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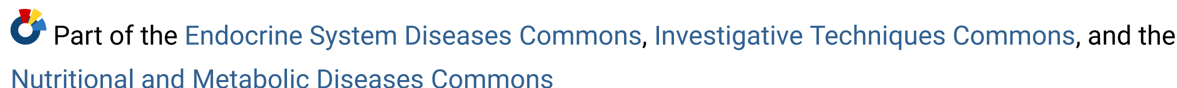

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To the Graduate Council:

I am submitting herewith a thesis written by Carlie Joelle Frydman entitled "Autoimmune-Mediated Beta-Cell Death & Dysfunction: Potential Role of Signaling through the Fas Receptor." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

J. Jason Collier, Major Professor

We have read this thesis and recommend its acceptance:

Michael D. Karlstad, Jay Whelan

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Autoimmune-Mediated Beta-Cell Death & Dysfunction: Potential Role of Signaling through the Fas Receptor

A Thesis
Presented for
the Master of Science Degree
The University of Tennessee, Knoxville

Carlie Joelle Frydman
August 2012

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is an endocrine disorder that continues to afflict a growing proportion of the U.S. population. Characterized by an autoimmune attack on the pancreatic [beta] cells that leads to their destruction, T1DM develops from absolute insulin deficiency resulting in chronic hyperglycemia. Although the disease requires lifelong insulin therapy and confers enhanced risk for long-term complications, the mechanism of [beta] cell death remains unclear. Fas receptor signaling is critical among cells of hematopoietic origin for its role in immune homeostasis and mediation of target cell death. Fas receptor-ligand interactions might also have a role in [beta] cell death leading to the development of T1DM; pro-inflammatory cytokines released from islet leukocytes can induce Fas receptor to the [beta] cell surface, and systemic loss-of-function mutations in Fas receptor and Fas ligand (FasL) abrogate disease in spontaneous diabetes-prone mice. However, systemic deficiency in Fas and FasL causes an alteration in the T cell repertoire that prevents diabetes, and thus cannot be attributed to absence of Fas [beta] cell signaling. Moreover, the use of distinct Fas mutations and transgenic models that produce dissimilar mechanisms of [beta] cell death leads to conflicting results reported in the scientific literature. Recent evidence using transgenic mouse models of diabetes has indicated a role for Fas in the insulitic phase but not the effector phase of [beta] cell death, while other studies have suggested that alteration of the T cell repertoire by Fas signaling is a causal factor in the autoimmune [beta] cell attack. Furthermore, ectopically-expressed FasL is a potential therapeutic tool for protection of islet transplants by its known ability to provide immune privilege in some tissues. This literature review collectively presents the diverse roles for Fas signaling in [beta] cell death and provides insight into why conflicting conclusions regarding Fas signaling currently exist. Thus, the goal of this literature review is to enable investigators interested in Fas-mediated signaling in the pancreatic [beta] cell to choose an appropriate model system for study design that ideally will translate to therapeutic interventions for T1DM.

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List of Abbreviations:

AAV, adeno-associated virus vector; Ab, antibody; AdFasL, replication-deficient adenoviral vector containing FasL; AICD, activation-induced cell death; ALPS, autoimmune lymphoproliferative syndrome; APAF-1, apoptotic protease activating factor-1; APC, antigen-presenting cell; APO-1, apoptosis antigen 1; BCR, B cell receptor; CD95/APO-1, Fas; cFLIP, cellular FLICE-like inhibitory protein; $cFLIP_L$, cFLIP long; CTL, cytotoxic T lymphocyte; CY, cyclophosphamide; DC, dendritic cell; DD, death domain; DN, double-negative; dnFADD, dominant-negative Fas-associated death domain; DP, double-positive; DR, death receptor; ETn, early transposable element; FADD, Fas-associated death domain; FasL/CD95L/APO-1L, Fas ligand; GDM, Gestational diabetes mellitus; GSIS, glucose-stimulated insulin secretion; HA, influenza virus hemagglutinin; hFas, human Fas; HIP, human insulin promoter; HLA, human leukocyte antigen; ICE, interleukin-1β-converting enzyme; IDDMI, insulin-dependent diabetes mellitus locus; IFN-γ, interferon gamma; IL-10R, interleukin-10 receptor; IL-1R, interleukin-1 receptor; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LCMV, lymphocytic choriomeningitis virus; lpr^{cg}, *lpr* complementing *gld*; LTR, long terminal repeat; mFasL, membrane-bound FasL; MLDS, multiple low-dose streptozotocin; MOMP, mitochondrial outer membrane permeabilization; MTCH2/MIMP, mitochondrial carrier homologue 2/Met-induced mitochondrial protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NHP, non-human primate; NK, natural killer; NMMA, N-methylarginine; NO, nitric oxide; NOD, non-obese diabetic; OVA, ovalbumin; RIP, rat insulin promoter; *scid*, severe combined immunodeficiency; sFasL, soluble FasL; siRNA, small interfering RNA; SLE, systemic lupus erythematosus; Smac/DIABLO, second mitochondria-derived activator of caspase/direct IAP binding protein with low pl; SNP, sodium nitroprusside; SOCS-1, suppressor of cytokine signaling-1; SP, single positive; T_C, cytotoxic T cell; TCR, T-cell receptor; T_H, helper T cell; TNF-R, tumor necrosis factor receptor; TNF-α, tumor necrosis factor alpha; T1DM, Type 1 diabetes mellitus; T2DM, Type 2 diabetes mellitus; wt, wild-type; XIAP, X-linked inhibitor of apoptosis;

