species regulate activation-induced T cell apoptosis. Immunity **10:**735-744.

Hildeman, D. A., Y. Zhu, T. C. Mitchell, P. Bouillet, A. Strasser, J. Kappler, and P. Marrack. 2002. Activated T cell death in vivo mediated by pro-apoptotic Bcl-2 family member Bim. Immunity **16:**759-767.

Kabir, A., M. Watanabe, Z. Takeda, T. Kizaki, and Y. Urano. 1983. Distribution of peanut agglutinin binding sites in rat lymphatic organs. Cell Struct. Funct. **8:**29-34.

Kao, C., M. M. Sandau, M. A. Daniels, and S. C. Jameson. 2006. The sialyltransferase ST3Gal-I is not required for regulation of CD8-class I MHC binding during T cell development. J. Immunol. **176:**7421-7430.

Kashio, Y., K. Nakamura, M. J. Abedin, M. Seki, N. Nishi, N. Yoshida, T. Nakamura, and M. Hirashima. 2003. Galectin-9 induces apoptosis through the calcium-calpain-caspase-1 pathway. J. Immunol. **170:**3631-3636.

Kitagawa, H., and J. C. Paulson. 1994. Differential expression of five sialyltransferase genes in human tissues. J Biol Chem **269:**17872-17878.

Kondo, M., K. Akashi, J. Domen, K. Sugamura, and I. L. Weissman. 1997. Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common γ chain-deficient mice. Immunity **7:**155-162.

Kono, M., Y. Ohyama, Y. C. Lee, T. Hamamoto, N. Kojima, and S. Tsuji. 1997. Mouse beta-galactoside alpha 2,3-sialyltransferases: comparison of in vitro substrate specificities and tissue specific expression. Glycobiology **7:**469-479.

Kuwabara, I., Y. Kuwabara, R. Y. Yang, M. Schuler, D. R. Green, B. L. Zuraw, D. K. Hsu, F. T. Liu. 2002. Galectin-7 (PIG1) exhibits pro-apoptotic function through JNK activation and mitochondrial cytochrome c release. J. Biol. Chem. **277:**3487-3497.

Landolfi, N. F., J. Leone, J. E. Womack, and R. G. Cook. 1985. Activation of T lymphocytes results in an increase in H-2-encoded neuraminidase. Immunogenetics **22:**159-167.

Lau, L. L., B. D. Jamieson, T. Somasundaram, and R. Ahmed. 1994. Cytotoxic T-cell memory without antigen. Nature **369:**648-652.

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

Liu, Y., R. Wada, H. Kawai, K. Sango, C. Deng, T. Tai, M. P. McDonald, K. Araujo, J. N. Crawley, U. Bierfreund, K. Sandhoff, K. Suzuki, and R. L. Proia. 1999. A genetic model of substrate deprivation therapy for a glycosphingolipid storage disorder. J. Clin. Invest. **103:**497-505.

Lohman, B. L., E. S. Razvi, and R. M. Welsh. 1996. T-lymphocyte downregulation after acute viral infection is not dependent on CD95 (Fas) receptor-ligand interactions. J. Virol. **70:**8199-8203.

Lohman, B. L., and R. M. Welsh. 1998. Apoptotic regulation of T cells and absence of immune deficiency in virus-infected IFN-gamma receptor knock-out mice. J. Virol. **72:**7815–7821.

Manjunath, N., M. Correa, M. Ardman, and B. Ardman. 1995. Negative regulation of T-cell adhesion and activation by CD43. Nature **377:**535-538.

Mansour, M. H., H. I. Negm, A. H. Saad, A. Y. Zahran, and N. Badir. 1995. Identification of peanut agglutinin-binding glycoproteins on lizard lymphocytes. Zoolog. Sci. **12:**79-85.

Maraskovsky, E., L. A. O'Reilly, M. Teepe, L. M. Corcoran, J. J. Peschon, and A. Strasser. 1997. Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1$^{-/-}$ mice. Cell **89:**1011-1019.

Marrack, P., and J. Kappler. 2004. Control of T cell viability. Ann. Rev. Immunol. **22:**765-787.

Marth, J. D. 1999. O-glycans. *In* A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart, and J. Marth (eds.), Essentials of Glycobiology. pp. 101-113. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Matloubian, M., M. Suresh, A. Glass, M. Galvan, K. Chow, J. K. Whitmire, C. M. Walsh, W. R. Clark, and R. Ahmed. 1999. A role for perforin in downregulating T-cell responses during chronic viral infection. J. Virol. **73:**2527-2536.

Moody, A. M., D. Chui, P. A. Reche, J. J. Priatel, J. D. Marth, and E. L. Reinherz. 2001. Developmentally regulated glycosylation of the CD8αβ coreceptor stalk modulates ligand binding. Cell **107:**501-512.

