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THE ROLE OF ENDOPLASMIC RETICULUM STRESS RESPONSE SIGNALING IN T CELLS

A Dissertation Presented

By

STEVEN C. PINO

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

July 8th, 2008

Interdisciplinary Graduate Program

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APPROVAL PAGE

THE ROLE OF ENDOPLASMIC RETICULUM STRESS RESPONSE SIGNALING IN T CELLS

A Dissertation Presented

By

Steven C. Pino

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ABSTRACT

T cells play a central role in cellular-mediated immunity and must become activated to participate as effector cells in the immune response. The activation process is highly intricate and involves stimulation of a number of downstream signaling pathways enabling T cells to proliferate and produce cytokines that are vital for proper effector function. This increase in protein production and protein folding activity adds to the normal physiological strain on cellular machinery. One cellular compartment that has generated a mechanism to mitigate the stress induced by increased protein production is the endoplasmic reticulum (ER).

In general, an increase in cellular production of proteins that overwhelms a cell's protein folding capability can alter ER homeostasis and lead to ER stress. To counteract this stress, an adaptive cellular mechanism known as the ER stress response (ERSR) is initiated. The ERSR allows a cell to cope with normal physiological stress within the ER caused by increased protein translation. In this dissertation, we show that *in vitro* and *in vivo* T cell activation involving T cell receptor (TCR) ligation in the presence of costimulation initiates the physiological ERSR. Interestingly, the ERSR was also activated in T cells exposed only to TCR ligation, a treatment known to induce the 'non-responsive' states of anergy and tolerance. We further identified a key component of the downstream TCR signaling pathway, protein kinase C (PKC), as an initiator of physiological ERSR signaling, thus revealing a previously unknown role for this serine/threonine protein kinase in T cells. Therefore, induction of the physiological ERSR

through PKC signaling may be an important 'preparatory' mechanism initiated during the early activation phase of T cells.

If ER stress is persistent and ER homeostasis is not reestablished, physiological ER stress becomes pathological and initiates cellular death pathways through ER stress-induced apoptotic signaling. We further present data demonstrating that absence of functional Gimap5, a putative GTPase implicated to play a role in TCR signaling and maintenance of overall T cell homeostasis, leads to pathological ER stress and apoptosis. Using the BioBreeding diabetes-prone (BBDP) rat, a model for type 1 diabetes (T1D), we link pathological ER stress and ER stress-induced apoptotic signaling to the observed T cell lymphopenic phenotype of the animal. By depleting the ER stress apoptotic factor CHOP with siRNA, we were able to protect Gimap5$^{-/-}$ BBDP rat T cells from ER stress-induced death. These findings indicate a direct relationship between Gimap5 and maintenance of ER homeostasis for T cell survival.

Overall, our findings suggest that the ERSR is activated by physiological and pathological conditions that disrupt T cell homeostasis. TCR signaling that leads to PKC activation initiates a physiological ERSR, perhaps in preparation for a T cell response to antigen. In addition, we also describe an example of pathological ERSR induction in T cells. Namely, we report that the absence of functional Gimap5 protein in T cells causes CHOP-dependent ER stress-induced apoptosis, perhaps initiated by deregulation of TCR signaling. This indicates a dual role for TCR signaling and regulation in the initiation of both the physiological and pathological ERSR. Future research that provides insights into the molecular mechanisms that govern ERSR induction in TCR signaling and regulation

may lead to development of therapeutic modalities for treatment of immune-mediated diseases such as T1D.

TABLE OF CONTENTS

COPYRIGHT INFORMATION II

APPROVAL PAGE III

ACKNOWLEDGEMENTS IV

ABSTRACT VI

TABLE OF CONTENTS IX

LIST OF TABLES XII

LIST OF FIGURES XIII

ABBREVIATIONS XIV

CHAPTER I: INTRODUCTION TO ENDOPLASMIC RETICULUM STRESS 1

- OVERVIEW OF ENDOPLASMIC RETICULUM STRESS 1
- COMPONENTS OF ER STRESS RESPONSE SIGNALING 1
- ATF6 AND TRANSCRIPTIONAL INDUCTION OF ERSR-INDUCED GENES 2
- AMPLIFICATION OF THE ERSR BY IRE1 SIGNALING 4
- PROTEIN TRANSLATIONAL ATTENUATION BY PERK 6
- ER STRESS-INDUCED APOPTOSIS 8
- ERSR SIGNALING DURING PHYSIOLOGICAL CELLULAR PROCESSES 11
- THE ERSR IN HEALTH AND DISEASE 12

CHAPTER II: T CELL ACTIVATION AND SIGNALING 14

- OVERVIEW OF T CELL ACTIVATION 14
- DEVELOPMENT OF T CELLS IN THE THYMUS 17
- T CELL RECEPTOR SIGNAL TRANSDUCTION 18
- PROTEIN KINASE C SIGNALING 20
- TOLERANCE MECHANISMS 21
- TRANSPLANTATION TOLERANCE 23
- T CELL RECEPTOR SIGNALING IMMUNOTHERAPIES 24

CHAPTER III: GIMAP5 AND THE BIOBREEDING DIABETES-PRONE RAT 25

- OVERVIEW OF GIMAP5 25
- THE FUNCTION OF GTPASES 25
- IDENTIFICATION OF GIMAP5 IN BCR/ABL ONCOGENE TRANSFECTED CELLS 26

THE GIMAP FAMILY OF GENES ... 27

THE BIOBREEDING DIABETES-PRONE AND DIABETES-RESISTANT RATS ... 29

CHAPTER IV: MATERIALS AND METHODS ... 31

ANIMALS ... 31

T CELL PREPARATION ... 31

IN VITRO T CELL STIMULATION ... 31

IL-2 ELISA ... 32

WESTERN BLOTTING ... 32

FLOW CYTOMETRY ... 33

ANTIBODIES ... 33

SYNCHIMERAS AND TOLERANCE INDUCTION ... 35

T CELL TRANSFECTION ... 35

APOPTOSIS DETECTION ... 36

STATISTICS ... 36

CHAPTER V: PROTEIN KINASE C SIGNALING DURING T CELL ACTIVATION INDUCES THE ENDOPLASMIC RETICULUM STRESS RESPONSE ... 37

INTRODUCTION ... 37

RESULTS ... 39

A. The ERSR is induced in T cells by thapsigargin ... 39

B. The ERSR occurs following *in vitro* T cell activation ... 41

C. The ERSR occurs following partial T cell activation *in vitro* via signal one alone ... 42

D. Upregulation of multiple ER chaperones following *in vitro* TCR signaling in the presence or absence of signal two ... 46

E. The ERSR is induced by *in vivo* activation of T cells ... 48

F. The ERSR characterizes *in vivo* tolerized T cells that receive signal one alone ... 49

G. Ionomycin stimulation of T cells does not lead to ERSR signaling ... 53

H. PKC signaling in T cells initiates the ERSR ... 57

I. PKC inhibition during T cell activation with PMA and Io diminishes ERSR signaling and IL-2 production ... 58

J. The expression of multiple ER chaperones increases following PKC activation ... 62

SUMMARY ... 64

CHAPTER VI: ABSENCE OF FUNCTIONAL GIMAP5 PROTEIN ACTIVATES ENDOPLASMIC RETICULUM STRESS-INDUCED APOPTOSIS IN T CELLS 66

INTRODUCTION 66

RESULTS 67

A. Increased expression of ERSR proteins in Gimap5$^{-/-}$ BBDP rat lymphocytes..... 67

B. GRP78 and CHOP expression levels are similar between Gimap5$^{+/+}$ and Gimap5$^{-/-}$ rat thymocyte CD4^{+}/CD8^{+} subpopulations 70

C. CD90^{+} recent thymic emigrants comprise the majority of peripheral CD4^{+}/CD8^{+} T cells in the Gimap5$^{-/-}$ rat.. 73

D. Gimap5$^{-/-}$ rat T cell populations in the periphery express increased levels of GRP78 and CHOP 76

E. Knockdown of CHOP expression in Gimap5$^{-/-}$ rat T cells leads to decreased apoptosis and cell death 81

SUMMARY.. 88

CHAPTER VII: DISCUSSION.. 89

PKC SIGNALING INDUCES THE ERSR IN T CELLS 89

ER STRESS-INDUCED APOPTOSIS IN GIMAP5$^{-/-}$ T CELLS 93

CONCLUSIONS AND REMAINING QUESTIONS 97

REFERENCES.. 104

List of Tables

Table 1: Phenotypic profile of thymocytes from BBDR and BBDP rat strains 85

Table 2: Phenotypic profile of lymphocytes from BBDR and BBDP rat strains 86

Table 3: Phenotypic profile of CD90 cell surface expression on lymphocytes from BBDR and BBDP rat strains 87

List of Figures

Figure 1: ATF6 activation by the ERSR results in transcription of ERSR-induced genes ... 3

Figure 2: ERSR signaling results in increased ER chaperone expression ... 5

Figure 3: IRE1 signaling amplifies the ERSR ... 7

Figure 4: PERK activation by the ERSR results in translational attenuation ... 10

Figure 5: Downstream components of TCR signaling ... 16

Figure 6: Thapsigargin treatment of T cells induces ER stress ... 40

Figure 7: TCR signaling *in vitro* induces the ERSR ... 45

Figure 8: ER chaperone expression increases during *in vitro* TCR signaling ... 47

Figure 9: Increased GRP78 expression with *in vivo* T cell activation and *in vivo* tolerance induction ... 52

Figure 10: Stimulation of T cells with Io does not activate ERSR signaling ... 56

Figure 11: Activating PKC with PMA treatment initiates ERSR signaling in T cells ... 61

Figure 12: PKC inhibition during T cell activation prevents ERSR signaling ... 63

Figure 13: A model for enhanced ER chaperone expression following PKC signaling downstream of TCR/CD3 ligation ... 65

Figure 14: The expression of ER chaperones increases in lymphocytes from the Gimap5$^{-/-}$ BBDP rat ... 69

Figure 15: ERSR signaling is similar in thymocytes from BBDR and BBDP rats ... 72

Figure 16: The majority of T cells in the periphery of the Gimap5$^{-/-}$ BBDP are CD90^{+} recent thymic emigrants ... 75

Figure 17: The ERSR is increased in all TCR^{+} lymphocyte populations within BBDP rats ... 78

Figure 18: ER stress-induced apoptotic signaling is activated within TCR^{+} lymphocyte populations in BBDP rats ... 79

Figure 19: B cell populations within BBDP and BBDR express similar ERSR signaling ... 80

Figure 20: Knockdown of CHOP protein expression enhances Gimap5$^{-/-}$ T cell viability ... 84

ABBREVIATIONS

ER	endoplasmic reticulum
Ca^{2+}	calcium
ERSR	endoplasmic reticulum stress response
ATF6	activating transcription factor
IRE1	inositol-requiring enzyme 1
PERK	PKR-like ER kinase
GRP78	glucose-regulated protein 78
S1P	site-1-protease
S2P	site-2-protease
bZIP	basic leucine zipper
GRP94	glucose-regulated protein 94
PDI	protein disulfide isomerase
ERp72	ER protein 72
XBP1	X-box binding protein 1
GLS	Golgi complex localization signal
UPR	unfolded protein response
eIF2	eukaryotic translation initiation factor 2
ATF4	activating transcription factor 4
GADD34	growth-arrest DNA-damage gene 34
PP1	protein phosphatase 1
CHOP	C/EBP-homologous protein
MEF	mouse embryonic fibroblast
TM	tunicamycin
TG	thapsigargin
MHC	major histocompatibility complex
T2D	type 2 diabetes
T1D	type 1 diabetes
TCR	T cell receptor
APC	antigen presenting cell
PMA	phorbol 12-myristate 13-acetate
Io	ionomycin
PKC	protein kinase C
IL-2	interleukin-2
PTK	protein tyrosine kinase

MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor κB
PLCγ	phospholipase Cγ
PIP_2	phosphatidyl inositol-4,5 biphosphate
DAG	diacylglycerol
IP_3	inositol-1,4,5-trisphosphate
CN	calcineurin
NFAT	nuclear factor of activated T cells
GEFs	guanine-nucleotide exchange factors
AP-1	activator protein-1
T_{regs}	regulatory T cells
DST	donor specific transfusion
mAb	monoclonal antibody
Gimap	GTPases of immunity-associated proteins
CML	chronic myeloid leukemia
BBDP	BioBreeding diabetes-prone
BBDR	BioBreeding diabetes-resistant
VAF	viral antibody free
poly I:C	polyinosinic:polycytidylic acid
KRV	Kilham rat virus
PBS	phosphate buffered saline
FBS	fetal bovine serum
DMSO	dimethyl sulfoxide
BCA	bicinchoninic acid
FCS	fetal clone serum
DES	Désiré
HRP	horseradish peroxidase
PBMC	peripheral blood mononuclear cells
7AAD	7-amino-actinomycin D
FSC	forward scattering
SSC	side scattering
MFI	mean fluorescence intensity
AU	arbitrary units
BCR	B cell receptor
Egr	early growth receptor
AICD	activation-induced cell death

CHAPTER I: INTRODUCTION TO ENDOPLASMIC RETICULUM STRESS

Overview of Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) is a multifunctional organelle which plays a vital role in the regulation of numerous cellular processes. The ER, which functions as the central processing plant for the synthesis, folding, and assembly of secretory and transmembrane proteins (1-3), also participates in calcium (Ca^{2+}) signaling, vesicle trafficking, drug metabolism, and lipid biogenesis (4-6). As the main component for protein processing within a cell, the ER has evolved numerous signaling pathways that monitor its protein folding capacity to ensure these pathways do not become overwhelmed (3). However, disturbances to normal cellular functioning and homeostatic conditions can interfere with the responsibilities of the ER and cause “stress” to the organelle. These perturbations that disrupt normal ER functioning and lead to ER stress include accumulations of unfolded or misfolded proteins, Ca^{2+} fluxes across the ER membrane, glucose depletion, and significant changes to the redox or ionic potential within the lumen of the ER (5-8). To restore ER homeostasis and alleviate ER stress, eukaryotic cells trigger an adaptive cellular mechanism known as the ER stress response (ERSR) (5,6).

Components of ER Stress Response Signaling

The ERSR was initially discovered in the budding yeast *Saccharomyces cerevisiae* where researchers uncovered a role for this signaling pathway in the maintenance and regulation of numerous secretory pathways (5,9). Within mammals, the

general features of the ERSR discovered in yeast are maintained, but function in a more complex manner and also include the responsibilities of protein translational control and programmed cellular death pathways (9,10). Component programs of the mammalian ERSR include: 1) transcriptional activation of ERSR-induced genes, 2) general attenuation of protein translation, 3) ER-associated degradation, and 4) ER stress-induced apoptosis. Coordination of the multifaceted ERSR relies upon the precise regulation of transducer molecules that are responsible for carrying out the initiation signals necessary for orchestration of the multiple complex programs initiated by ERSR signaling (11). The three major ERSR transducers responsible for activation of the ERSR include activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and PKR-like ER kinase (PERK) (12).

ATF6 and Transcriptional Induction of ERSR-induced genes

The constitutively expressed ATF6 is localized to the ER membrane of all cells and remains bound to glucose-regulated protein 78 (GRP78), an ER-resident molecular chaperone which functions as the central regulator of the ERSR (11,13). Upon conditions that cause ER stress, GRP78 releases from ATF6, unveiling a Golgi complex localization signal. This allows the 90 kDa ATF6 molecule to migrate to the Golgi complex where it is proteolytically cleaved by site-1-protease (S1P) and site-2-protease (S2P), thus generating a 50 kDa nuclear form of ATF6, known as pATF6(N) (11,14,15). This nuclear form of ATF6 is a basic leucine zipper (bZIP) transcription factor that shuttles to the

FIGURE 1

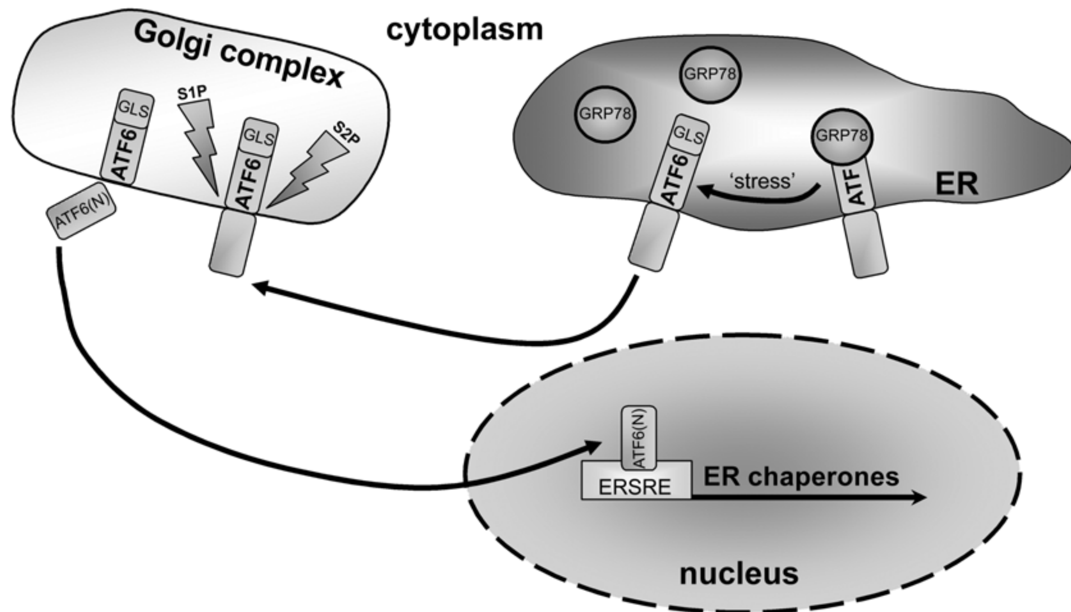

Figure 1: ATF6 activation by the ERSR results in transcription of ERSR-induced genes.

The release of ATF6 from GRP78 unveils a Golgi complex localization signal (GLS) on ATF6. This allows ATF6 to shuttle to the Golgi complex where the protein undergoes proteolytic cleavage by S1P and S2P. This cleavage results in a nuclear form of ATF6, which as a transcription factor, travels to the nucleus and binds to the ERSR element in the promoter of ER chaperone genes (adapted from (16)).

nucleus and binds to the ERSR element within the promoter sequence of target genes thereby driving their expression (Figure 1) (11,17). In this manner, activation of ERSR signaling is capable of utilizing pre-existing ATF6 protein for the transcription of genes regulated by the ERSR element without synthesis of de novo proteins (11). Many of the target genes for pATF6(N) encode ER chaperone proteins, including GRP78, glucose-regulated protein 94 (GRP94), protein disulfide isomerase (PDI), and ER protein 72 (ERp72). Chaperones reside in the lumen of the ER and participate in protein folding activities as well as quality control mechanisms that ensure retention of misfolded or unfolded proteins to the ER (18,19). Upon activation of the ERSR, expression of ER chaperones is increased to enhance the protein folding capacity of the ER in order to alleviate the "stress" caused by an accumulation of unfolded proteins (Figure 2).

Amplification of the ERSR by IRE1 Signaling

In cells that are not undergoing ER stress, the second main ERSR transducer, IRE1, remains bound to GRP78 in an unphosphorylated and monomeric state (20). IRE1 is an ER transmembrane protein that contains both serine-threonine kinase and endoribonuclease domains in it cytoplasmic region (11,21). When ER stress occurs, GRP78 releases from IRE1's lumenal domain allowing IRE1 to activate via oligomerization and trans-autophosphorylation (20). Activated IRE1 through its endoribonuclease activity performs a site-specific cleavage on its substrate molecule, X-box binding protein 1 (*XBP1*) mRNA, thus removing a 26-nucleotide intron (11,22). Spliced *XBP1* mRNA is ligated by an unidentified RNA ligase and allows for the

FIGURE 2

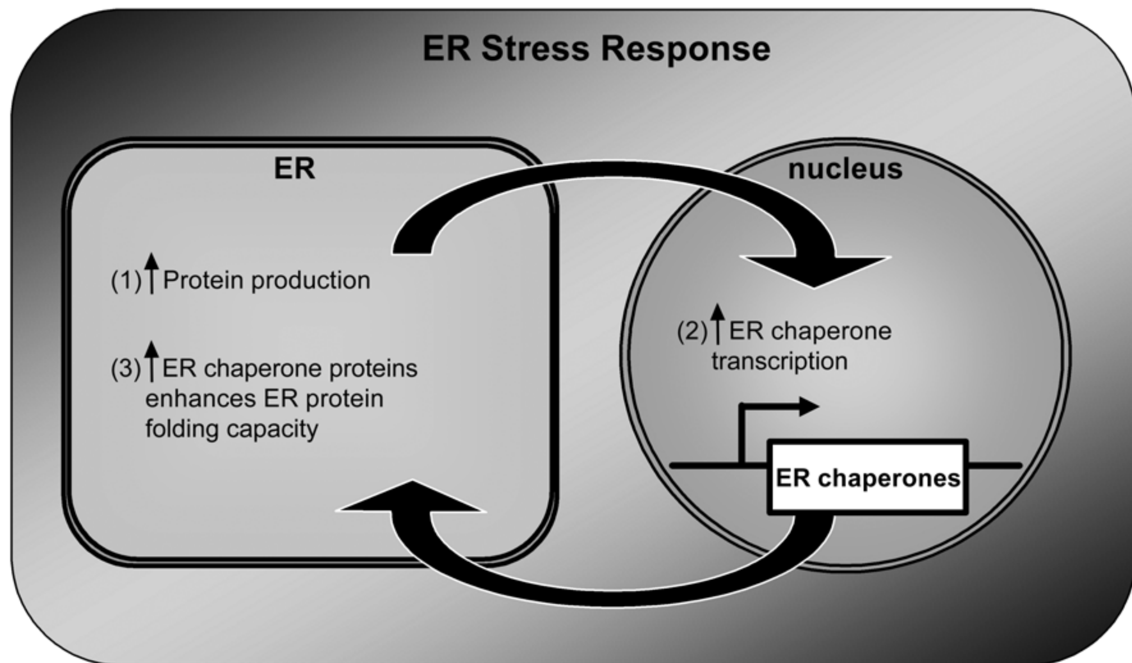

Figure 2: ERSR signaling results in increased ER chaperone expression.

An increase in protein production into the ER results in signaling from the ER to the nucleus leading to enhanced transcription of ER chaperones. The ER chaperones are translated into the ER causing an increase in ER chaperone proteins which enhances the protein folding capacity of the ER.

formation of the mature *XBP1* mRNA. Mature *XBP1* mRNA encodes for a bZIP transcription factor, pXBP1(S), which travels to the nucleus and binds to the ERSR element of target genes, as well as to another cis-acting element, known as the unfolded protein response (UPR) element (Figure 3) (22). Through this complex mechanism, activation of IRE1 is capable of increasing the amount of ER chaperones and further amplifying the ERSR to assist in the return of the cell to homeostasis.

Protein Translational Attenuation by PERK

Although the three ERSR transducer molecules are activated simultaneously when ER stress conditions begin, the third ERSR transducer molecule, PERK, is thought to handle the immediate response (23). PERK is a transmembrane protein that is activated when GRP78 dissociates from its lumenal domain (24). PERK activation is comparable to the activation process of IRE1, in that the loss of GRP78 allows for PERK to oligomerize and trans-autophosphorylate (6,24). Activated PERK phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2) on serine 51 which causes attenuation of general protein translation through decreased recognition of the AUG initiation codon (6,12,15). Therefore, through a translational control mechanism, PERK decreases the workload of the ER by decreasing protein synthesis (25).

Paradoxically, the translation of certain mRNAs occurs more efficiently in the presence of phosphorylated eIF2α (15,26). Because a global attenuation of protein translation would disrupt the synthesis of ERSR-induced gene products, it is important for the cell to maintain regulatory networks to balance the opposing effects of ERSR

FIGURE 3

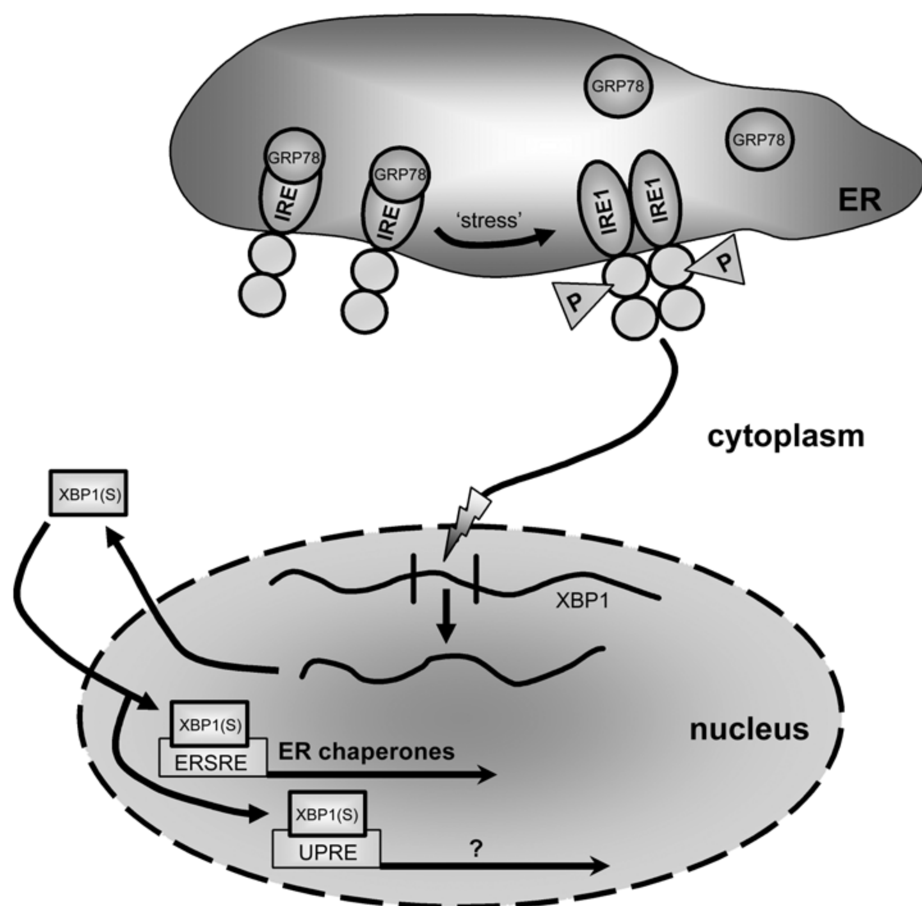

Figure 3: IRE1 signaling amplifies the ERSR.

When ER stress occurs, GRP78 releases IRE1 which oligomerizes and *trans*-autophosphorylates to become activated. The activated form of IRE1 cleaves *XBP1* mRNA allowing for formation of a mature form of *XBP*1 mRNA which encodes a transcription factor. This transcription factor enters into the nucleus and binds to elements within the promoter sequence of ERSR and UPR-regulated genes (adapted from (11)).

activation (11,25). One such mRNA that is preferentially translated by phosphorylated eIF2α encodes for activating transcription factor 4 (ATF4) (12,26). Upon PERK activation, ATF4 translation increases leading to the induction of growth-arrest DNA-damage gene 34 (GADD34). GADD34, through an association with protein phosphatase 1 (PP1), acts to dephosphorylate eIF2α, thus alleviating the repression of protein translation (11,27,28). If persistent ER stress exists within the cell, ATF4 induces the expression of C/EBP-homologous protein (CHOP), a transcription factor involved in ER stress-induced apoptotic signaling (Figure 4) (24). Consequently, activation of PERK and its downstream signaling pathways enable the ERSR to affect processes necessary for determining cell survival (11).

ER Stress-Induced Apoptosis

Normally, stress within the ER triggers the ERSR in an attempt to return the cell to homeostasis (20,22). However, if ER stress persists and cellular homeostasis can not be restored, the ERSR can initiate cell death stimuli via ER stress-induced apoptotic signaling (5,29). Initiation of ER stress-induced apoptosis through ERSR signaling involves the transcriptional activation of the bZIP transcription factor *chop* (30). CHOP protein acts to repress the promoter of the *bcl-2* gene, thus downregulating antiapoptotic Bcl-2 protein and rendering cells sensitive to the proapoptotic effects of BH3-only proteins (31,32). $CHOP^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibit significantly lower cell death when faced with agents that perturb ER function (33). Furthermore, targeted disruption of the *chop* gene in the Akita mouse protected beta cells from

FIGURE 4

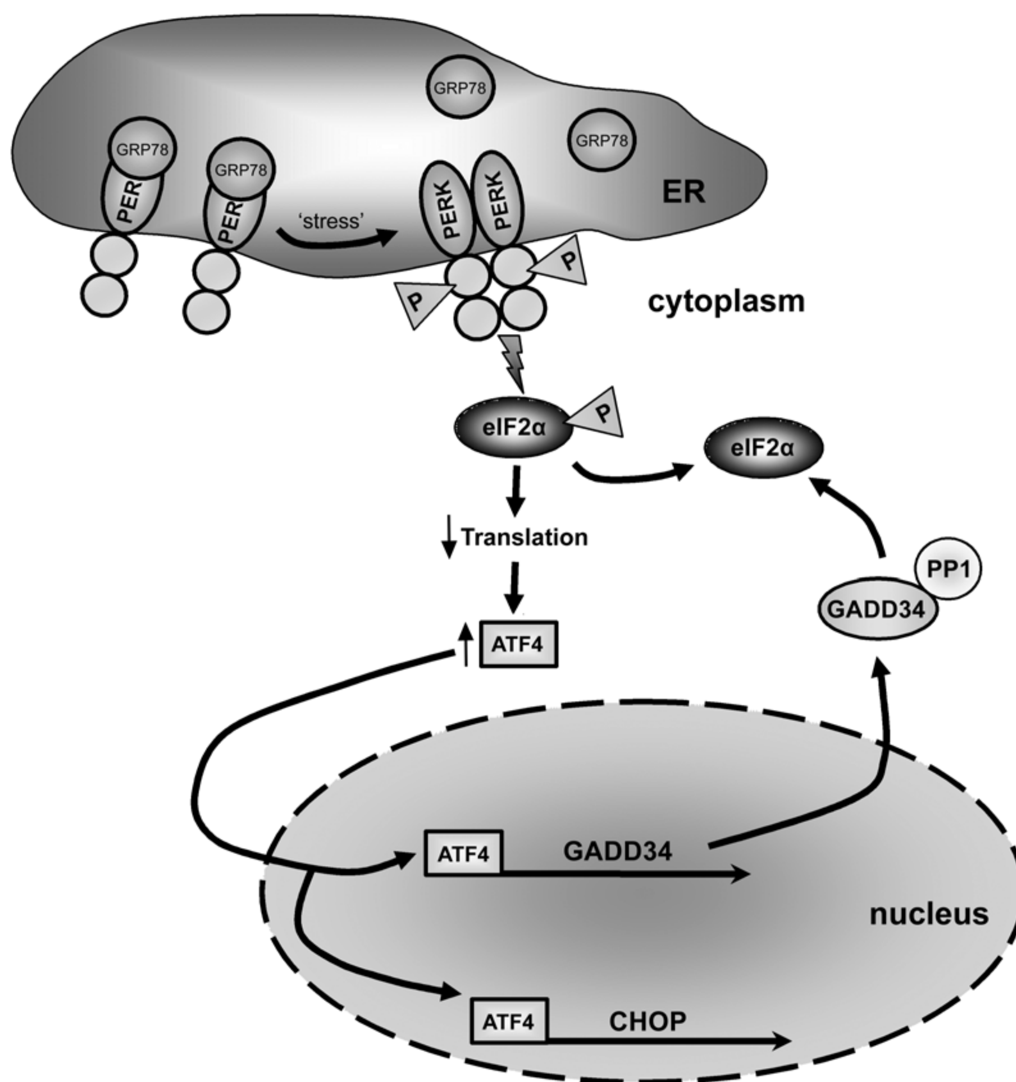

Figure 4: PERK activation by the ERSR results in translational attenuation.

The release of GRP78 from PERK following ERSR signaling allows for oligomerization and *trans*-autophosphorylation of the protein. Activated PERK phosphorylates eIF2α thereby effectively decreasing protein translation. However, certain mRNAs, such as *atf4* mRNA, are preferentially translated by phosphorylated eIF2α. ATF4 induces the expression of GADD34 and CHOP. GADD34 together with PP1 act to dephosphorylate eIF2α to relieve the cell of protein translational attenuation (adapted from (11)).

apoptosis normally caused by an accumulation of mutant insulin in the ER (34,35). These data suggest that CHOP acts as a regulatory protein in the initiation of ER stress-induced apoptosis.

ERSR Signaling during Physiological Cellular Processes

ERSR signaling may be initiated by pathological events, and has classically been associated with exposure to deleterious chemical stressors. Chemical agents such as tunicamycin (TM), an inhibitor of N-linked glycosylation (36), and thapsigargin (TG), an inhibitor of the ER $Ca2^{+}$-ATPase (37), disrupt ER homeostasis and lead to ERSR signaling. More recently, ERSR signaling has also been shown to occur during numerous cellular processes. During muscle development, expression of GRP78 and CHOP is induced in both differentiating myoblasts and apoptotic cells, indicating a role for ERSR signaling during myoblast differentiation (38). Additionally, the differentiation of B lymphocytes into antibody-producing plasma cells involves a physiological ERSR to permit high levels of antibody production (11,39,40). Furthermore, ERSR signaling has been found to be essential for the proper functioning of hepatocytes and osteoblasts (23).

Recently, priming of $CD4^{+}$ T cells with peptides in the context of major histocompatibility complex (MHC) molecules has been reported to utilize components of physiological ERSR signaling, including expression of stress response-induced genes (41). Investigations of pancreatic beta cells have also led to the discovery of the initiation of multiple components of ERSR signaling during their production and secretion of insulin (42). IRE1 signaling has been found to play several roles in maintaining ER

homeostasis and regulated insulin production in pancreatic beta cells (43,44). Thus, physiological ER stress appears to be an intrinsic cellular state that may be initiated by distinct activators involved in the precise regulation of cellular differentiation and function.

The ERSR in Health and Disease

Misfolding of cellular proteins leads to ER stress and has been speculated as the cause of numerous conformational diseases, such as Parkinson's disease and Alzheimer's disease (3). Formation of protein aggregates, caused by an accumulation of unfolded or misfolded proteins, is thought to disrupt proteasome function, thereby inhibiting the ER-associated degradation pathway. This malfunction of cellular machinery is thought to interfere with ERSR induction and has been implicated in the development of Parkinson's disease (3,24,45). In patients with Alzheimer's disease, the ERSR has been shown to be activated (46,47) and it is believed that its induction is due to an accumulation of amyloid beta-peptides that cause cerebral neuritic plaques (24).

The ERSR has also been implicated in the pathogenesis of type 1 and type 2 diabetes (T2D) (48). Beta cells are highly susceptible to ER stress and disturbances in ER homeostasis have been shown to lead to ER stress-induced apoptotic signaling within these cells (34). Alternatively, ER stress may result in misfolding of insulin produced by the beta cell in such a way that the generation of "neo-autoantigens" occurs (44,49). In either case, both scenarios may initiate an autoimmune response and lead to the pathogenesis of type 1 diabetes (T1D). Additionally, ER stress has been implicated in insulin resistance development and over activation of ERSR signaling components may

contribute to the progression of T2D (8,48). Mice deficient in XBP1 develop peripheral insulin resistance and T2D (50); however, chemical chaperones that enhance the folding capacity of the ER normalize glycemic levels, restore insulin sensitivity, and enhance insulin action in tissues throughout the body (51). Therefore, understanding the relationship between ERSR activation and diabetes induction may lead to new therapeutic approaches capable of preventing the pathogenesis of this disease.

CHAPTER II: T CELL ACTIVATION AND SIGNALING

Overview of T Cell Activation

T cells are a critical component of the adaptive immune response and participate in the removal of foreign pathogens from the body. Typically, two signals are required for a T cell to become fully activated. Signal one occurs when the T cell receptor (TCR) recognizes and binds to a peptide presented by a MHC molecule expressed on an antigen presenting cell (APC) (52,53). Signal two is generated by ligation of costimulatory molecules on the T cell and APC (54). If signal one occurs in the absence of signal two, T cells enter a state of antigen-specific tolerance or become 'non-responsive', a state often termed anergy (55,56).

Alternatively, T cells can be activated *in vitro* by stimulating with a combination of phorbol 12-myristate 13-acetate (PMA) along with the Ca^{2+} ionophore, ionomycin (Io) (57,58). PMA specifically activates protein kinase C (PKC) upon entering the cell while Io acts as a Ca^{2+} channel regulator by raising the intracellular level of Ca^{2+} (57,58). Activation of T cells by signal one and signal two via surface mediated receptors or by direct stimulation with PMA and Io results in numerous downstream signals that activate pathways enabling T cells to proliferate and produce cytokines, such as interleukin-2 (IL-2) (Figure 5) (59,60). Therefore, the process of T cell activation is a major determinant in T cell fate and together with cytokines, such as IL-2, drives T cell proliferation and differentiation programs (53).

FIGURE 5

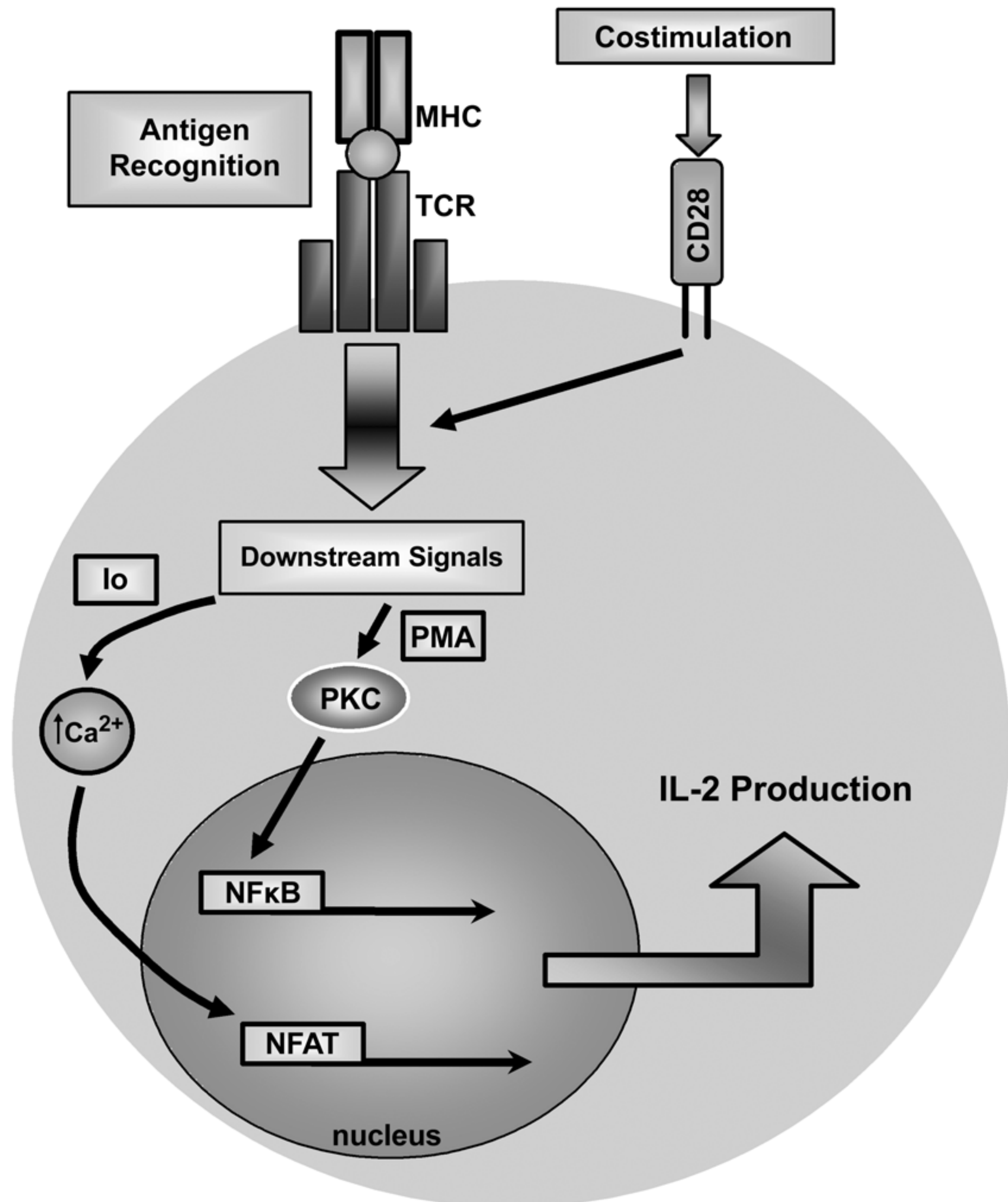

Figure 5: Downstream components of TCR signaling.

TCR recognition and ligation to a MHC-antigen complex in the presence of costimulation result in numerous downstream signaling events. Two of these signaling events, an influx of Ca^{2+} and activation of PKC, lead to activation of transcription factors necessary for production of the important cytokine, IL-2. Downstream TCR signaling events can be mimicked *in vitro* through treatment with PMA and Io.