Moody, A. M., S. J. North, B. Reinhold, S. J. Van Dyken, M. E. Rogers, M. Panico, A. Dell, H. R. Morris, J. D. Marth, and E. L. Reinherz. 2003. Sialic capping of CD8β Core-1-O glycans controls thymocyte-major histocompatibility complex class I interaction. J. Biol. Chem. **278:**7240-7246.

Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevalulation of bystander activation during viral infection. Immunity **8:**177-187.

Newton, K., A. W. Harris, M. L. Bath, K. G. C. Smith, and A. Strasser. 1998. A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. EMBO J. **17:**706-718.

Nguyen, L. T., K. McKall-Faienza, A. Zakarian, D. E. Speiser, T. W. Mak, and P. S. Ohashi. 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.

Onami, T. M., L. E. Harrington, M. A. Williams, M. Galvan, C. P. Larsen, T. C. Pearson, N. Manjunath, L. G. Baum, B. D. Pearce, and R. Ahmed. 2002. Dynamic regulation of T cell immunity by CD43. J. Immunol. **168:**6022-6031.

Ohtsubo, K., S. Takamatsu, M. T. Minowa, A. Yoshida, M. Takeuchi, and J. D. Marth. 2005. Dietary and genetic control of glucose transporter 2 glycosylation promotes insulin secretion in suppressing diabetes. Cell **123:**1307-1321.

Ohtsubo, K., and J. D. Marth. 2006. Glycosylation in cellular mechanisms of health and disease. Cell **126:**855-867.

Ong, C. J., D. Chui, H. S. Teh, and J. D. Marth. 1994. Thymic CD45 tyrosine phosphatase regulates apoptosis and MHC-restricted negative selection. J. Immunol. **152:**3793-3805.

Ou, R., S. Zhou, L. Huang, and D. Moskophidis. 2001. Critical role for alpha/beta and gamma interferons in persistence of lymphocytic choriomeningitis virus by clonal exhaustion of cytotoxic T cells. J. Virol. **75:**8407-8423.

Paulson, J. C., and K. J. Colley. 1989. Glycosyltransferases. Structure, localization, and control of cell type-specific glycosylation. J. Biol. Chem. **264:**17615-17618.

Pellegrini, M., G. Belz, P. Bouillet, and A. Strasser. 2003. Shutdown of an acute T cell immune response to viral infection is mediated by the pro-apoptotic Bcl-2 homology 3-only protein Bim. Proc. Natl Acad. Sci. USA **100:**14175-14180.

Pellegrini, M., P. Bouillet, M. Robati, G. T. Belz, G. M. Davey, and A. Strasser. 2004. Loss of Bim increases T cell production and function in interleukin 7 receptor-deficient mice. J. Exp. Med. **200:**1189-1195.

Pereira, M. E., E. A. Kabat, R. Lotan, and N. Sharon. 1976. Immunochemical studies on the specificity of the peanut (Arachis hypogaea) agglutinin. Carbohydr. Res. **51:**107-118.

Perillo, N. L., K. E. Pace, J. J. Seilhamer, and L. G. Baum. 1995. Apoptosis of T cells mediated by galectin-1. Nature **378:**736-739.

Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, J. D. Meyer, and B. L. Davison. 1994. Early lymphocyte expansion is severly impaired in interleukin 7 receptor-deficient mice. J. Exp. Med. **180:**1955-1960.

Petschner, F., C. Zimmerman, A. Strasser, D. Grillot, G. Nunez, and H. Pircher. 1998. Constitutive expression of Bcl-xL or Bcl-2 prevents peptide antigen-induced T cell deletion but does not influence T cell homeostasis after a viral infection. Eur. J. Immunol. **28:**560-569.

Piller, F., V. Piller, R. I. Fox, and M. Fukuda. 1988. Human T-lymphcyte activation is associated with changes in O-glycan biosynthesis. J. Biol. Chem. **263:**15146-15150.

Priatel, J. J., D. Chui, N. Hiraoka, C. J. Simmons, K. B. Richardson, D. M. Page, M. Fukuda, N. M. Varki, and J. D. Marth. 2000. The ST3Gal-I sialyltransferase controls $CD8^+$ T lymphocyte homeostasis by modulating O-glycan biosynthesis. Immunity **12:**273-283.

Raedler, A., E. Raedler, R. Arndt, and H. G. Thiele. 1981. Terminal galactosyl residues of cell-surface glycoconjugates exposed on both human and murine immature T- and B-cells. Cell Tissue Res. **218:**219-226.

Razvi, E. S., Z. Jiang, B. A. Woda, and R. M. Welsh. 1995. Lymphocyte apoptosis during the silencing of the immune response to acute viral infections in normal, lpr, and Bcl-2-transgenic mice. Am. J. Pathol. **147:**79–91.

Reich, A., H. Körner, J. D. Sedgwick, and H. Pircher. 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.