Development of T cells in the Thymus

T cells develop in the bone marrow from a common lymphoid progenitor cell that travels from the bone marrow to the thymus (61). Upon entering the thymus, the progenitor cell proliferates and begins gene arrangement for production of γδ and αβ T cells (62). A common feature of T cell development is the assembly process of the TCR consisting of germline variable (V), diversity (D), and joining (J) gene segments. This process of V(D)J recombination results in random generation of a diverse and clonally distributed repertoire set of T cell antigen receptors, capable of recognizing a diverse array of antigenic patterns (63,64).

Production of a successful V(D)J recombination results in a pre-TCR that is required for $CD4^{-}CD8^{-}$ thymocytes to proceed to the $CD4^{+}CD8^{+}$ double positive thymocyte stage. After thymocytes proceed to the double positive stage, subsequent developmental decisions are mediated by peptide-MHC complex ligands found on the stromal cells within the thymic milieu (65). These cellular interactions of receptors on thymocytes with peptide-MHC complexes cause thymocytes to undergo positive and negative selection to eliminate potentially self-reactive cells by apoptosis or cause differentiation into mature $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ thymocytes (64,66). If a TCR interacts with peptide-MHC ligands with a low, but measurable affinity, the thymocyte will be "positively" selected; however, if there is no or too high of affinity during the interaction, death by neglect or "negative" selection will occur, respectively (65,67). A TCR that interacts with peptide-class II MHC complexes develops into a $CD4^{+}CD8^{-}$

thymocyte while a TCR that has a specificity for peptide-class I MHC complexes develops into a $CD4^-CD8^+$ thymocyte (67).

Positively selected thymocytes continue to travel throughout the thymus toward the centrally located medulla region (67). Consequently, the medulla contains few, single positive thymocytes with many characteristics of mature T cells. This population of cells includes single positive thymocytes that will leave the thymus, enter the bloodstream, and populate the secondary lymphoid tissues. The medulla also contains populations of mature T cells that participate in the elimination of any foreign antigens in the thymus (61). Although thymocytes undergo positive and negative selection, it is possible for cells to express a TCR with affinity for self-peptide-MHC complexes. If these cells escape the thymus and enter into the circulation, they pose a threat to the body through the capability of mounting a self-reactive immune response (67). Therefore, the body has developed tolerance mechanisms to render these potential self-reactive T cells unresponsive.

T Cell Receptor Signal Transduction

For an antigen-specific immune response to begin, a TCR must recognize and bind to a peptide and MHC molecule on the surface of an APC (52,53). Upon ligation of the TCR with the MHC molecule, another protein interacts with the receptor complex to complete the signal transduction (68,69). The CD8 protein binds to epitopes that are part of MHC class I molecules and therefore participates in responses by cytotoxic T cells, while the CD4 protein present on the surface of helper T cells binds to epitopes presented exclusively by MHC class II molecules (69). Additionally, for complete activation to

occur, a second costimulatory signal is required to be delivered to the T cell. The most common molecules responsible for this secondary signal is the CD28 molecule on the T cell and the B7 ligand on the APC (70). If a T cell receives both signals, production of the potent growth factor cytokine IL-2 begins (71).

After a T cell receives signaling through its TCR in the presence of costimulation, induction of protein tyrosine kinase (PTK) activity is initiated (72). PTK activation is mediated by Lck and Fyn, both Src kinases, as well as members of the Tec kinase family (72). PTK activity leads to numerous downstream signaling events, including an influx of intracellular Ca^{2+} and activation of Ras-mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), and PKC (72,73). These pathways lead to expression of genes responsible for T cell proliferation and differentiation.

Following PTK signaling, an influx of extracellular Ca^{2+} into the T cell occurs. For this biochemical event to happen, phospholipase Cγ (PLCγ) must be activated to cleave inner membrane phosphatidyl inositol-4,5 biphosphate (PIP_2) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) (74). IP_3 leads to release of ER Ca^{2+} stores as well as influx of extracellular Ca^{2+}, causing a sustained increased intracellular Ca^{2+} level, which activates a protein phosphatase known as calcineurin (CN). Calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT), a transcription factor that travels into the nucleus and drives transcription of multiple genes, including IL-2 (75).

Protein Kinase C Signaling

The other product of PIP_2 cleavage by PLCγ, DAG, activates the signaling molecule PKC (76). The PKC serine/threonine family contains multiple isoforms that in their inactive conformation localize to the cytoplasm (77). Signaling from upstream components activate PKC and cause its translocation to the plasma membrane (78). PKC phosphorylates proteins that inhibit NF-κB, thus targeting these inhibitory proteins for proteasome-mediated degradation. This process allows NF-κB to travel to the nucleus, where as a transcription factor it regulates expression of genes necessary for T cell proliferation and differentiation (79).

Amongst the PKC isoforms, PKCθ has been extensively studied and shown to play an important role in numerous TCR downstream signaling pathways (80). Research indicates that Jurkat T cells receiving signaling through the TCR in the presence of costimulation specifically activates PKCθ and downstream NF-κB (80-82). Also, *in* vitro studies in PKCθ-deficient mice indicated T cell proliferation and IL-2 production was diminished in the absence of PKCθ (83). Therefore, PKCθ has been identified as an important component in the regulation of T cell survival and proliferation, in addition to IL-2 production.

In addition to PKC and CN activation, TCR signaling leads to an accumulation of the active form of p21ras (Ras) (72). Activation of Ras protein is mediated by guanine-nucleotide exchange factors (GEFs), which exchange GDP to GTP for activation of Ras (84). In turn, Ras activates the MAP kinase cascade including ERK, JNK, and the p38 MAPK (72). This kinase cascade leads to production and nuclear localization of activator

protein-1 (AP-1), which is comprised of fos and jun constituents (85). AP-1 is a transcriptional regulator of the IL-2 gene and is capable of driving IL-2 protein expression (72). These signaling pathways are crucial for the activation of T cells so that they can participate as effector cells in an immune response.

Tolerance Mechanisms

Within the immune system, the establishment of immunological tolerance involves two mechanisms, central and peripheral tolerance. Central tolerance is the term used for the intrathymic deletion of self-reactive T cells (86). The chief mechanisms utilized by central tolerance techniques involve clonal deletion or inactivation of self-reactive T cells (87). Clonal deletion involves the triggering of apoptosis in T cell progenitors whose TCR has too high of an affinity for self-antigens presented on MHC molecules (88). The clonal deletion of potentially self-reactive T cells is a highly efficient process, but T cells that are capable of recognizing self-antigens do escape the thymus to the periphery and most individuals harbor a population of self-reactive T cells. However, pathogenesis of an autoimmune response is a rare incident, thus indicating the body has developed other means to keep self-reactive T cells in check (89). Peripheral tolerance is the term used to describe mechanisms within the periphery that inhibit the ability of self-reactive T cells to mount an autoimmune response.

Peripheral tolerance is maintained through numerous mechanisms, including anergy, dominant immune suppression by regulatory T cells, extrathymic deletion of T cells, and immunological ignorance of self tissues (90). These tolerance mechanisms are

necessary because not all self-antigens are expressed in the thymus to cause clonal deletion (67). Anergy is a form of peripheral tolerance that causes self-reactive T cells to become unresponsive (56). This is accomplished when T cells receive activation signals in the absence of costimulation (91). These anergic T cells fail to produce IL-2 and become unresponsive to subsequent antigen stimulation, even in the presence of costimulation (85,91). The induction of anergy has been shown to be an active process that requires synthesis of new proteins because cycloheximide can block the anergic state (69,92).

Another form of peripheral tolerance is maintained through a specialized group of T cells that modulate the activity of self-reactive T cells (89). These specialized T cells, known as regulatory T cells (T_{regs}), suppress self-reactive T cells through an active mechanism. This mechanism is thought to function in a cell-cell contact manner, possibly requiring the transcription factor Foxp3, and cytokines such as IL-10 and TGF-β (93). T_{regs} have been shown to be a major factor for peripheral tolerance, as their depletion results in systemic autoimmunity (93).

A third form of peripheral tolerance that prevents autoimmune activity is the process of extrathymic deletion. This mechanism causes effector T cells to disappear through apoptosis after contact with their specific antigen (94). Disappearance of the T cells normally occurs in the presence of an abundance of antigens in the periphery following over stimulation of antigenic activation (95). A final mechanism of peripheral tolerance that inhibits self-reactive T cells is the ignorance of self tissues. There are numerous ways that this form of maintenance prevents T cells from becoming activated,

even in the presence of their cognate antigen. First, anatomic barriers that are limiting factors for T cell migration and activation maintain self-reactive T cells in an ignorant state. Secondly, receptors on naïve T cells trigger their homing to lymphoid organs, thus preventing the detection of certain self-antigens. Thirdly, class II MHC molecules and certain costimulatory molecules are not expressed on all tissues, thus preventing activation. Lastly, T cell mediated attacks on immune-privileged tissues is prevented from self-reactive responses by the use of inhibitory surface molecules and immunosuppressive cytokines (67). Overall, these central and peripheral tolerance mechanisms work in combination to prevent self-reactive immune responses from occurring in the body.

Transplantation Tolerance

The principles of peripheral tolerance mechanisms underlie the foundation of techniques used to induce transplantation tolerance for the long term survival of foreign tissues. For complete T cell activation to occur, it has been established that a minimum of three signaling events are needed. First, the T cell must receive an initial signal through the TCR. This leads to upregulation of the cell surface molecule CD154 on the T cell which engages and maturates the APC through ligation of CD40. Signaling to the APC through CD40 leads to upregulation of costimulatory molecules on the APC that deliver the final activation signal to the T cell. Our laboratory has developed a two-step costimulation blockade involving donor specific transfusion, termed DST, and a brief course of anti-CD154 mAb which blocks CD40-CD154 interaction, thereby preventing

costimulatory molecule upregulation on the APC (96,97). This protocol causes deletion of alloreactive $CD8^+$ T cells (98) and has been shown *in vivo* to induce tolerance and enhance long term allograft survival in mice, rats, and non-human primates (99-102).

T Cell Receptor Signaling Immunotherapies

Besides developing strategies to disrupt T cell activation through inhibition of cell surface signaling between the T cell and APC, research is underway to develop immunotherapies based on interfering with specific downstream TCR signaling pathways. These novel immunotherapies can be developed for treatment of T cell mediated diseases, including allergic reactions, autoimmunity, malignancies, and transplant rejection (103). For example, targeting the TCR/CD3 complex through utilization of an anti-CD3 monoclonal antibody (mAb) has been used to deplete T cells and cause tolerization for the purpose of organ transplantation (103,104). Additionally, agents such as CTLA-4Ig, which are designed to block costimulatory receptors on the T cell from interacting with an APC, are successful in preventing allograft rejection and graft-versus-host disease, as well as delaying autoimmune responses (103). Downstream of the TCR and costimulatory molecules, disruption of Ca^{2+} signaling with the drug cyclosporin A, disrupts CN and inhibits NFAT translocation to the nucleus (105). This interferes with IL-2 production and proliferation by the T cell and is used as an immunosuppressive agent during organ and bone marrow transplantation (103). Therefore, targeting signaling pathways and components downstream of the TCR offers a promising means of modulating and regulating T cell function.

CHAPTER III: GIMAP5 AND THE BIOBREEDING DIABETES-PRONE RAT

Overview of Gimap5

The regulation of T cell survival has been linked to expression of Gimap5, a member of the GTPase of the immunity-associated protein family of genes (formerly known as IANs, immune-associated nucleotide-binding proteins) (106-108). Although the exact role for Gimap5 in the maintenance of T cell homeostasis remains unknown, research indicates Gimap5 regulates mitochondrial apoptotic signaling through interactions with members of the Bcl-2 family of proteins (106,109,110). Additionally, the localization of Gimap5 remains controversial, with localization linked to the mitochondria (111), as well as the ER, Golgi complex, and centrosome (112). More recently, Gimap5 was found to exclusively localize to the ER (113).

The Function of GTPases

Gimap5 belongs to a family of genes that encode putative GTPases of unknown function in immune tissues (109). GTPases are GTP-binding proteins that function in numerous cellular activities, including transmission of messages across the plasma membrane to intracellular messaging systems, vesicle formation and fusion, and protein trafficking within the cell (114-116). GTP-binding proteins have been discovered in many intracellular organelles, including the ER, Golgi complex, and mitochondria (116). Gimap family members are characterized by a GTP-binding motif, known as the AIG1 domain, and multiple coiled-coil motifs (110).

GTP-binding proteins cycle through three conformational states as they bind and hydrolyze GTP: GTP-bound, empty, and GDP-bound (117). This occurs by conversion of the GTP-bound state through hydrolysis of GTP to the GDP-bound state utilizing GTPase activity (117-119). To reactivate the protein, the GDP is replaced by GTP in the inactive GDP-bound form, a switch mediated by guanine nucleotide exchange proteins (117,118). The GTP-bound form of the signaling protein is considered the active form of the protein because it is capable of activating effector enzymes, unlike the GDP-bound form (117).

Identification of Gimap5 in Bcr/Abl Oncogene Transfected Cells

Gimap5 was first identified in Bcr/Abl-transformed hematopoietic precursor 32D cells (111). The *Bcr/Abl* oncogene is a fusion formed from a truncated breakpoint cluster region (*Bcr*) gene with the cellular homolog of the transforming gene from the Abelson murine leukemia virus, *c-Abl* (111). The protein product of this oncogene, Bcr/Abl, displays constitutive tyrosine kinase activity and is responsible for the activation of numerous signaling molecules (120-123). Additionally, the Bcr/Abl fusion protein is found in >95% of chronic myeloid leukemia (CML) cases and is capable of converting cell lines to a growth factor independent state (124,125).

Several mechanisms have been proposed to explain the role for Bcr/Abl in the accumulation of leukemic cells that leads to the pathogenesis of CML. Bcr/Abl has been linked to the inhibition of apoptosis and is also connected to the expression of antiapoptotic molecules, such as several members from the Bcl-2 family of proteins (126-128). Also, the role of Bcr/Abl in several signaling pathways may contribute to its ability

to control proliferation and an ability to prevent apoptosis (111). It has been determined that the tyrosine kinase activity is essential for Bcr/Abl to maintain its antiapoptotic abilities (129).

The Gimap Family of Genes

The expression of the *Gimap* family of genes has been implicated in the regulation of T cell survival through modulation of TCR signaling and interactions with Bcl-2 family members (109,110). The *Gimap* family of genes are found in higher plant forms and vertebrates and tend to be encoded within short spanning sequences within the genome (109). Expression of the Gimap family in humans has been detected in multiple tissues, but remains highest in immune cells (109). However, in the mouse, the Gimap family is mainly expressed in T cells and B cells (109,130). Overall, the expression of the *Gimap* family of genes appears to be regulated during an immune response in lymphocytes (109,110).

Gimap4 (formerly known as Ian1) which shares homology to a pathogen-induced plant protein, AIG1, appears to lie downstream of TCR signaling (131). Gimap4, unlike most Gimap family members, lacks a putative transmembrane domain, and seems to localize predominantly to the cytoplasm (108,109). Gimap4 is primarily found in T cells, but its expression is turned on during thymic selection events (132). Despite its expression in T cells, Gimap4 does not appear to play a significant role in T cell development as *Gimap4*-deficient mice show normal T cell development (110). However, peripheral T cells from these mice exhibit a delay in apoptosis in response to different

cellular disturbances, indicating Gimap4 acts to accelerate T cell death (132). Thus, Gimap4 appears to function as a positive regulator of cell death.

Gimap1 (formerly known as *Ian2*, *Iap38*, and *Imap38*) expression is induced in spleens of mice that develop immunity to the experimental malarial parasite *Plasmodium chabaudi* (130,133). Gimap1 contains membrane anchoring hydrophobic regions at the C-terminus and localizes to the ER (110,134). In the thymus, *Gimap1* expression is highest in double positive thymocytes, but is minimally expressed in peripheral T cells (110). Similar to other members of the *Gimap* family of genes, including *Gimap9*, *Gimap6*, *Gimap7*, and *Gimap2*, the exact molecular function remains unclear (110).

Both Gimap8 and Gimap3 have been shown to localize to the ER, Golgi complex, and mitochondria (111,135). Gimap8 appears to function as an apoptotic inhibitor while Gimap3 may participate in thymic selection (111,135). Analogous to Gimap8, Gimap5 appears to function as an antiapoptotic factor because the natural knockout of *Gimap5* in T cells from BioBreeding Diabetes-Prone (BBDP) rats has been shown to result in mitochondrial dysfunction and apoptosis of recent thymic emigrants leading to a peripheral T cell lymphopenia (106). Gimap5 is further believed to modulate TCR signaling by inhibiting the MAP kinase pathway, thus promoting T cell quiescence (136). Overall, the exact mechanisms by which Gimap5 participates in the regulation of T cell survival is still unknown.

The BioBreeding Diabetes-Prone and Diabetes-Resistant Rats

The BBDP rat contains a frameshift mutation in *Gimap5*, which has been implicated as the cause of the profound peripheral T cell lymphopenia seen in the rat (106,137,138). Because of this mutation, the function of Gimap5 has been extensively studied using the BBDP rat, which also serves as an animal model of T1D. The BBDP rat, the oldest known rat model for T1D, was discovered in a colony in 1974 at BioBreeding Laboratories in Canada (139). Selective breeding has resulted in BBDP rats that spontaneously develop T1D with >90% frequency (139). BBDP rats have various T1D susceptibility loci and exhibit a severe T cell lymphopenia. This lymphopenia in the peripheral T cell compartment is characterized by reductions in $CD4^+$ T cells and a near absence of $CD8^+$ T cells and the regulatory $RT6^+$ T cell population; however, the development of thymocytes is generally normal (131,137,140).

In contrast, BioBreeding diabetes-resistant rats (BBDR), which were derived from outbred BBDP rat forebears, are *Gimap5*$^{+/+}$, non-lymphopenic, and do not develop spontaneous autoimmune diabetes when housed in a viral antibody free (VAF) facility (139). They circulate normal numbers of $CD4^+$ T cells, $CD8^+$ T cells, and $RT6^+$ regulatory T cells (141). However, BBDR rats have a proclivity to develop diabetes in response to environmental perturbations. Treating VAF housed BBDR rats with a depleting anti-$RT6^+$ antibody combined with polyinosinic:polycytidylic acid (poly I:C), a synthetic double stranded polyribonucleotide and immune system activator, leads to diabetes pathogenesis (141). Additionally, viral infection with Kilham rat virus (KRV) causes diabetes development in approximately 30% of BBDR rats, but pretreatment with

poly I:C induces diabetes in 100% of KRV-infected rats. However, it remains unclear how KRV infection combined with poly I:C leads to diabetes (142).

Multiple diabetes susceptible loci have been identified in the BB rat. To acquire T1D in rat models, there appears to be a necessity for at least one gene that is associated with the rat MHC (141). In the BB rat, diabetes development requires at least one MHC class II $RT1^{u}$ allele, named *Iddm2* (141,143). Additionally, analyses determined *Iddm4*, which maps to chromosome four, has been found to be a major non-MHC determinant of diabetes development in the BB rat (141,144). Lastly, the lymphopenia in the BBDP rat is linked to a recessive mutation in *Iddm1*, which encodes for Gimap5 protein. However, this mutation is not found in the BBDR rat (141).

Similar to human T1D, BBDP rats develop spontaneous diabetes during adolescence (60-100 days of age) that is characterized by lymphocytic infiltration into the islets of the animals (139). Although the exact event that incites autoreactivity toward the beta cell remains elusive, researchers speculate that the process involves presentation of an unknown autoantigen by the MHC class II $RT1^{u}$ allele (139,141). Additionally, a role for regulatory T cells has been established in the BB rat model because transfusion of regulatory $RT6^{+}$ T cells into the BBDP rat prevents pathogenesis of T1D. Furthermore, depletion of regulatory $RT6^{+}$ T cells from BBDR rats, together with a low dose of poly I:C, leads to T1D within four weeks of treatment (139). Collectively, these data suggest a complex interplay between lymphopenia (generated by *Gimap5*$^{-/-}$ or transiently introduced by $RT6^{+}$ T cell depletion or KRV infection) and diabetes susceptibility genes for the pathogenesis of T1D.

CHAPTER IV: MATERIALS AND METHODS

Animals. For T cell activation experiments, six- to ten-week-old male BALB/c (*H2*^{d}) (Charles River Laboratories, Wilmington, MA) mice and C57BL/6 (*H2*^{b}) and CBA (*H2*^{k}) mice (The Jackson Laboratory, Bar Harbor, ME) were used. For Gimap5 studies, BBDR and BBDP rats were obtained from Biomedical Research Models (Worcester, MA). Eight week-old male or female rats were used and all of the rats were nondiabetic at the time of study. All animals were housed in a VAF and maintained in accordance with the guidelines of the University of Massachusetts Medical School Institutional Animal Care and Use Committee and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

T cell preparation. Spleen and mesenteric lymph nodes were removed from BALB/c mice and processed aseptically for T cell activation experiments. BALB/c T cells were purified (93-97% TCRβ^{+}) as previously described (145). For Gimap5 studies, cervical and mesenteric lymph nodes were aseptically removed from BBDR and BBDP rats and extruded through mesh wire to prepare single cell suspensions for flow cytometry and Western blot analyses.

***In vitro* T cell stimulation.** Six-well culture plates (BD Falcon, Bedford, MA) were incubated overnight with 10 µg of anti-CD3 mAb (clone 17A2; BD Pharmingen, San Jose, CA) per well in phosphate buffered saline (PBS) at 4°C. BALB/c purified T cells

were cultured in plates rinsed with PBS at 6×10^6 cells per well in 3 mL of RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 1% Pen/Strep/Glut (Gibco, Carlsbad, CA), and 0.1% β-mercaptoethanol (Gibco) at 37°C. Where indicated, soluble anti-CD28 mAb (1 μg/mL, clone 37.51; BD Pharmingen) was added. Control T cells were incubated with immobilized isotype matched IgG mAb. For PMA (Calbiochem, San Diego, CA) and Io (Calbiochem) stimulation, T cells were stimulated with 100 ng/mL PMA and 1 μg/mL or 2 μg/mL Io for 20 h. For TG (Calbiochem) treatment, T cells were plated at 6×10^6 cells per well in 6 mL culture media with 2 μM TG. Calphostin C (Calbiochem) was added at 500 nM for the duration of activation for specified treatment groups. Control T cells were treated with the vehicle dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

IL-2 ELISA. BALB/c T cells were cultured as described (above). Supernatants were harvested after 17 or 20 hrs and assayed for IL-2 production using a mouse IL-2 ELISA set (BD Pharmingen) according to manufacturer's instructions.

Western blotting. BALB/c or rat lymphocytes were lysed (145) and protein concentrations determined by bicinchoninic acid (BCA) protein assay (Sigma-Aldrich). Protein (30 μg) was mixed with 4X SDS-PAGE loading buffer and Western blot analyses were performed as described (145). Actin was used as a loading control. Band densities were measured by densitometry (ImageJ software, NIH, Bethesda, MD). Density values

are expressed as a ratio normalized to actin, and the 'fold change' is compared to control samples.

Flow cytometry. BALB/c or rat single cell suspensions were washed and suspended in PBS containing 1% fetal clone serum (FCS) (HyClone) and 0.1% sodium azide (Sigma-Aldrich). Samples from BALB/c mice were incubated in anti-CD16/32 for 10 min at 4°C. KB5 synchimera splenic cells were incubated with clonotypic Désiré (DES) mAb for 20 min., washed, incubated for 20 min with secondary mAb for DES, and labeled with fluorescent mAbs to cell-surface markers. BALB/c and rat single cell suspensions were labeled with fluorescent mAbs to various cell-surface markers as described in the text. To detect intracellular GRP78 or CHOP, cells were permeabilized using Cytofix/Cytoperm (BD Pharmingen) according to the manufacturer's directions. Cells were washed and incubated with Alexa Fluor 647-conjugated anti-GRP78 or anti-CHOP mAbs for 20 min. Labeled cells were washed, fixed with 1% paraformaldehyde (Polysciences, Warrington, PA) in PBS and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA) or LSR II (BD Biosciences) and FlowJo Software (PC version 7.2.2; Tree Star, Ashland, OR). Lymphoid cells were gated according to their light-scattering properties.

Antibodies. For T cell activation studies, IgG2a developing reagent for DES (clone R19-15), CD44 (clone IM7), CD8α (clone 53-6.7), CD69 (clone H1.2f3), 7-AAD, TCRβ (clone H57-597), GRP78 (clone 40), PDI (clone 34), and isotype control unconjugated or fluorochrome-conjugated anti-mouse mAbs were from BD Pharmingen. Zenon Mouse

IgG2a and IgG1 Labeling Kits, Alexa Fluor 647 (Invitrogen, Carlsbad, CA), were used to label GRP78 or CHOP mAb per the manufacturer's directions. A mouse hybridoma cell line secreting the KB5-specific clonotypic DES mAb (146) was a gift from Dr. J. Iacomini (Harvard Medical School, Boston, MA). Anti-actin (clone C4), anti-ERp72, and anti-GRP94 (clone 9G10) antibodies were obtained from Chemicon International (Temecula, CA), Calbiochem, and Stressgen (San Diego, CA), respectively. Anti-rabbit, anti-rat, and anti-mouse IgG horseradish peroxidase (HRP) conjugates and anti-CHOP mAb were from Santa Cruz Biotechnology (Santa Cruz, CA). MR1 hamster anti-mouse CD154 mAb was produced as tissue culture supernatant and purified by affinity chromatography (National Cell Culture Center, Minneapolis, MN). Antibody concentration was determined by measurement of optical density and confirmed by ELISA (98). The concentration of contaminating endotoxin was determined commercially (Charles River Endosafe, Charleston, SC) and was uniformly <10 units/mg of mAb (147). For Gimap5 experiments, anti-rat CD8a-PE (clone Ox-8), PerCp-conjugated anti-rat TCRαβ (clone R73), anti-rat CD90-FITC (clone HIS51), Biotin-conjugated anti-rat CD25 (clone OX-39), and Streptavidin-conjugated APC-Cy7 mAbs were obtained from BD Pharmingen. Anti-rat CD4-Pacific Blue mAb and its corresponding isotype control Ab were obtained from Serotec (Raleigh, NC). Cleaved capase-3 antibody was obtained from Cell Signaling Technology (Danvers, MA). Polyclonal antiserum to Gimap5 was generated as described (106).

Synchimeras and tolerance induction. To determine the *in vivo* ERSR in antigen-activated alloreactive $CD8^+$ T cells in a normal microenvironment, we used KB5 TCR transgenic hematopoietic "synchimeric" mice generated as described (148). The transgenic T cells that develop in the animals express an anti-H2-K^b specific TCR recognized by the mAb DES. These procedures have been documented to generate a stable population of DES^+CD8^+ cells comprising 5-8% of peripheral blood mononuclear cells (PBMCs) within 8 weeks of bone marrow transplantation (148,149). Male KB5 synchimeric mice were treated with a single transfusion of C57BL/6 (H2-K^b*)* mouse splenocytes, known as a donor specific transfusion (DST), for full activation of transgenic DES^+CD8^+ T cells or DST plus anti-CD154 mAb for induction of tolerance as described (148,150).

T cell transfection. T cells from BBDP rats were purified as previously described (145). T cells were plated at $6x10^6$ cells per well in 3 mL of RPMI medium (Sigma-Aldrich) containing 10% FBS (Hyclone), 1% Pen/Strep/Glut (Gibco, Carlsbad, CA), and 0.1% β-mercaptoethanol (Gibco) at 37 degrees Celsius in the presence of 10 ng/mL of PMA (Calbiochem) and 100 ng/mL of ionomycin (Calbiochem). After 12 h, T cells were resuspended in 100 μL Nucleofector solution using human T cell Nucleofector kit (VPA-1002, Amaxa Biosystems, Gaithersburg, MD) with 100 nM CHOP siRNA (catalogue no. L-088282-01, Dharmacon, Lafayette, CO) or 100 nM control siRNA (catalogue no. D-001210-02, Dharmacon) and nucleofected using program U-014 in the Amaxa

Nucleofector apparatus. Following nucleofection, T cells were plated in 3 mL complete media and harvested at 30 h for protein preparation and flow cytometry analysis.

Apoptosis Detection. To determine cellular apoptotic signaling, transfected T cells were harvested, washed twice with PBS, and stained with FITC-conjugated anti-annexin V and 7-amino-actinomycin D (7AAD) according to the manufacturer's directions (BD Pharmingen). At least 20,000 cells per sample were analyzed with a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Statistics. Statistical analyses were performed with GraphPad Prism software (Graphpad Software, San Diego, CA). Differences were compared by two-tailed unpaired *t*-tests. Values of $p<0.05$ were considered statistically significant.

CHAPTER V: PROTEIN KINASE C SIGNALING DURING T CELL ACTIVATION INDUCES THE ENDOPLASMIC RETICULUM STRESS RESPONSE

Introduction

Ligation of the TCR (signal one) in the presence of costimulation (signal two) results in downstream signals that increase protein production enabling naïve T cells to fully activate and gain effector function. Enhanced production of proteins by a cell requires an increase in ER chaperone expression which is accomplished through activation of a cellular mechanism known as the ERSR. The ERSR is initiated during the cascade of events that occur for the activation of many cells; however, this process has not been comprehensively studied for T cell function. We hypothesized that full T cell activation (signals one and two) resulting in IL-2 cytokine production generates ERSR induction.

In this study, we used primary T cells and mice circulating TCR transgenic $CD8^+$ T cells to investigate ER chaperone expression in which TCR signaling was initiated in the presence or absence of costimulation. In the presence of both signals, *in vitro* and *in vivo* analyses demonstrated induction of the ERSR, as evidenced by elevated expression of GRP78 and other ER chaperones. Unexpectedly, ER chaperones were also increased in T cells exposed only to signal one, a treatment known to cause T cells to enter the 'non-responsive' states of anergy and tolerance. Treatment of T cells with an inhibitor to PKC, a signaling molecule specifically activated by TCR ligation, indicated this serine/threonine protein kinase is involved in the induction of the ERSR during T cell activation, thus revealing a previously unknown role for this signaling protein in T cells.

Collectively, these data suggest that induction of the ERSR through PKC signaling is an important component for the preparation of a T cell response to antigen.

Results

A. The ERSR is induced in T cells by thapsigargin.

We first confirmed the ability of T cells to generate an effective ERSR in the presence of a known ER stress inducer, TG. To do so, BALB/c mouse lymph node and splenic T cells (>93% purity) were treated with TG, and the expression of various ER chaperone proteins was quantified. Thapsigargin selectively inhibits the ER Ca^{2+}-ATPase, disrupting ER Ca^{2+} stores and leads to ER stress (37). In T cells treated with TG, expression levels of the ER chaperones GRP78, GRP94, PDI, and ERp72 increased within 2h (1.3- to 1.7-fold above the expression of vehicle (DMSO) treated control cells) and remained elevated (1.7- to 3.3-fold) through 24h of treatment (Figure 6). These data confirm that T cells can generate a robust ERSR when treated with a known ER stress inducing chemical, TG.

Figure 6

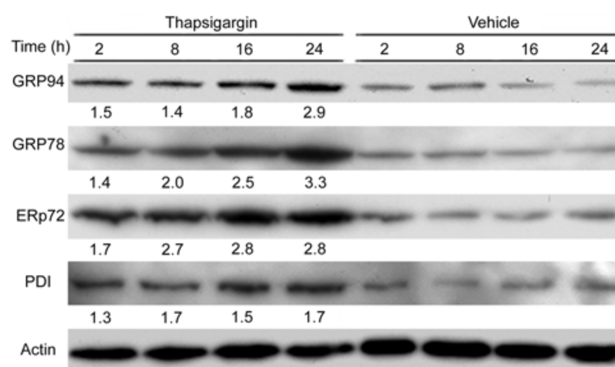

Figure 6: Thapsigargin treatment of T cells induces ER stress.

Purified T cells were treated with 2 μM TG or DMSO (Vehicle) for 2, 8, 16, and 24 h. TG- and DMSO-treated T cell protein lysates were analyzed by Western blot for ER chaperone expression. Band densities were measured by densitometry and values shown are 'fold changes' relative to control (=1.0) after actin normalization at the same time point. Data represent one of two independent time course experiments.

B. The ERSR occurs following *in vitro* T cell activation.

Having established that freshly isolated T cells exhibit a functional ERSR when treated with a chemical stressor, we next investigated whether this response would be induced by activation through the TCR with costimulation. To do so we incubated purified T cells with both a mAb to CD3 (a TCR subunit that, when ligated, provides signal one) and the costimulatory anti-CD28 mAb for 17 h. As a negative control, a portion of the purified T cells were incubated with an immobilized isotype matched IgG mAb. Lymphocytes were gated according to their forward scattering (FSC) and side scattering (SSC) light properties, a means of distinguishing viable cells from their non-viable counterparts. The percentage of viable lymphocytes following co-treatment with anti-CD3 plus anti-CD28 mAbs was 71.7%±0.6% (Figure 7A). This treatment increased the percentage of T cells expressing the early activation marker CD69 from 3.8%±1.2% in cells treated with an immobilized isotype matched IgG control mAb to 45.2%±4.2% in co-treated cells (Figure 7B, shaded region and black line, respectively).

To determine whether T cell activation via signal one plus signal two also led to the induction of a physiological ERSR, intracellular expression of the ER chaperone GRP78, an indicator of ER stress (13), was quantified by flow cytometry. Because only ~50% of T cells are stimulated by the plate-bound mAbs in these *in vitro* incubations, GRP78 expression levels were separately measured in $CD69^{high}$ and $CD69^{low}$ sub-populations of T cells. After 17 h of co-treatment with anti-CD3 and anti-CD28 mAbs, intracellular GRP78 expression was significantly higher in T cells expressing high levels of CD69 ($CD69^{high}$) than in $CD69^{low}$ cells (Figure 7C). The relative amount of

intracellular GRP78 protein in the CD69^{high} and CD69^{low} sub-populations is quantified by mean fluorescence intensity (MFI; Figure 7D). As expected, T cells co-treated with anti-CD3 plus anti-CD28 mAbs secreted a robust amount of the IL- 2 cytokine (Figure 7E). These data indicate that full T cell activation resulting in cytokine secretion induces ERSR signaling.

C. The ERSR occurs following partial T cell activation *in vitro* via signal one alone.

We next investigated the ERSR in T cells exposed only to signal one (TCR ligation alone). Because such cells are generally thought to become 'non-responsive', we hypothesized that this process would *not* invoke a classical ERSR or, alternatively, would induce one qualitatively and/or quantitatively different from that associated with fully activated (signals one and two) T cells.

We first determined the expression of CD69 on T cells that received only signal one through treatment with anti-CD3 mAb alone. This treatment is known to lead to the expression of the CD69 activation marker (151), and we found a similar increase in CD69 expression on T cells incubated with anti-CD3 mAb alone (48.2%±2.5%) as compared to co-treated T cells (Figure 7B). To ensure these T cells had a functional phenotype of cells only receiving signal one, we measured their production of IL-2. As expected, T cells incubated with anti-CD3 mAb alone produced significantly less IL-2 compared to T cells exposed to both signal one and signal two (Figure 7E).

We then directly tested our hypothesis that the ERSR in these T cells would be different from that observed in the fully activated, cytokine-secreting T cells. Because

apoptosis is a possible outcome of anti-CD3 mAb alone treatment, 7-AAD was used to exclude the contribution of any ERSR from non-viable T cells (data not shown). At 17 h, the intracellular GRP78 expression in T cells incubated with anti-CD3 mAb alone was significantly higher in $CD69^{high}$ than in $CD69^{low}$ T cells (Figures 7C,D). Unexpectedly, however, intracellular GRP78 expression was not significantly different between $CD69^{high}$ T cells in anti-CD3 plus anti-CD28 mAbs and anti-CD3 mAb alone treatment groups (Figure 7D). Therefore, GRP78 expression correlates with signaling through the TCR but, at least initially, does not distinguish between incomplete activation (anti-CD3 mAb alone) and full T cell activation (anti-CD3 plus anti-CD28 mAbs) in which high levels of IL-2 are produced.

Figure 7

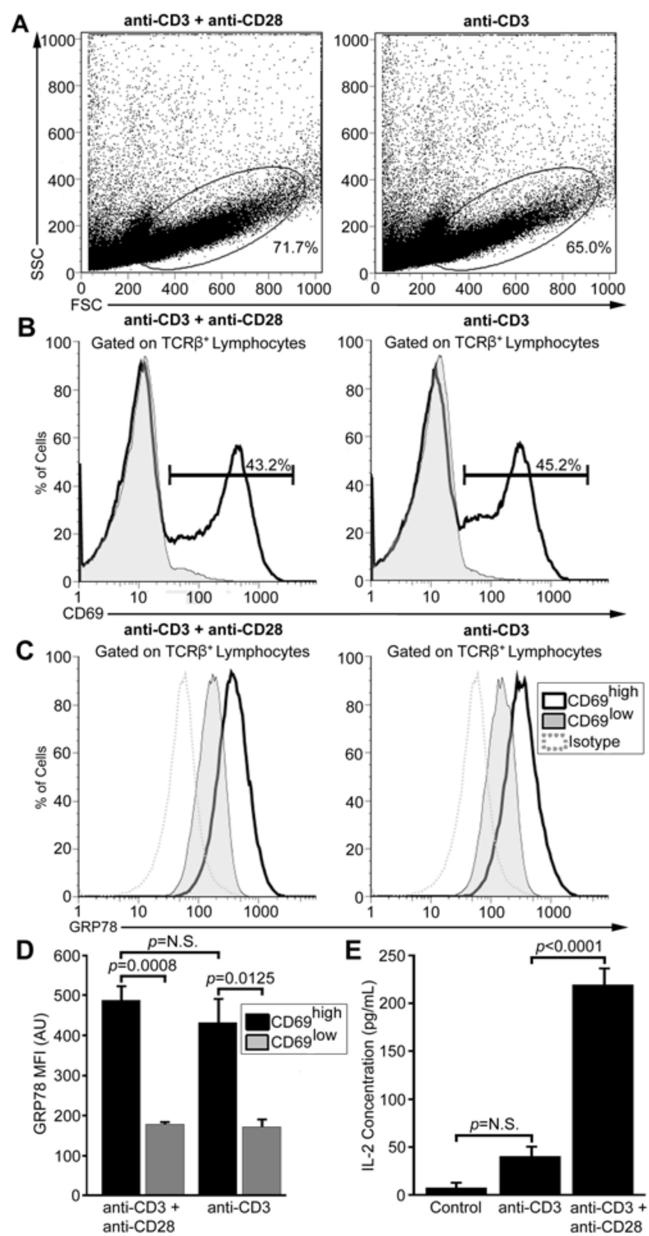

Figure 7: **TCR signaling *in vitro* induces the ERSR.**

Purified T cells were co-treated with anti-CD3 plus anti-CD28 mAbs (*left*, n=3) or treated with anti-CD3 mAb alone (*right*, n=3) for 17 h as described in Methods. (A) Representative flow dot plots depicting the forward scattering (horizontal axis) and side scattering (vertical axis) light properties of lymphocytes. Viable lymphocyte populations are encircled. (B) Flow cytometric analyses of the activation marker CD69 in treated T cells (black line). Shaded regions represent CD69 expression in T cells incubated with immobilized isotype matched IgG mAb (Control). (C) Intracellular GRP78 expression in $CD69^{high}$ (black line) and $CD69^{low}$ (shaded region) T cells. The GRP78 expression for isotype control staining (dotted line) is shown in each histogram. (D) Bar graphs displaying the mean of intracellular GRP78 protein expression (arbitrary units; AU) for $CD69^{high}$ and $CD69^{low}$ T cells. Error bars represent the s.e.m. of triplicate samples. (E) IL-2 production was determined by ELISA on T cell supernatants obtained from three independent experiments for each treatment group. Samples were examined in triplicate and error bars represent s.e.m. of experimental replicates. Data shown are from one of three independent experiments.

D. Upregulation of multiple ER chaperones following *in vitro* TCR signaling in the presence or absence of signal two.

To confirm the initiation of the ERSR in activated T cells, protein lysates of purified T cells incubated for 17 h with anti-CD3 mAb alone or anti-CD3 plus anti-CD28 mAbs were analyzed by Western blot. Analogous to the results obtained using flow cytometry, Western blot analyses revealed that GRP78 protein expression was increased in T cells incubated with anti-CD3 mAb alone (2.1-fold) or with anti-CD3 plus anti-CD28 mAbs (2.1-fold) when compared to that observed in T cells incubated with the immobilized isotype matched IgG mAb control (Figure 8). The expression levels of the ER chaperone proteins GRP94, ERp72, and PDI were also increased to a similar amount following stimulation with either anti-CD3 mAb alone or in the presence of anti-CD3 plus anti-CD28 mAbs (Figure 8). These data further reveal that the ERSR leading to increased production of ER stress associated chaperone proteins occurs *in vitro* in T cells activated with anti-CD3 mAb, irrespective of anti-CD28 mAb costimulation.

Figure 8

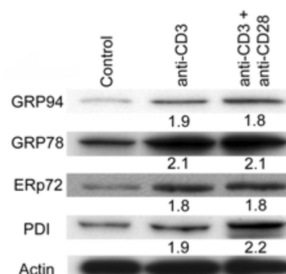

Figure 8: ER chaperone expression increases during *in vitro* TCR signaling.

Purified T cells were treated with immobilized isotype matched IgG mAb (Control), anti-CD3 mAb alone, or with anti-CD3 plus anti-CD28 mAbs for 17 h as described in Methods. Western blot analyses of ER chaperone proteins were performed on lysates from T cells treated as indicated. Numbers represent densitometry of protein band densities after normalization to actin and comparison to control (=1.0). Data shown are from one of three independent experiments.