Reisner, Y., M. Linker-Israeli, and N. Sharon. 1976. Separation of mouse thymocytes into two subpopulations by the use of peanut agglutinin. Cell. Immunol. **25:**129-134.

Reisner, Y., M. Biniaminov, E. Rosenthal, N. Sharon, and B. Ramot. 1979. Interaction of peanut agglutinin with normal human lymphocytes and with leukemic cells. Proc. Nat. Acad. Sci. USA **76:**447-451.

Rose, M. L., and F. Malchiodi. 1981. Binding of peanut lectin to thymic cortex and germinal centers of lymphoid tissue. Immunology **42:**583-591.

Salmena, L., B. Lemmers, A. Hakem, E. Matysiak-Zablocki, K. Murakami, P. Y. Au, D. M. Berry, L. Tamblyn, A. Shehabeldin, E. Migon, A. Wakeham, D. Bouchard, W. C. Yeh, J. C. McGlade, P. S. Ohashi, and R. Hakem. 2003. Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. Genes Dev. **17:**883-895.

Schachter, H., and I. Brockhausen. 1989. The biosythesis of branched O-glycans. Symp. Soc. Exp. Biol. **43:**1-26.

Seder, R. A., and R. Ahmed. 2003. Similarities and differences in $CD4^+$ and $CD8^+$ effector and memory T cell generation. Nat. Immunol. **4:**835-842.

Sharma, V., M. Delgado, and D. Ganea. 2006. Granzyme B, a new player in activation-induced cell death, is down-regulated by vasoactive intestinal peptide in Th2 but not Th1 effectors. J. Immunol. **176:**97-110.

Siegel, R. M., F. K. Chan, H. J. Chun, and M. J. Lenardo. 2000. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. Nat. Immunol. **1:**469-474.

Snapp, K. R., C. E. Heitzig, L. G. Ellies, J. D. Marth, and G. S. Kansas. 2001. Differential requirements for the O-linked branching enzyme core 2 β1-6-N-glycosaminyltransferase in biosynthesis of ligands for E-selectin and P-selectin. Blood **97:**3806-3811.

Strasser, A., A. W. Harris, and S. Cory. 1991. Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. Cell **67:**889-899.

Strasser, A. 2005. The role of BH3-only proteins in the immune system. Nat. Rev. Immunol. **5:**189-200.

Sturm, A., M. Lensch, S. Andre, H. Kaltner, B. Wiedenmann, S. Rosewicz, A. U. Dignass, and H. J. Gabius. 2004. Human galectin-2: novel inducer of T cell apoptosis with distinct profile of caspase activation. J. Immunol. **173:**3825-3837.

Taira, S., and H. Nariuchi. 1988. Possible role of neuraminidase in activated T cells in the recognition of allogeneic Ia. J. Immunol. **141:**440-446.

Uni, Z., and E. D. Heller. 1991. Peanut agglutinin as a marker of maturation and activation of chicken thymic-derived lymphocytes. Poult. Sci. **70:**1516-1520.

von Freeden-Jeffry, U., P. Vieira, L. A. Lucian, T. McNeil, S. E. G. Burdach, and R. Murray. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J. Exp. Med. **181:**1519-1526.

Wang, X. Z., S. E. Stepp, M. A. Brehm, H. D. Chen, L. K. Selin, and R. M. Welsh. 2003. Virus-specific CD8 T cells in peripheral tissues are more resistant to apoptosis than those in lymphoid organs. Immunity **18:**631-642.

Weinstein, J., E. U. Lee, K. McEntee, P.-H. Lai, and J. C. Paulson. 1987. Primary structure of β-galactoside α2,6-sialyltransferase. J. Biol. Chem. **262:**17735-17743.

Walsh, C. M., B. G. Wen, A. M. Chinnaiyan, K. O'Rourke, V. M. Dixit, and S. M. Hedrick. 1998. A role for FADD in T cell activation and development. Immunity **8:**439-449.

Wu, W., P. H. Harley, J. A. Punt, S. O. Sharrow, and K. P. Kearse. 1996. Identification of CD8 as a peanut agglutinin (PNA) receptor molecule on immature thymocytes. J. Exp. Med. **184:**759-764.

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

Zhang, M., S. M. Park, Y. Wang, R. Shah, N. Liu, A. E. Murmann, C. R. Wang, M. E. Peter, and P. G. Ashton-Rickardt. 2006. Serine protease inhibitor 6 protects cytotoxic T cells from self-inflicted injury by ensuring the integrity of cytotoxic granules. Immunity **24:**451-461.

Zhou, S., R. Ou, L. Huang, and D. Moskophidis. 2002. Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T cells during persistent viral infection. J. Virol. **76:**829-840.

Zhumabekov, T., P. Corbella, M. Tolaini, and D. Kioussis. 1995. Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. J. Immunol. Methods **185:**133-140.