E. The ERSR is induced by *in vivo* activation of T cells.

We next sought to determine if a functional ERSR also occurs with T cell activation *in vivo*. To do so, we required a system in which we could readily identify a population of activated cells with well defined kinetics. We elected to use KB5 synchimeric mice to investigate T cell activation *in vivo* (148,152). These KB5 synchimeric mice were modified (as described in Methods) to circulate a trace population (~5-8%) of TCR transgenic $CD8^+$ T cells that specifically recognize the H2-K^b antigen. To induce T cell activation *in vivo*, the KB5 synchimeric mice were given a DST of H2-K^b-expressing splenocytes from C57BL/6 mice. This *in vivo* DST provides both signals one and two and uniformly activates only the transgenic population of $CD8^+$ T cells. The transgenic $CD8^+$ T cells can be distinguished from endogenous T cells by use of the DES mAb.

Spleen cells were recovered from KB5 synchimeric mice two days after injection of C57BL/6 splenocytes. Representative histograms illustrate that transgenic DES^+CD8^+ T cells can be readily distinguished from endogenous (non-transgenic) DES^-CD8^+ lymphocytes from the KB5 synchimeric mice (Figure 9A). Transgenic DES^+CD8^+ T cells from DST-treated mice exhibited an increased expression of the activation marker CD44 (91.2±2.4%) compared to the DES^-CD8^+ lymphocyte population (29.3±6.7%; Figure 9B, black line and shaded region, respectively). As expected, few DES^+CD8^+ T cells in untreated KB5 synchimeric mice expressed high levels of CD44 (14.4±5.7%; Figure 9B).

To investigate the *in vivo* ERSR in activated $CD8^+$ T cells, expression of intracellular GRP78 protein was determined by flow cytometry. We observed that

intracellular GRP78 protein expression was not significantly different between DES$^+$CD8$^+$ T cells and non-transgenic DES$^-$CD8$^+$ lymphocytes from untreated KB5 synchimeric mice (Figures 9C,D). However, intracellular GRP78 protein expression was significantly greater in DES$^+$CD8$^+$ T cells than in non-transgenic DES$^-$CD8$^+$ lymphocytes from DST treated mice (Figures 9C,D). Additionally, intracellular GRP78 protein expression of DES$^+$CD8$^+$ T cells from KB5 synchimeric mice injected with the allogeneic cells was significantly greater than in DES$^+$CD8$^+$ T cells from untreated mice (Figure 9D). These data provide evidence of a physiological ERSR after *in vivo* activation of antigen-specific CD8$^+$ T cells.

F. The ERSR characterizes *in vivo* tolerized T cells that receive signal one alone.

The KB5 synchimera mouse system has been used to study transplantation tolerance induction. In such studies, in addition to an injection of DST, recipient mice also receive an anti-CD154 mAb to block CD40-CD154 interaction, thus preventing signal two by inhibiting expression of costimulatory molecules on the surface of the APC (148,152). When anti-CD154 mAb is given, the majority of the alloresponsive KB5 transgenic T cells (approximately 66%) disappear (148,152). Little is known, however, about the molecular phenotype of the T cells that are not deleted. We hypothesized that residual KB5 transgenic T cells in this tolerance induction system would display an ‘activated’ ER stress phenotype analogous to that which we had observed in T cells exposed *in vitro* to signal one alone.

Transgenic DES^+CD8^+ T cells from these co-treated mice were stimulated as documented by an increased expression of the activation marker CD44 (80.6±1.6%; Figure 9B, black line), while intracellular GRP78 protein expression in the DES^+CD8^+ T cells was significantly increased as compared to non-transgenic DES^-CD8^+ lymphocytes (Figures 9C,D). Furthermore, intracellular GRP78 protein expression in activated DES^+CD8^+ T cells from KB5 synchimeric mice co-treated with DST plus anti-CD154 mAb was significantly greater than in DES^+CD8^+ T cells from untreated KB5 synchimeric mice (Figure 9D). Interestingly, the increase of GRP78 chaperone protein expression by flow cytometry in *in vivo* tolerized alloreactive $CD8^+$ T cells (2.2-fold) was similar to that observed following *in vitro* activation using anti-CD3 mAb alone (2.5-fold), another protocol that activates T cells that fail to become fully functional (Figure 9C). These data demonstrate the presence of a physiological ERSR following *in vivo* tolerance induction ('non-responsiveness') in $CD8^+$ T cells. These observations suggest that allogeneic cells that are not actually deleted by the tolerance induction protocol have a molecular phenotype consistent with partial activation.

Figure 9

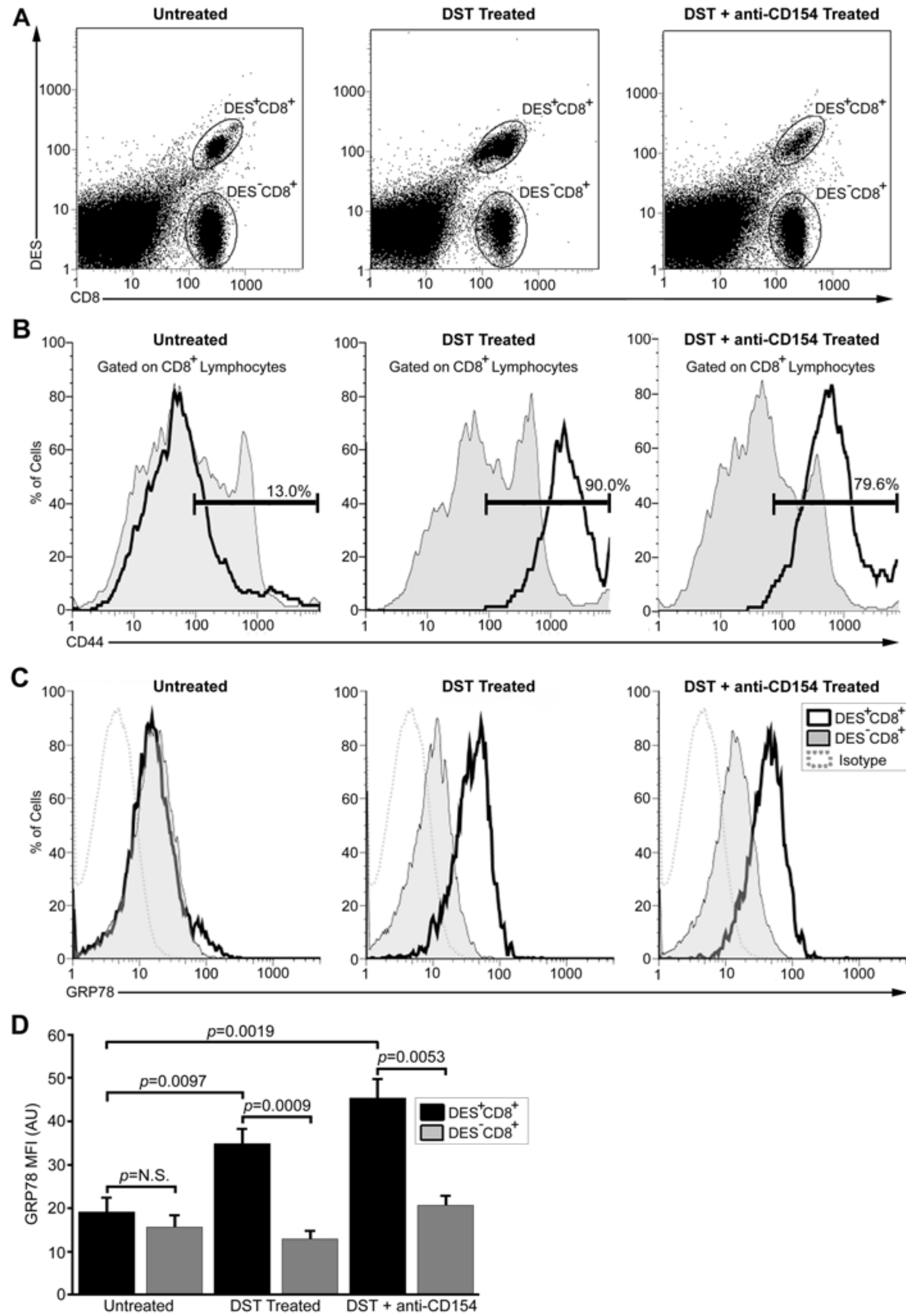

Figure 9: **Increased GRP78 expression with *in vivo* T cell activation and *in vivo* tolerance induction.**

KB5 synchimeric mice were untreated (*left*, n=5), DST treated (*middle*, n=4) to induce *in vivo* activation, or DST plus anti-CD154 mAb co-treated (*right*, n=3) to achieve tolerance induction of transgenic $DES^{+}CD8^{+}$ T cells as described in Methods. (A) Representative flow dot plots depicting CD8 expression (horizontal axis) and the anti-H2-K^{b} specific TCR recognized by the mAb DES (vertical axis) on lymphocytes obtained from the indicated treatment groups. $DES^{+}CD8^{+}$ T cells and $DES^{-}CD8^{+}$ lymphocyte populations are encircled. (B) Flow cytometric analyses of the activation marker CD44 on gated $DES^{+}CD8^{+}$ T cells (black line) and $DES^{-}CD8^{+}$ lymphocytes (shaded region). (C) Intracellular GRP78 expression in $DES^{+}CD8^{+}$ T cells (black line) and $DES^{-}CD8^{+}$ (shaded region) lymphocyte populations. The isotype control for GRP78 staining is indicated by a dotted line. (D) The mean of GRP78 protein expression displayed as bar graphs with error bars representing the s.e.m. Data are representative of four independent experiments.

G. Ionomycin stimulation of T cells does not lead to ERSR signaling.

T cell activation via TCR (or CD3) ligation is known to signal through pathways that regulate Ca^{2+} levels within the T cell (153). Because fluxes in Ca^{2+} levels are known to cause ER stress (37) and we earlier showed TG treatment caused increases in ER chaperone expression (Figure 6), we investigated whether stimulating T cells with Io, a Ca^{2+} channel regulator which raises intracellular Ca^{2+} levels and mimics certain components of TCR signaling, leads to activation of the ERSR. To accomplish this, we stimulated purified T cells with DMSO (vehicle) or Io alone for 20 h.

The percentage of viable lymphocytes following control (vehicle treated) or Io (1 μg/mL) treatment was 73.0%±0.4% and 67.6%±3.7%, respectively (Figure 10A). Only 5.2%±4.3% of T cells had increased expression of CD69 following Io treatment, a number statistically similar to the percentage of cells (4.3%±0.3%) in the vehicle treatment (p=0.6135; Figure 10B). This suggests that the small percentage of $CD69^{high}$ T cells found in the vehicle and Io alone treatment groups may have been activated *in vivo* previous to their isolation. As expected, the small percentage of $CD69^{high}$ T cells within the vehicle and Io alone treated populations had increased intracellular GRP78 protein expression compared to the $CD69^{low}$ T cells (Figures 10C,D). Interestingly, 47.5%±0.2% of T cells incubated with a high dose of Io (2 μg/mL) showed increased expression of CD69 indicating this treatment is above the threshold necessary to initiate activation (Figure 10B). However, the intracellular GRP78 protein expression within these $CD69^{high}$ T cells was not statistically different from that of $CD69^{low}$ T cells (Figures 5C,D), demonstrating that upregulation of this activation marker by Io treatment can occur in the

absence of ERSR signaling. Additionally, as anticipated, vehicle or Io treated T cells did not produce high levels of IL-2 (Figure 10E). These data indicate that Ca^{2+} signaling downstream of TCR or CD3 ligation is not responsible for ERSR signaling during T cell activation.

Figure 10

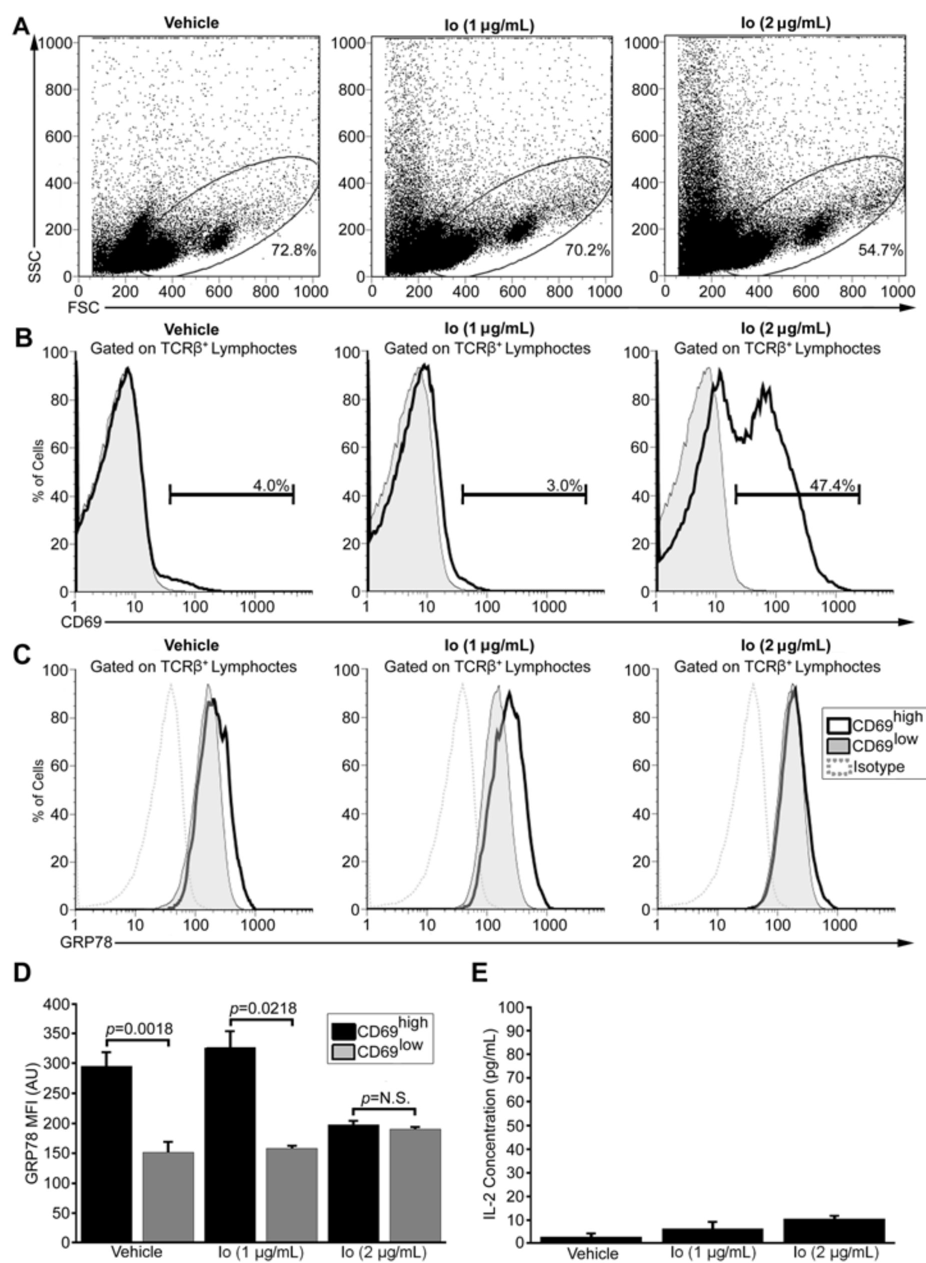

Figure 10: Stimulation of T cells with Io does not activate ERSR signaling.

Purified T cells were treated with Vehicle (*left*, n=4), Io (1 µg/mL, *middle*, n=2), and Io (2 µg/mL, *right*, n=2) for 20 h as described in Methods. (A) Representative flow dot plots depicting the forward scattering (horizontal axis) and side scattering (vertical axis) light properties of lymphocytes. Viable lymphocytes are encircled. (B) Surface expression of CD69 activation marker in treated T cells (black line) or CD69 isotype matched IgG control expression (shaded region). (C) The expression of intracellular GRP78 protein in $CD69^{high}$ (black line) and $CD69^{low}$ (shaded region) T cells. The isotype IgG mAb control for GRP78 is depicted as a dotted line in each histogram. (D) The mean of intracellular GRP78 protein expression in $CD69^{high}$ and $CD69^{low}$ T cells with error bars representing the s.e.m. of duplicate samples. (E) Secretion of IL-2 was measured by ELISA on T cell supernatants harvested from two independent experiments. Samples were examined in triplicate and error bars represent s.e.m. of experimental replicates. Data shown are representative of two experiments.

H. PKC signaling in T cells initiates the ERSR.

In addition to Ca^{2+} fluxes occurring downstream of TCR or CD3 ligation, signaling pathways that specifically activate PKC are initiated (153). We next investigated whether PKC activation could initiate ERSR signaling. For these studies, we stimulated T cells for 20 h with PMA, a specific activator of PKC, in the presence or absence of the PKC inhibitor, calphostin C.

In T cells treated with PMA alone (100 ng/mL), the majority of cells (95.7%±0.4%) showed increased expression of the activation marker CD69 (Figure 11B). Additionally, the $CD69^{high}$ T cells had an increased expression of intracellular GRP78 protein that was statistically different from their $CD69^{low}$ T cell counterparts (Figures 11C,D). These data indicate that PKC activation alone is sufficient to induce the ERSR. To validate the specificity of this response, T cells were next stimulated with PMA in the presence of the PKC specific inhibitor, calphostin C (500 nM). The percentage of cells that were $CD69^{high}$ remained elevated (91.6%±0.07%; Figure 11B). In contrast, however, expression of intracellular GRP78 protein in these $CD69^{high}$ T cells was not elevated and did not differ significantly from that of the $CD69^{low}$ T cells (Figures 11C,D). In addition, the intracellular GRP78 protein expression in $CD69^{high}$ T cells from the PMA treatment group was significantly higher than in the presence of calphostin C (Figure 11D), indicating that inhibition of PKC blocks the ERSR. As expected, PMA treatment in the presence or absence of calphostin C did not result in high levels of IL-2 secretion from the T cells (Figure 11E). These data demonstrate that activating PKC in T cells results in ERSR signaling, even in the absence of IL-2 cytokine production.

I. PKC inhibition during T cell activation with PMA and Io diminishes ERSR signaling and IL-2 production.

Treatment of T cells with PMA combined with Io has been shown to cause cellular proliferation and enhanced IL-2 secretion (154). Within the lymphocyte gate (77.5%±2.5%; Figure 11A) for PMA and Io treated cells, 95.8%±0.4% of the T cells increased expression of CD69 (Figure 11B), indicating the majority of the T cells were activated. These T cells secreted very high levels of IL-2 (Figure 11E) and the $CD69^{high}$ T cells displayed an intracellular GRP78 protein level that was significantly higher than the few $CD69^{low}$ T cells within the population (Figures 11C,D).

Similar to T cells treated with PMA in the presence of calphostin C, treatment of T cells with PMA and Io in the presence of the PKC inhibitor had little effect on the expression of the activation marker CD69 (92.3%±0.8%; Figure 11B), thus indicating these cells were receiving the initial T cell activation stimuli. However, blocking PKC activation with the inhibitor caused the cells to secrete significantly less IL-2 into the supernatant compared to PMA and Io treated cells in the absence of the inhibitor (Figure 11E). This was not a secretory defect caused by the inhibitor because an intracellular protein ELISA also revealed a dramatic decrease in intracellular IL-2 within T cells that were treated with PMA and Io in the presence of the inhibitor (data not shown). Furthermore, intracellular GRP78 expression within the $CD69^{high}$ T cells did not differ significantly from that in the $CD69^{low}$ T cells (Figures 11C,D). These data indicate PKC activation in the initiation of ERSR signaling in T cells and suggest that signaling

downstream of the TCR (signal one) leading to PKC activation is sufficient to cause increased ER chaperone expression, independent of IL-2 production and secretion.

Figure 11

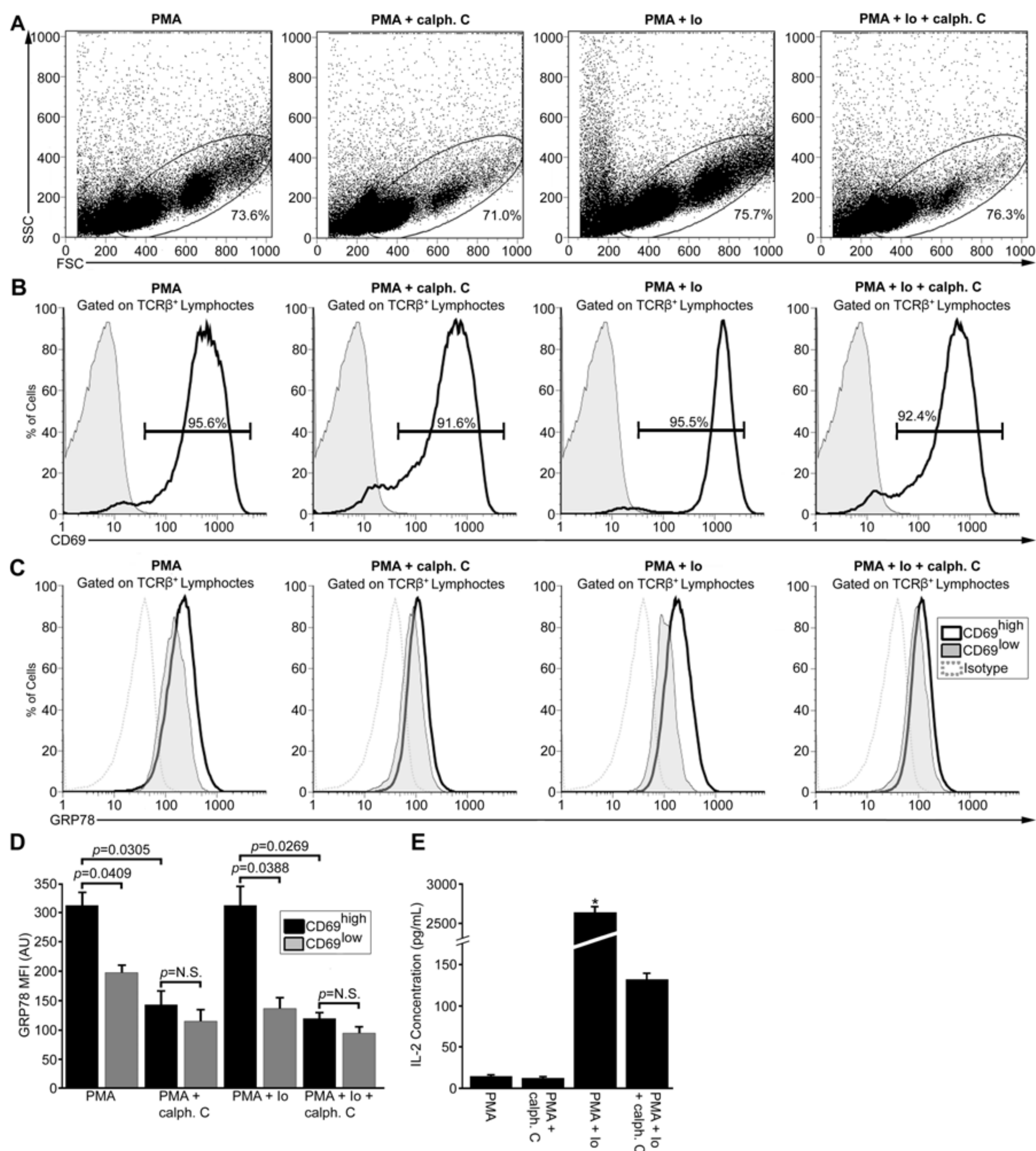

Figure 11: Activating PKC with PMA treatment initiates ERSR signaling in T cells. Purified T cells were treated with PMA (n=2), PMA + calphostin C (n=2), PMA + Io (n=2), or PMA + Io + calphostin C (n=2) for 20 h as described in Methods. (A) Representative flow dot plots depicting the forward scattering (horizontal axis) and side scattering (vertical axis) light properties of lymphocytes. Viable lymphocyte populations are encircled. (B) Representative histograms indicating expression of the activation marker CD69 (black line) and isotype IgG control for CD69 expression (shaded region). (C) Intracellular GRP78 expression for $CD69^{high}$ (black line) and $CD69^{low}$ (shaded region) T cells. Depicted in each histogram is the isotype IgG mAb control for GRP78 (dotted line). (D) Bar graphs displaying the mean of GRP78 protein expression in $CD69^{high}$ and $CD69^{low}$ T cell populations shown with error bars representing the s.e.m. of duplicate samples. (E) IL-2 secretion by T cells measured by ELISA on supernatants from two independent experiments for each treatment group. *, $p<0.0001$ for PMA + Io vs. all other treatment groups. Shown are mean and s.e.m. of triplicate samples. Data shown are representative of two experiments.

J. The expression of multiple ER chaperones increases following PKC activation.

To further demonstrate ERSR signaling in T cells following PKC activation, we examined the expression of multiple ER chaperones by Western blot analyses. The expression of multiple ER chaperones was not substantially increased during Io treatment in comparison to the expression in vehicle treated T cells (Figure 12). However, quantification of ER chaperone expression revealed that PKC activation with PMA or PMA combined with Io increases GRP94, GRP78, ERp72, and PDI (1.5- to 2.3-fold above the expression of vehicle (DMSO) treated T cells). Interestingly, simultaneous treatment of T cells with PMA or PMA combined with Io and the PKC inhibitor, calphostin C, effectively decreases the expression of multiple ER chaperones to levels comparable with vehicle treated T cells (Figure 12). These data further reveal PKC activation leads to increased production of multiple ER stress chaperone proteins.

Figure 12

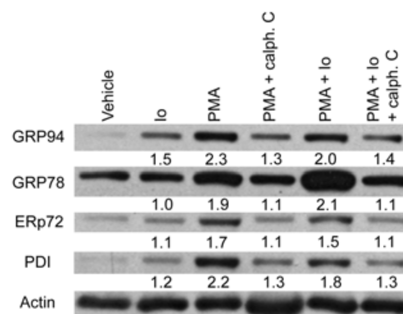

Figure 12: PKC inhibition during T cell activation prevents ERSR signaling.

Western blot analyses of ER chaperone proteins in lysates obtained from T cells stimulated with DMSO (vehicle), Ionomycin (Io), PMA, PMA with calphostin C (PMA + calph. C), PMA + Ionomycin (PMA + Io), and PMA + Ionomycin with calphostin C (PMA + Io + calph. C). Numbers represent densitometry of protein band densities after normalization to actin and comparison to control (=1.0). Data shown are from one of two independent experiments.

Summary

The ERSR is an adaptive cellular mechanism that has been shown to be important during many physiological cellular processes. Our data illuminates the contribution of physiological ERSR signaling to modulation of T cell function during various physiologically relevant immune states. From these studies we have identified PKC as an intracellular signal that is involved in the induction of the ERSR in T cells (Figure 13). Furthermore, we demonstrate that signaling through the TCR alone is sufficient to generate the ERSR, thus supporting evidence that 'non-responsive' immune states are active processes that require increased expression of ER chaperones for maintenance of the tolerant state. Taken together, our data reveal an underlying, but largely unappreciated role for the physiological ERSR in the initial activation phase of T cells following immune stimulation. Furthermore, these findings suggest a new pathway for potential therapeutic intervention in the modulation of immune cell function.

Figure 13

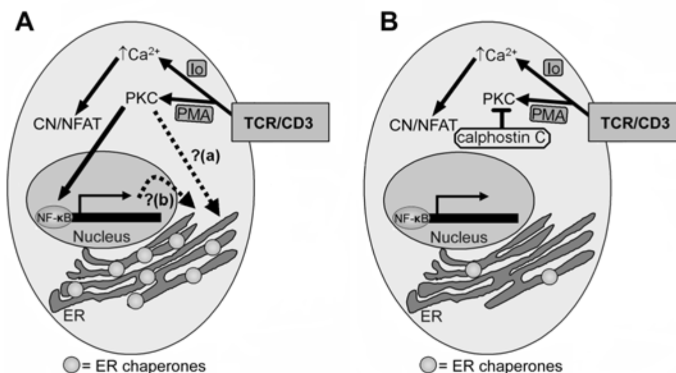

Figure 13: A model for enhanced ER chaperone expression following PKC signaling downstream of TCR/CD3 ligation.

(A) Activating T cells through the TCR/CD3 complex initiates downstream signaling events that activate PKC and increases the intracellular Ca^{2+} concentration. PKC activation and increased intracellular Ca^{2+} in T cells can be mimicked through *in vitro* treatment with PMA and Io treatment, respectively. Increasing intracellular Ca^{2+} activates CN and the transcription factor, NFAT, but did not initiate ERSR signaling in T cells. However, the ERSR did occur with PKC activation, as evidenced by enhanced ER chaperone expression. It remains to be determined if (a) activated PKC directly signals or interacts with ER molecules to initiate ERSR signaling or (b) PKC through activation of the transcription factor NF-κB (155) indirectly enhances ER chaperone expression. (B) The highly specific PKC inhibitor, calphostin C, caused a significant reduction in ER chaperone expression, thus indicating PKC signaling is necessary for initiation of the ERSR during T cell activation.

CHAPTER VI: ABSENCE OF FUNCTIONAL GIMAP5 PROTEIN ACTIVATES ENDOPLASMIC RETICULUM STRESS-INDUCED APOPTOSIS IN T CELLS

Introduction

The expression of the GTPase of the immunity-associated protein family of genes (*Gimap*) has been implicated in the regulation of T cell survival through modulation of TCR signaling. A member of the family, *Gimap5*, has been identified as having a significant role in T cell survival in BBDP rats. BBDP rats have a profound T cell lymphopenia resulting from a frameshift mutation in the *Gimap5* gene. Because Gimap5 has been shown to localize to the ER and previous reports demonstrated an increase of the ER chaperone GRP94 in purified Gimap5$^{-/-}$ T cells, we hypothesized that absence of a functional Gimap5 protein disrupts ER homeostasis.

In this study, we found that the absence of functional Gimap5 protein in T cells of the BBDP rat activates ERSR signaling. We further discovered that ER stress-induced apoptotic signaling occurs in Gimap5$^{-/-}$ T cells and plays a role in the observed lymphopenia in the BBDP rat. By using siRNA-mediated knockdown of the ER stress apoptotic factor CHOP, we were able to protect Gimap5$^{-/-}$ T cells from ER stress-induced death. However, ER stress was not found in thymocytes or B cells from the BBDP rat. These finding indicate a direct relationship between Gimap5 and maintenance of ER homeostasis in the regulation of T cell survival.

Results

A. Increased expression of ERSR proteins in Gimap5$^{-/-}$ BBDP rat lymphocytes.

To examine levels of ER stress in lymph nodes and thymuses from Gimap5$^{+/+}$ BBDR and Gimap5$^{-/-}$ BBDP rats, expression of various ER chaperone proteins was examined by Western blot. These analyses revealed that the expression of ER chaperones GRP94, GRP78, and ERp72 was increased in lymphocytes from Gimap5$^{-/-}$ BBDP rats when compared to that in Gimap5$^{+/+}$ BBDR rats (Figure 14). However, in thymocytes from Gimap5$^{+/+}$ BBDR and Gimap5$^{-/-}$ BBDP rats, ER chaperones were expressed at a basal level (Figure 14). These results indicate that the ERSR is upregulated in lymphocytes from Gimap5$^{-/-}$ BBDP rats leading to increased ER chaperone expression.

To determine if activation of ER stress-induced apoptosis was also occurring in Gimap5$^{-/-}$ BBDP rats, we examined expression of the ER stress apoptotic factor, CHOP. Upregulation of CHOP has been reported to signal the activation of ER stress-mediated apoptotic signals (30) which ultimately culminate in cell death through proteolytic cleavage of caspase-3 (156). Under normal physiological conditions, CHOP expression is minimal and difficult to detect (157). Induction of CHOP was seen by Western blot analyses in lymphocytes from Gimap5$^{-/-}$ BBDP rats, but was not detected in thymocytes from Gimap5$^{-/-}$ BBDP or in thymocytes and lymphocytes from Gimap5$^{+/+}$ BBDR rats (Figure 14). Furthermore, the presence of cleaved caspase-3, the executor of apoptosis downstream of ER stress-induced apoptotic signaling, was only detected in Gimap5$^{-/-}$ BBDP lymphocytes (Figure 14). As a control, Gimap5 protein was confirmed by Western blot analysis and was only detected in tissues from Gimap5$^{+/+}$ BBDR rats (Figure 14).

These data provide evidence of ERSR and ER stress-induced apoptotic signaling in lymphocytes from Gimap5$^{-/-}$ BBDP rats.

Figure 14

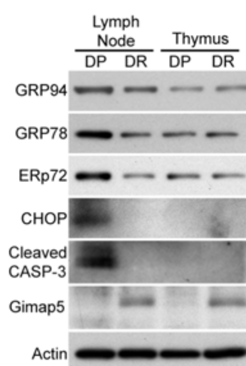

Figure 14: The expression of ER chaperones increases in lymphocytes from the Gimap5$^{-/-}$ BBDP rat.

Western blot analyses of various ER chaperone proteins (GRP94, GRP78, and ERp72), the ER stress apoptotic factor CHOP, and cleaved caspase-3 (CASP-3) in lymphocytes and thymocytes from BBDP and BBDR rats. Gimap5 protein expression was confirmed in lysates from BBDR rats and actin was used as a loading control. Data shown are from one of three independent experiments.

B. GRP78 and CHOP expression levels are similar between Gimap5$^{+/+}$ and Gimap5$^{-/-}$ rat thymocyte CD4^{+}/CD8^{+} subpopulations.

Lymphopenia in the Gimap5$^{-/-}$ BBDP rat is characterized by reductions in peripheral T cells and has been suggested to occur post-thymically; however, development of thymocytes is generally normal (131). Analysis of thymocyte populations in Gimap5$^{+/+}$ BBDR and Gimap5$^{-/-}$ BBDP rats revealed similar percentages of CD4^{+}, CD8^{+}, and CD4^{+}CD8^{+} thymocytes (Figure 15A, Table 1), analogous to previous reports (158). To determine if there are differences in ERSR signaling between the various thymocyte populations in Gimap5$^{+/+}$ BBDR and Gimap5$^{-/-}$ BBDP rats, we examined expression of GRP78 using intracellular flow cytometry. Quantification of intracellular GRP78 expression revealed no significant difference in the thymocyte populations between Gimap5$^{+/+}$ BBDR and Gimap5$^{-/-}$ BBDP rats (Figures 15B,D). Additionally, we analyzed for induction of ER stress-induced apoptosis by examining intracellular CHOP expression using flow cytometry. Similar to intracellular GRP78 expression, the MFI of intracellular CHOP expression was not significantly different in thymocyte populations between Gimap5$^{+/+}$ BBDR and Gimap5$^{-/-}$ BBDP rats (Figures 15C,D). Similar to data obtained by Western blot analyses (Figure 14), these data provide evidence that ERSR signaling during thymocyte development in Gimap5$^{-/-}$ BBDP rats does not differ significantly from that in thymocytes of Gimap5$^{+/+}$ BBDR rats.

Figure 15

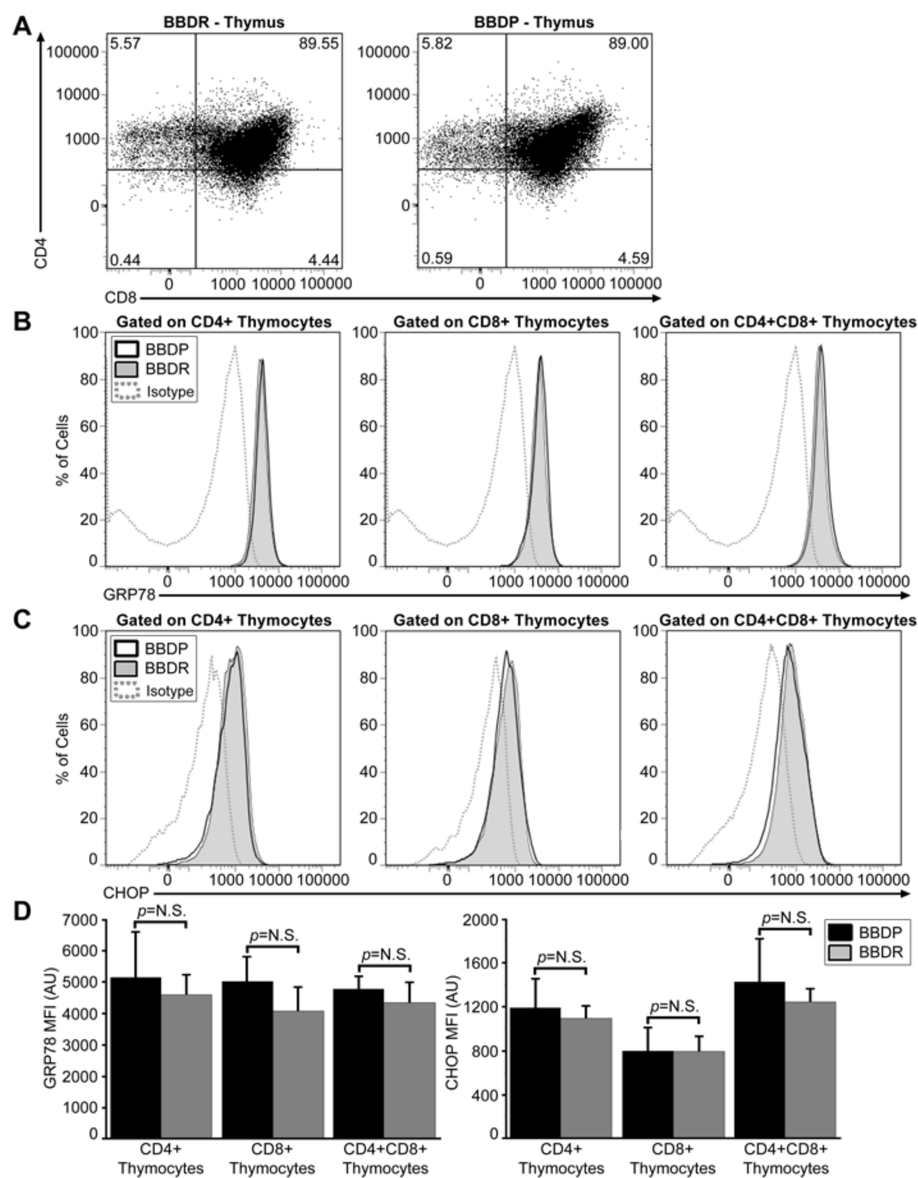

Figure 15: ERSR signaling is similar in thymocytes from BBDR and BBDP rats.

(A) Representative flow dot plots depicting CD8 expression (horizontal axis) and CD4 expression (vertical axis) on thymocytes. (B,C) Intracellular GRP78 and CHOP expression was determined in thymocyte populations from BBDP (black line) and BBDR (shaded region) rats. Isotype control for GRP78 staining (dotted line) is shown in each histogram. (D) Bar graphs displaying the mean of intracellular GRP78 protein or CHOP protein expression (arbitrary units; AU). Error bars represent the S.D. of triplicate samples. Data shown are representative of three independent experiments.

C. $CD90^+$ recent thymic emigrants comprise the majority of peripheral $CD4^+$/$CD8^+$ T cells in the $Gimap5^{-/-}$ rat.

$Gimap5^{-/-}$ BBDP rats undergo a peripheral T cell lymphopenia that is attributed to a mutation in the *Gimap5* gene (137,138). We first confirmed the peripheral T cell lymphopenia (158) by flow cytometry analyses and found reductions in TCR positive cells in gated lymphocytes from the $Gimap5^{-/-}$ BBDP rat (Figures 16A,B, Table 2). Comparable to a previous report (158), we also verified that the peripheral T cell compartment of $Gimap5^{-/-}$ BBDP rats is comprised of reductions in $CD4^+$ T cells and a near absence of $CD8^+$ T cells (Figure 16C, Table 2).

We next examined expression of CD90, a cell surface molecule found on T cells recently exported to the periphery, amongst $CD4^+$ and $CD8^+$ T cells from the $Gimap5^{+/+}$ BBDR and $Gimap5^{-/-}$ BBDP rats. In $Gimap5^{-/-}$ BBDP rats the majority of $CD90^+$ T cells, known as recent thymic emigrants, disappear from the periphery within seven days of their export from the thymus (159). Flow cytometry analyses revealed that the preponderance of gated $CD4^+$ and $CD8^+$ T cells from $Gimap5^{-/-}$ BBDP rats still express CD90 on the cell surface, indicating few mature T cells survive (Figure 16D, Table 3). However, the majority of $CD4^+$ and $CD8^+$ T cells from the $Gimap5^{+/+}$ BBDR rats have down-regulated CD90 and are phenotypically characteristic of mature T cells (Figure 16E, Table 3). These data support previous reports that $Gimap5^{-/-}$ BBDP rats suffer from a profound T cell lymphopenia consistent with the disappearance of recent thymic emigrants from the periphery prior to CD90 down-regulation and maturation.

Figure 16

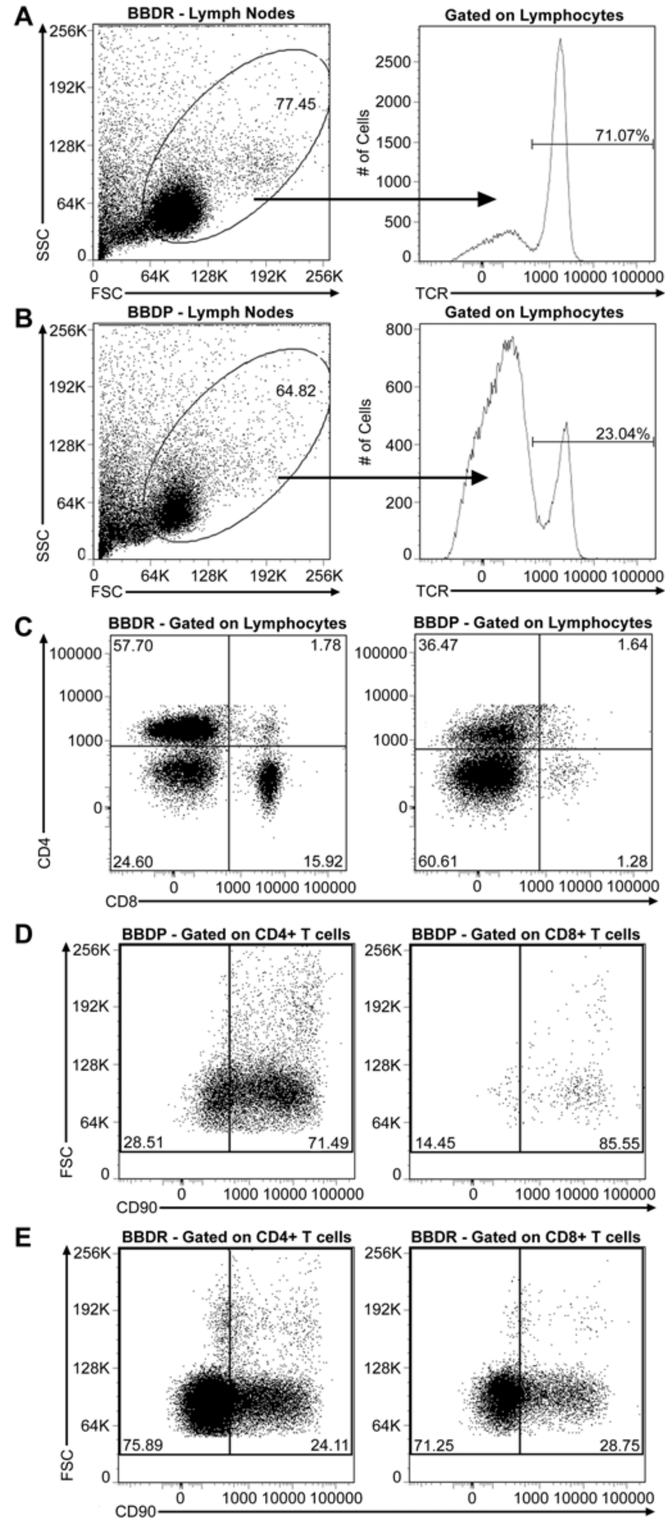

Figure 16: The majority of T cells in the periphery of the Gimap5$^{-/-}$ BBDP are CD90^{+} recent thymic emigrants.

(A,B) Representative flow dot plots depicting the forward scattering (horizontal axis) and side scattering (vertical axis) light properties of lymphocytes. Viable lymphocytes are encircled. The arrow points to histograms representing gating of TCR^{+} cells within the lymphocyte population. (C) Quadrant gating of flow dot plots portraying CD8 expression (horizontal axis) and CD4 expression (vertical axis) on gated lymphocytes. Numbers indicate the percentage of cells in each quadrant. (D,E) Representative flow dot plots depicting CD90 expression (horizontal axis) and forward scattering (vertical axis) of lymphocytes within gated CD4^{+} and CD8^{+} T cell populations. Numbers represent the percentage of corresponding CD90^{-} (left quadrant gate) and CD90^{+} (right quadrant gate) T cells within each dot plot. Data shown are representative of three independent experiments.

D. Gimap5$^{-/-}$ rat T cell populations in the periphery express increased levels of GRP78 and CHOP.

We and others have shown that recent thymic emigrants disappear from the periphery of Gimap5$^{-/-}$ BBDP rats (159). However, the mechanism that causes the number of recent thymic emigrants to diminish remains elusive. We next tested our hypothesis that absence of functional Gimap5 protein in Gimap5$^{-/-}$ BBDP rat T cells disrupts ER homeostasis and integrity leading to the ERSR and ER stress-induced apoptosis, thus accounting for recent thymic emigrant death. Examining intracellular GRP78 expression on gated recent thymic emigrants (TCR^{+}CD8^{+}CD90^{+} and TCR^{+}CD4^{+}CD90^{+} lymphocytes) revealed an increase in GRP78 expression in cells from Gimap5$^{-/-}$ BBDP rats as compared with that in cells from Gimap5$^{+/+}$ BBDR rats (Figures 17A,C). Additionally, intracellular GRP78 expression was increased to a similar extent in T cell populations from Gimap5$^{-/-}$ BBDP rats that down-regulated CD90 from the cell surface (TCR^{+}CD8^{+}CD90^{-} and TCR^{+}CD4^{+}CD90^{-} lymphocytes) over the same population of T cells from Gimap5$^{+/+}$ BBDR rats (Figures 17B,C). These data provide evidence that ERSR signaling occurs not only in recent thymic emigrant populations from Gimap5$^{-/-}$ BBDP rats, but also in phenotypically mature CD90^{-} T cells.

To investigate activation of ER stress-induced apoptotic signaling, we examined intracellular expression of CHOP within Gimap5$^{-/-}$ BBDP rat T cell populations. The MFI of intracellular CHOP expression was increased in recent thymic emigrants from Gimap5$^{-/-}$ BBDP rats over that in Gimap5$^{+/+}$ BBDR rats (Figures 18A,C). Similar to GRP78 expression, the enhanced expression of intracellular CHOP was not limited to

recent thymic emigrants and was detected in TCR^+ subpopulations of $CD90^-$ lymphocytes from $Gimap5^{-/-}$ BBDP rats (Figures 18B,C). Importantly, in gated $TCR^-CD45RA^+$ cells, indicative of B cell populations, intracellular GRP78 and CHOP expression did not differ significantly between $Gimap5^{-/-}$ BBDP and $Gimap5^{+/+}$ BBDR rats (Figures 19A,B). These data reveal that the *Gimap5* mutation in BBDP rats results in specific activation of ERSR signaling that triggers ER stress-induced apoptosis not only in recent thymic emigrants, but also in T cells that have down-regulated CD90.

Figure 17

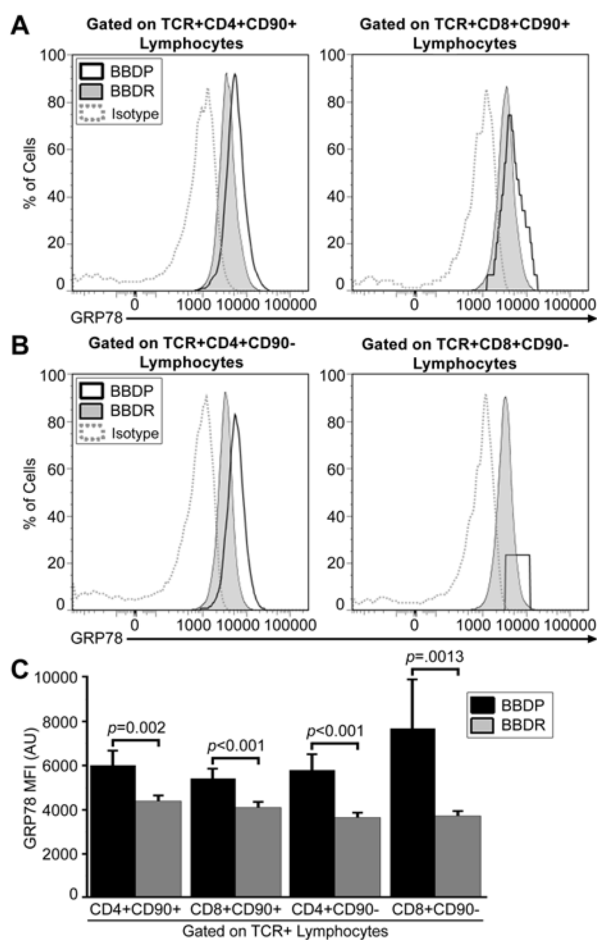

Figure 17: The ERSR is increased in all TCR$^+$ lymphocyte populations within BBDP rats.

(A,B) The expression of intracellular GRP78 protein in BBDP (black line) and BBDR (shaded region) rat T cell populations. The isotype control for GRP78 staining is depicted as a dotted line in each histogram. (C) The mean of intracellular GRP78 protein expression in BBDP and BBDR TCR$^+$ lymphocytes with error bars representing the S.D. of multiple samples. Data shown are representative of three independent experiments.

Figure 18

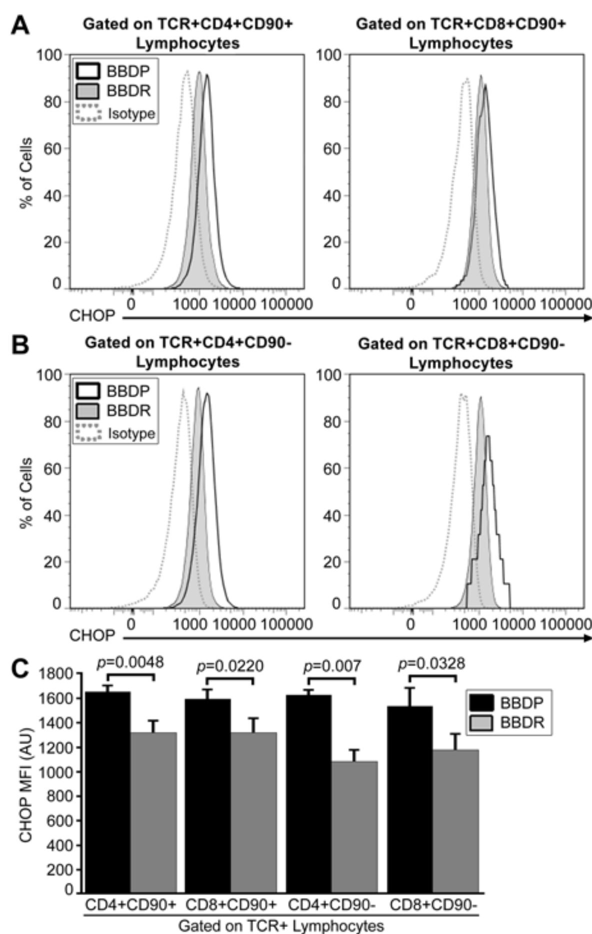

Figure 18: ER stress-induced apoptotic signaling is activated within TCR$^+$ lymphocyte populations in BBDP rats.

(A,B) Intracellular CHOP expression for BBDP (black line) and BBDR (shaded region) rat TCR$^+$ lymphocytes. Depicted in each histogram is the isotype control for GRP78 staining (dotted line). (C) Bar graphs displaying the mean of CHOP protein expression in gated BBDP and BBDR T cell populations. Error bars represent the S.D. of triplicate samples. Data shown are representative of three independent experiments.

Figure 19

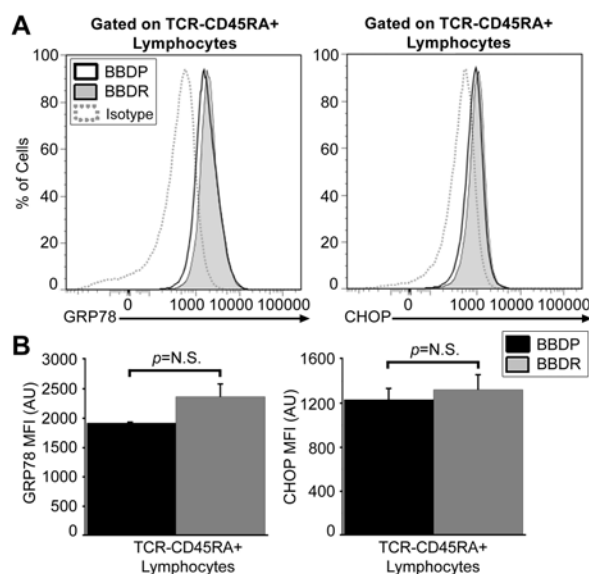

Figure 19: B cell populations within BBDP and BBDR express similar ERSR signaling.

(A) Intracellular GRP78 (left) and CHOP (right) expression in $TCR^{-}CD45RA^{+}$ populations from BBDP (black line) and BBDR (shaded region) rats. The dotted line represents the GRP78 isotype control staining. (B) Bar graphs displaying the mean of intracellular GRP78 protein (left) and CHOP protein (right) expression for BBDP and BBDR $TCR^{-}CD45RA^{+}$ lymphocytes with error bars representing the S.D. of duplicate samples. Data shown are representative of two independent experiments.

E. Knockdown of CHOP expression in Gimap5$^{-/-}$ rat T cells leads to decreased apoptosis and cell death.

CHOP is a major regulator of ER stress-induced apoptotic signaling (30) and inhibition of CHOP is known to decrease ER stress-mediated apoptosis (160). To determine if decreasing CHOP expression in T cells from Gimap5$^{-/-}$ BBDP rats reduces apoptosis, Gimap5$^{-/-}$ BBDP rat T cells were purified, stimulated for 12 h with 10 ng/mL of PMA and 100 ng/mL of Io, then transfected by nucleofection with 100 nM siRNAs targeted to CHOP (siCHOP) or non-specific control siRNA (siControl). Western blot analyses revealed a significant reduction in CHOP protein expression in T cells transfected with siCHOP as compared to T cells transfected with siControl (Figure 20A). The effects of reducing CHOP protein expression on induction of ER stress-induced apoptosis were examined through flow cytometry. Using annexin V and 7AAD staining, flow cytometric analyses revealed three distinct populations of T cells: viable (annexin V$^-$/7AAD$^-$; lower left quadrant), early apoptotic (annexin V$^+$/7AAD$^-$; upper left quadrant), and both late apoptotic and necrotic (annexin V$^+$/7AAD$^+$; upper right quadrant) (Figures 20B,C).

As compared to T cells transfected with siControl, reducing CHOP expression significantly decreased the percentage of T cells that enter into a late apoptotic stage of death, thus allowing for an increased percentage of T cells to remain viable (Figures 20B,D). However, there was no significant difference in the percentage of cells that were in the early stages of apoptosis between T cells transfected with siCHOP or siControl (Figures 20B,D). To ensure the results of CHOP inhibition were specific to T cells, the

effects of siCHOP transfection were examined in gated TCR^- lymphocytes. In contrast to T cells, there were no statistical differences in the percentages of viable, early apoptotic, and late apoptotic TCR^- lymphocytes between siCHOP and siControl transfected cells (Figures 20C,E). Therefore, these studies suggest that CHOP protein actively participates in apoptotic signaling in $Gimap5^{-/-}$ T cells and reduction of this ER stress apoptotic factor decreases T cell death.

Figure 20

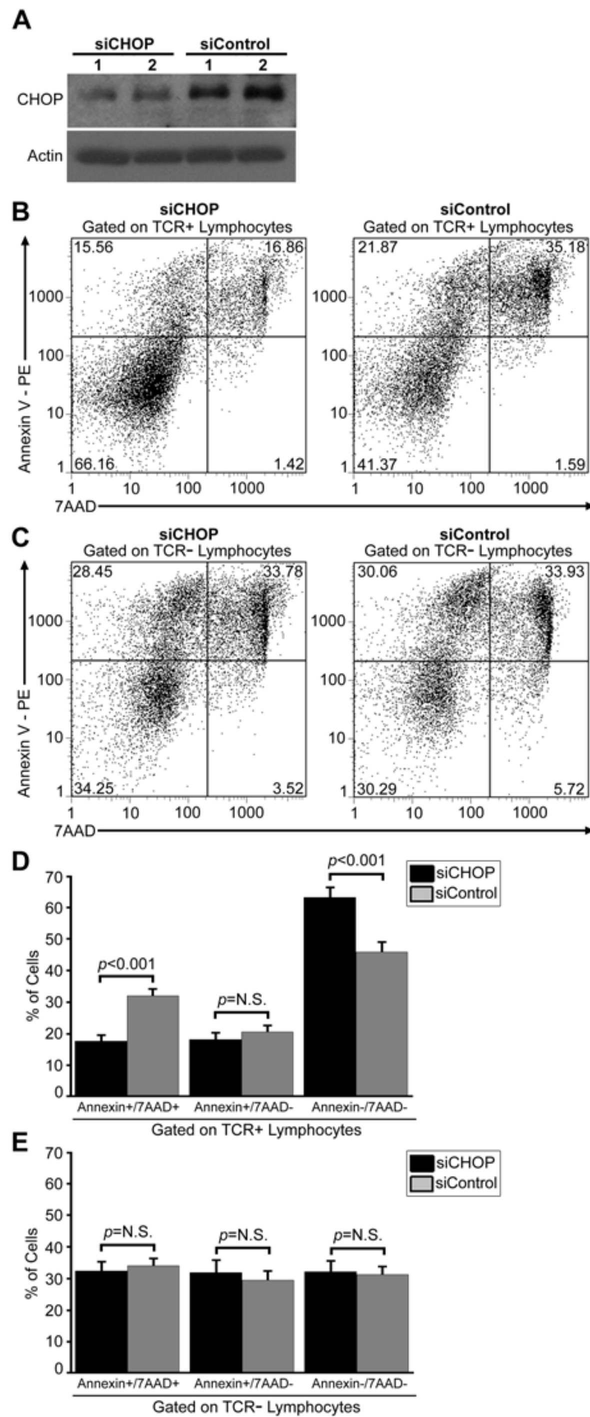

Figure 20: Knockdown of CHOP protein expression enhances Gimap5$^{-/-}$ T cell viability.

(A) Western blot analyses of the ER stress apoptotic factor CHOP in purified T cells transfected with either CHOP siRNA (siCHOP) or non-specific control siRNA (siControl). Actin was used as a loading control. (B,C) Representative flow dot plots depicting cells positive for annexin V (vertical axis) and 7AAD staining (horizontal axis) on gated TCR^{+} or TCR^{-} lymphocytes. (D,E) Bar graphs displaying the percentage of TCR^{+} or TCR^{-} lymphocytes that were positive for both annexin V and 7AAD staining (left), positive for annexin V staining alone (middle), and negative for both staining (right). Error bars represent the S.D. of quadruplicate samples and data shown are representative of two independent experiments.

Table 1

Table 1: Phenotypic profile of thymocytes from BBDR and BBDP rat strains

Animal Strain	$CD4^+$ Thymocytes	$CD8^+$ Thymocytes	$CD4^+CD8^+$ Thymocytes
BBDR	6.36 ± 0.71[a]	4.95 ± 1.03	88.34 ± 1.19
BBDP	5.16 ± 1.16	4.78 ± 2.02	89.81 ± 2.05

[a]Indicated data are means ± S.D. (n = 4).

Table 2

Table 2: Phenotypic profile of lymphocytes from BBDR and BBDP rat strains

Animal Strain	Lymphocytes	TCR^+ Lymphocytes	$CD4^+$ Lymphocytes[b]	$CD8^+$ Lymphocytes[b]
BBDR	78.95 ± 1.92[a]	68.51 ± 2.44	57.04 ± 0.59	16.88 ± 1.44
BBDP	67.91 ± 3.48	22.79 ± 0.71	36.97 ± 1.54	1.24 ± 0.07

[a]Indicated data are means ± S.D. (n = 3).
[b]Gated on total lymphocytes.

Table 3

Table 3: Phenotypic profile of CD90 cell surface expression on lymphocytes from BBDR and BBDP rat strains

Animal Strain	$TCR^+CD4^+CD90^+$ Lymphocytes	$TCR^+CD4^+CD90^-$ Lymphocytes	$TCR^+CD8^+CD90^+$ Lymphocytes	$TCR^+CD8^+CD90^-$ Lymphocytes
BBDR	22.83 ± 8.14[a]	77.16 ± 8.14	28.01 ± 9.72	71.99 ± 9.71
BBDP	67.54 ± 3.54	32.47 ± 3.46	86.23 ± 0.65	13.72 ± 0.58

[a]Indicated data are means ± S.D. (n = 3).

Summary

The absence of functional Gimap5 protein has been linked to the lymphopenic phenotype of the BBDP rat. Our data indicates that $Gimap5^{-/-}$ T cells have increased levels of the ER chaperone GRP78 and the ER stress apoptotic factor CHOP. Furthermore, disrupting the production of CHOP protein protects $Gimap5^{-/-}$ T cells from death, thus demonstrating the participation of ER stress-induced apoptosis in the T cell lymphopenia of the BBDP rat. However, the involvement of the ERSR in $Gimap5^{-/-}$ BBDP rats is limited to T cells as thymocytes and B cells did not display ERSR signaling that differed significantly from $Gimap5^{+/+}$ BBDR rats. Overall, these data indicate that Gimap5 regulates T cell survival through maintenance of ER homeostasis.

CHAPTER VII: DISCUSSION

PKC Signaling Induces the ERSR in T cells

Activation of T cells results in the upregulated expression of numerous proteins essential for differentiation and for effector functions (161). Many of these proteins, such as cytokines, chemokines, and cell surface molecules, participate in the activation process, and thus distinguish activated T cells from their naïve precursors (69). Considering the exigency on protein folding and production, it is conceivable that fully activated T cells require more efficient protein handling in the ER. Therefore, initiation of the ERSR and upregulation of ER chaperones as a physiological consequence of TCR signaling is a logical step to augment the protein folding ability of the ER. Consistent with this, our data from Chapter V demonstrate upregulation of ER chaperones during *in vitro* and *in vivo* activation of T cells through signal one and signal two. Since 'partial' activation of T cells (signal one only) resulted in reduced production of the important cytokine, IL-2, we hypothesized that the ERSR in this case would be qualitatively or quantitatively different. Unexpectedly, however, we found that ER chaperones were also increased in cells that we purposefully treated to omit (in our *in vitro* studies) or block (in our *in vivo* tolerance model) signal two. Lastly, we demonstrate that activation of PKC, a serine/threonine protein kinase found downstream of TCR signaling, is involved in the initiation of ERSR signaling during the T cell activation process (Figure 13).

The importance of PKC during T cell activation has been comprehensively investigated and PKC has been found to play key roles in activating numerous downstream signaling pathways of the TCR (80,162). Following TCR or CD3 ligation,

downstream signaling events leading to the production of DAG and IP_3 occurs. It has been shown that these molecules play an active regulatory role in PKC activation (153). By using calphostin C, a highly specific inhibitor of PKC that functions by competing for the DAG binding site on PKC's regulatory domain (163), we now show a previously uncharacterized link between PKC activation and ERSR signaling in T cells. Although full activation of T cells is required for production of IL-2, our data establish that the initial stimuli through the TCR leading to PKC activation, or direct activation of PKC with PMA treatment, is sufficient to initiate activation of the ERSR. Since TCR signaling is well characterized, further insights into T cell functioning may be employed by dissection of the ERSR in these cells.

In a recent study, APC-stimulated $CD4^+$ T cells utilized elements of the ERSR, including increased expression of ERSR genes, during their differentiation into effector cells (41). The authors speculated that ERSR signaling, as part of a general integrated stress response, may be induced by imbalances in nutrients following $CD4^+$ T cell clonal expansion and differentiation or, alternatively, that TCR stimulation activates ERSR signaling in preparation for effector function. In Chapter V we extend those findings and demonstrate that stimulation of T cells through the TCR alone is sufficient to induce the expression of ERSR proteins, even in the absence of differentiation. Although consistent with a potential preparatory function of the ERSR prior to T cell differentiation and proliferation, ERSR signaling may alternatively be activated due to the misfolding of proteins upregulated by TCR stimulation or secondary factors that disrupt ER

homeostasis. Further studies are necessary to determine the exact role for ERSR induction following TCR engagement.

During the differentiation of B cells into high-rate immunoglobulin secreting plasma cells, the expression of ER chaperones is increased in preparation for antibody secreting activity (39,40). Signaling through the B cell receptor (BCR) alone, while insufficient to allow B cell differentiation into plasma cells, is also a physiological inducer of the ERSR and causes an increase in ER chaperone expression (164). In an analogous manner, we found that ER chaperone expression in T cells was elevated by TCR signaling. Although PKC has been shown to be required for BCR signal transduction (165), the relative contribution of PKC to the increase of ER chaperones in B cells has not been linked. We show that inhibiting PKC function during T cell activation results in a decreased amount of ER chaperones. Therefore, initiation of the ERSR by signaling through the TCR or BCR appears to play an essential preparatory role in the adaptive immune response, and PKC signaling downstream of these receptors may be intimately involved in the preparation.

Besides activating PKC and subsequent induction of the ERSR, signaling through the TCR complex without costimulation in naïve T cells can lead to anergy in $CD4^+$ T cells (55) or tolerance in $CD8^+$ T cells (98). Anergy in $CD4^+$ T cells is a form of tolerance in which antigen-exposed $CD4^+$ T cells become unresponsive to subsequent antigenic stimulation, even in the presence of costimulation (91). Maintenance of this anergic state has been shown to depend on the *de novo* synthesis of distinct proteins, including early growth response (Egr)-2 and Egr-3 proteins (85,166). Our data from Chapter V indicate

that expression of ER chaperones in T cells only receiving signal one does not differ from the expression in fully activated T cells. Therefore, we suggest that tolerant T cells are in an immunological 'active' state that requires increased levels of ER chaperones for the folding and assembly of proteins necessary for initiating and maintaining a 'non-responsive' phenotype. These studies further indicate that increased expression of ER chaperones may be a general mechanism employed by numerous cell types in response to extracellular and intracellular signaling for modulation of cellular functioning.

To investigate the induction of ERSR signaling in T cells in the context of physiologically relevant immune events, we evaluated *in vivo* ERSR signaling during activation and tolerance induction. We used an established mouse model in which administration of DST has been shown to fully activate TCR transgenic $CD8^+$ T cells (148,152). Our results show for the first time that *in vivo* antigen-exposed $CD8^+$ T cells that are destined to become either activated or tolerant undergo a physiological ERSR. The importance of PKC during the *in vivo* induction of a physiological ERSR are currently under investigation to determine if qualitative or quantitative differences are observed in antigen-exposed T cells that differ in their ultimate fate.

TCR ligation leads to signals that are necessary, but not sufficient, for fully functional T cell activation. In Chapter V we reveal an induction of the ERSR in T cells following TCR ligation (signal one), even in the absence of costimulation (signal two). Induction of the ERSR in activated and tolerant T cells may play an integral, underlying role in the initiation of an immune response. In addition, we now link PKC, an important mediator of downstream TCR signaling, as a key molecule in the initiation of the ERSR,

as inhibiting PKC function failed to induce ERSR signaling. Further studies characterizing ERSR induction and PKC activation downstream of TCR ligation may uncover novel therapeutic targets in this signaling pathway. These targets could be used to suppress or promote activation of T cells for the treatment of immune-mediated diseases and disorders.

ER Stress-Induced Apoptosis in Gimap5$^{-/-}$ T cells

In Chapter VI we examined the ERSR in T cells from Gimap5$^{-/-}$ BBDP rats. We demonstrated that absence of functional Gimap5 protein in T cells leads to ERSR signaling as evidenced by increased GRP78 protein expression. Furthermore, Gimap5$^{-/-}$ BBDP T cells initiate ER stress-induced apoptotic signaling through upregulation of CHOP protein. By decreasing the expression of this ER stress apoptotic factor with siRNA, we were able to protect Gimap5$^{-/-}$ BBDP T cells from ER stress-induced death. These observations suggest that Gimap5 protein plays a role in the maintenance of ER homeostasis and integrity in T cells.

Lymphopenia in the BBDP rat was originally linked to a recessive mutation in a diabetes susceptible locus, known as *lyp/Iddm1*, which encodes for Gimap5 protein (141). Although spontaneous diabetes development in the BBDP rat depends on the presence of *lyp/Iddm1*, diabetes susceptibility and lymphopenia are traits that can be inherited independently (141). To ensure that ERSR signaling in T cells from the BBDP rat was due to absence of functional Gimap5 protein and not a byproduct of diabetes development, we only used nondiabetic BBDP rats for studies. Furthermore, flow

cytometry and Western blot analyses on Gimap5$^{-/-}$ T cells from congenic Wistar Furth rats that do not develop spontaneous diabetes but carry the *lyp/Iddm1* and *Iddm4* (a major non-MHC determinant of diabetes development in the BB rat) have increased expression of ERSR proteins, including GRP78 and CHOP (data not shown). Overall, this indicates that ERSR signaling in T cells from the BBDP rat is a result of the absence of functional Gimap5 protein product and not a secondary consequence of spontaneous diabetes development.

Within the immune system, the establishment of immunological tolerance involves mechanisms to delete self-reactive T cells in order to avoid autoimmune diseases, such as T1D (86). The interactions of T cell receptors on thymocytes with thymic stromal cells are important for proper T cell development and eventually allow for $CD4^+CD8^+$ double positive thymocytes to differentiate into mature $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive thymocytes (64,66). Our data from Chapter VI reveal that the percentage of double positive and single positive thymocytes does not differ between BBDR and BBDP rats, even though thymocytes from the BBDP rat contain a mutation in their *Gimap5* gene. Furthermore, ERSR signaling was similar between the thymocyte populations from the BBDR and BBDP rats, thus indicating the main ER regulatory functions for Gimap5 protein may occur during T cell post-thymic development. In general, our data indicate that ERSR and ER stress-mediated signaling may only occur minimally during thymocyte development as levels of ER chaperones remained low as double positive thymocytes matured to the single positive stage.

Following thymic development, T cells still expressing CD90, known as recent thymic emigrants, enter into the circulation. Within the BBDP rat, we and others have shown that a limited number of recent thymic emigrants live to down-regulate CD90 expression and become mature T cells (Figure 16) (159). Because recent thymic emigrants undergo apoptosis more rapidly in the Gimap5$^{-/-}$ BBDP rat, we originally hypothesized that the level of ERSR signaling would be enhanced compared to that in CD90^{-} mature T cells from the same animal. Our data suggest that ER chaperone expression is comparable amongst the various CD90^{+} and CD90^{-} Gimap5$^{-/-}$ T cell populations from the BBDP rat, indicating that a global absence of the protein is sufficient to initiate ERSR signaling throughout peripheral T cell development.

ER stress response signaling has been shown to be required for numerous physiological functions in several cell types (23,38,39,41). More recently, pathological signals from the ER have been attributed to cell death and apoptosis and linked to many diseases, including diabetes (5,160). In this report, we show that absence of functional Gimap5 protein leads to these pathological signals from the ER and subsequent ER stress-induced apoptosis in T cells from the BBDP rat. We suggest that triggering of the ER stress-induced apoptotic pathway in the T cells of the BBDP rat is involved with the corresponding lymphopenic phenotype of the animal. This observation is further supported by data that demonstrate a reduction in the number of apoptotic T cells following knockdown of CHOP protein by siRNA. Interestingly, our data indicate ERSR signaling does not differ between B cells from the Gimap5$^{+/+}$ BBDR and Gimap5$^{-/-}$

BBDP rat (Figure 19). Therefore, these data suggest a direct relationship between Gimap5 and ERSR signaling for the maintenance of T cell survival.

In Chapter VI we demonstrate that Gimap5$^{-/-}$ T cells are prone to cell death through ER stress-induced apoptosis. However, we and others have previously shown a role for Gimap5 protein in maintenance of mitochondrial integrity for T cell survival (106,167). These two pathways are linked through the participation of CHOP protein in ER stress-induced cell death by sensitizing the mitochondria to numerous apoptotic factors (168). These apoptotic factors include downregulation of antiapoptotic Bcl-2 protein (31), activation of JNK and its associated proapoptotic downstream kinases (5), and activation of caspase-12 (169). We hypothesize that CHOP protein through its role in ER stress-induced apoptotic signaling potentially leads to the disruption of mitochondrial integrity that is observed in Gimap5$^{-/-}$ T cells (106,167). Consequently, loss of mitochondrial integrity in Gimap5$^{-/-}$ T cells may be secondary to ER dysfunction and subsequent ER stress-induced apoptotic signaling.

The localization of Gimap5 protein still remains controversial, however, recent research indicates an exclusive localization to the ER (113). Our data support an intimate link between Gimap5 and the ER because the ERSR is initiated in Gimap5$^{-/-}$ T cells. It has also been reported that in the BBDP rat the frameshift mutation in *Gimap5* results in production of a truncated protein that lacks its transmembrane domain (113). Therefore, we can not rule out the possibility that the presence of this nonfunctional protein product may accumulate in the ER and lead to the pathological ER stress that is observed in Gimap5$^{-/-}$ T cells. A similar observation is made in pancreatic beta cells of the Akita

mouse where accumulation of mutant insulin causes ER stress and eventual apoptosis through CHOP protein induction (34). In both cases, disruption of CHOP has been effective in delaying apoptosis and preventing cell death (34), thus indicating the importance of CHOP in ER stress-associated cell death signaling.

Our findings in Chapter VI suggest a role for ER stress-induced apoptosis in the T cells of the Gimap5$^{-/-}$ BBDP rat. We link for the first time a relationship between Gimap5 protein and maintenance of ER homeostasis and integrity. Additionally, our results indicate that proper ER functioning in T cells is a critical component of their survival, as ER stress caused by a mutation in the *Gimap5* gene leads to cellular death pathways and lymphopenia in the BBDP rat. Further studies characterizing the role of Gimap5 protein in T cell survival may uncover novel pathways capable of modulating T cell functioning.

Conclusions and Remaining Questions

T cells are a critical component of the adaptive immune response and are designed to protect the human body from disease and viral infection. In order for T cells to participate as effector cells in this response, they must be activated through their TCR. Therefore, understanding the molecular mechanisms that govern their activation process may provide a means of regulating T cell function. Furthermore, insight into the T cell activation process has the potential to advance our knowledge of autoimmune diseases, such as T1D.

The research presented in this dissertation was originally designed to further the understanding of signaling pathways involved in the T cell activation process

downstream of the TCR. In Chapter V we presented data linking a key downstream mediator of TCR signaling, PKC, to activation of the adaptive cellular mechanism known as the ERSR. Originally, the ERSR was thought to become activated in professional secretory cells such as plasma cells and beta cells which require an increased capacity of protein processing due to enhanced levels of protein synthesis and secretion (23). Here we present a model for activation of the ERSR in the T cell, a cell not classified as a professional secretory cell. Full activation of T cells *in vitro* and *in vivo* results in upregulation of the expression of ER chaperones, thus augmenting the protein folding capacity of the ER for maintenance of ER homeostasis. Unexpectedly, partial T cell activation through TCR signaling alone also induced the ERSR and an increase of ER chaperones. Although we were able to identify the downstream component of TCR signaling that activates the ERSR, the exact functions of the ERSR in T cells remains unknown.

Importantly, however, we have now identified the physiological process of T cell activation as an inducer of ERSR signaling. Specifically, our data indicate that activation of PKC, a molecule essential for TCR-induced T cell activation (162), as an initiator of physiological ERSR signaling in T cells. Determining the PKC isoform involved in the initiation of the ERSR in T cells may provide insight into the importance of this molecule in ERSR induction in numerous cell types. Recently, a report linked PKCθ activation to ERSR induction during autophagy in hepatocytes (170). Based on the significance of this PKC isoform in the differentiation and survival of T cells (162,171), a logical hypothesis for future analysis would examine PKCθ and ERSR activation in T cells.

Activation of PKC has been implicated to be important for proper functioning in various immune cells, including macrophage activation and B cell survival (172). Additionally, PKC signaling has been linked to inhibition of insulin receptor signaling and found to be increased along with protein levels in skeletal muscle from T2D patients (173,174). Our data specifically links PKC activation with ERSR induction, suggesting this association may occur in and be important for multiple cell types. Recent reports demonstrate that knockdown of PKC expression did not interfere with TG-induced eIF2α phosphorylation and *XBP1* mRNA splicing. Furthermore, deletion of ATF6 did not disrupt PKC activation (170). These data suggest that ERSR induction can occur independently of PKC activation and indicates that the resultant increases in transcription and translation of genes caused by PKC signaling may be the initiator of the ERSR in many cell types.

The physiological ERSR allows a cell to handle increased protein biosynthesis through adaptation of ER chaperone capacity (175). An increase in the expression of ER chaperones in activated T cells may be necessary for numerous cellular activities, including proper folding of effector cytokines. Induction of ER chaperones has been shown to have both beneficial and negative consequences in development of diseases. For example, ER chaperones have been shown to ameliorate the accumulation of misfolded proteins to protect against the neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease (176). However, upregulation of ER chaperones has been shown to promote cancer progression by preventing ER stress-induced cell apoptosis in tumor cells (176). Therefore, modulation of ERSR and ER chaperone induction in T cells

may serve as a target for therapeutic invention to enhance or inhibit the immune response.

Besides playing a role in disease prevention and development, a requirement for physiological ERSR signaling has been shown in cellular activation and differentiation. In pancreatic beta cells, physiological functioning requires a regulated activation of IRE1, a major ERSR upstream molecule (43). Additionally, B cell differentiation into antibody-secreting plasma cells requires physiological activation of ERSR signaling to handle increased immunoglobulin assembly (11,39,40). Our findings in T cells support these observations that the function and activation of normal cellular activities induces and requires physiological ERSR initiation. Our research also indicates the ERSR did not differ in magnitude between T cells that are fully or partially activated, suggesting a preparatory function for ERSR induction. Further investigation into physiological ERSR signaling in T cells destined to develop an effector or tolerant phenotype may reveal differences that regulate cellular progression.

As aforementioned, the physiological ERSR is activated to allow a cell to handle normal stress within the ER. If ER stress conditions that disturb ER homeostasis beyond restoration persist, pathological ERSR signaling is initiated to eliminate damaged cells (169). In Chapter VI we link absence of Gimap5, a GTPase that modulates TCR signaling and participates in maintenance of T cell homeostasis, to ER stress-induced apoptosis through pathological ERSR activation. Therefore, in addition to a role in physiological ERSR activation, deregulation of TCR signaling through Gimap5 protein absence initiates apoptosis through pathological ERSR induction. This may occur through loss of

TCR regulation by Gimap5 and the interactions of both the TCR and Gimap5 with Bcl-2 family members (109). Because CHOP induction leads to apoptosis through Bcl-2 downregulation (31), and absence of functional Gimap5 leads to an increase of CHOP protein, TCR deregulation caused by Gimap5 absence may initiate pathological ER stress. However, the possibility still exists that loss of TCR regulation by absence of Gimap5 affects Bcl-2 family members independent of CHOP induction. In general, these data indicates a dual role for TCR signaling and regulation in activation of both physiological and pathological ERSR induction.

Normally, T cell development in the thymus involves a series of differential TCR interactions with thymic stromal cells (64,66). This process also mediates the survival-or-death decision of thymocytes through the principles of positive and negative selection (109). Our data indicates that ERSR signaling may play only a minimal role in thymocyte development because ER chaperone expression remains low. However, our data also indicates that peripheral T cells have an established pathological ERSR that is activated by loss of functional Gimap5 protein, a TCR regulator molecule. Consequently, pathological ERSR signaling that leads to ER stress-induced apoptosis may therefore play a role in various TCR-mediated activities, such as activation-induced cell death (AICD). Identifying a correlation between AICD and pathological ERSR signaling may provide another outlet for immune intervention due to the critical role AICD plays in eliminating self-reactive T cells.

Despite the pathological ERSR signaling that occurs in T cells from the Gimap5$^{-/-}$ BBDP rat, a subset of T cells survive to a mature stage. It is these remaining T cells that

are also responsible for infiltrating the islets and specific destruction of beta cells in the BBDP rat. Previous data indicates that antigen activation of peripheral T cells from the Gimap5$^{-/-}$ BBDP rat rescues the cells from programmed cell death (140). This information combined with our data presented in both Chapter V and VI suggests that T cell activation, a known physiological ERSR inducer, has the capability of downregulating pathological ER stress, as not all T cells die in the Gimap5$^{-/-}$ BBDP rat. Thus, in T cells there may exist a dynamic relationship that balances physiological and pathological ER stress. The mechanisms that govern this relationship remain in question not only in T cells, but also in the majority of cell types that utilize ER stress to accomplish cellular activities.

In spite of these remaining questions, our work indicates that ERSR induction may play multiple roles in TCR signaling and regulation. We have shown that T cells are capable of activating both a physiological and a pathological ERSR, suggesting the cellular machinery for ERSR induction is not limited to professional secretory cells. As investigations further our knowledge into the role of the physiological ERSR in T cells, we may uncover novel means of regulating T cell responses to antigens. Additionally, control of pathological ERSR induction may provide a means to limit an ‘excessive’ immune response, *e.g.*, as in certain inflammatory or autoimmune diseases. In the future, the role of PKC signaling should be investigated in T cells to determine the exact function of this molecule in ERSR induction. To explore a link between the ERSR and the downstream signaling of PKC, experiments involving the inhibition of the transcription factor NF-κB through siRNA or inhibitors should be performed. It will also

be important to examine the role of Gimap5 in TCR regulation to investigate whether deregulated TCR signaling leads to cell death through ER stress-induced apoptosis. To accomplish this, components of the TCR signaling pathway that have been found to be deregulated in T cells from the Gimap5$^{-/-}$ BBDP rat should be examined for a link to ER stress and CHOP induction. Overall, advancements into the molecular mechanisms behind ERSR activation in T cells may provide potential targets for therapeutic intervention in immune-related diseases.

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