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CHAPTER I

General Overview

I. Characterization of Diabetes Mellitus

Diabetes mellitus is the umbrella term for the most common endocrine disorder affecting people in the United States. The rise in prevalence of this disease is accompanied by numerous studies devoted to understanding the molecular mechanism of action with the hope of generating novel therapeutic interventions. A much larger proportion of people now know at least one individual affected by diabetes and are dedicated to increasing awareness in an effort to gain funding for diabetes research. To appreciate the implications of living with diabetes and the critical need for effective therapies, it is important to understand the clinical characterization and pathogenesis of the disease.

Two major forms of diabetes exist: These are Type 1 (T1DM) and Type 2 (T2DM) and they share a common phenotype of overall losses in functional β-cell mass leading to hyperglycemia. Diminution of the pancreatic β-cell mass results in insulin deficiency. Insulin is a hormone secreted by β-cells of the pancreatic islet of Langerhans, and is critical to blood glucose homeostasis via its ability to promote cellular glucose entry and exit from the circulation. Insulin secretion is controlled by a number of different fuel substrates, with glucose providing the biggest overall effect. Glucose-stimulated insulin secretion (GSIS) is directed by a complex interaction of multiple metabolic pathways that begins with β-cell glucose entry and metabolism, and concludes with exocytosis of insulin-containing granules [1]. Loss of endogenous insulin signaling leads to alterations in fuel metabolism in a variety of tissues, and drastically impacts blood glucose homeostasis; the clinical manifestation is development of diabetes mellitus.

T2DM is the most prevalent form of diabetes, accounting for over 90% of total cases [2]. Although most commonly diagnosed in adults over 40 years of age, T2DM incidence continues to

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increase in young adults and adolescents. Development of the disorder is initiated with insulin resistance, whereby a decrease in insulin-mediated glucose uptake causes the pancreatic β-cell to respond by expanding its mass and increasing insulin secretion. However, once the β-cell can no longer expand or secrete sufficient amounts of insulin to match the rising insulin demands, occurrence of β-cell deficiency leads to impaired glucose tolerance and overt T2DM [3]. A number of factors are associated with increased risk for development of T2DM, and include an individual's genetic background as well as environmental factors. For example, visceral fat accumulation has progressively increased among the US population in parallel with T2DM incidence. Although insulin resistance is the initial step in development of T2DM, only about one-third of obese insulin-resistant individuals progress to overt T2DM; only when the β-cell can no longer meet the body's insulin demands do chronic hyperglycemia and diabetes develop.

Gestational diabetes mellitus (GDM) is a form of diabetes that is significantly less prevalent than either T1DM or T2DM, affecting approximately 7% of pregnant women, but whose pathogenesis is similar to T2DM. GDM is most commonly diagnosed in the third trimester of gestation and can be treated with dietary intervention and exercise, although insulin therapy may be required for more severe cases. Although the disorder often resolves at birth, women treated for GDM are at a significantly increased risk for development of T2DM, and to a much lesser extent T1DM. Maternal GDM increases the offspring risk for adverse outcomes, most notably macrosomia [4]; intrauterine exposure to hyperglycemia has also been suggested to create metabolic memory that results in T2DM development later in life. Indeed, children born to mothers with GDM have a significantly enhanced risk for development of T2DM relative to children born to mothers who do not carry the disorder.

T1DM is less prevalent than T2DM, yet currently affects approximately 900,000 people in the United States and is increasing at an alarming rate. Over 75% of these cases are onset at the age of 18 or younger, hence the antiquated terminology of Juvenile onset diabetes. T1DM develops when an

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individual's immune system loses the ability to differentiate foreign microorganisms from self [5]; this results in immune dysregulation and expansion of autoreactive CD4+ and CD8+ T cells, as well as B lymphocytes and other cells of hematopoietic origin. The disorder is characterized by complete insulin deficiency secondary to autoimmune-mediated β-cell death, which occurs via a two-step process **(Figure 1)**. The first phase is known as insulitis, in which dendritic cells, macrophages, and lymphocytes (most abundantly $CD8^+$ T lymphocytes), infiltrate the islet of Langerhans and cause inflammation upon release of pro-inflammatory cytokines. The islet of Langerhans contains multiple cell types: α, β, δ, ε, and PP cells, which exhibit endocrine function by their secretion of hormones directly into the bloodstream. Interestingly, however, T lymphocytes selectively target β-cells for death, leaving all other cell types residing within the islet free from the cytotoxic effect of these autoreactive cells. The second and final effector phase of β-cell death is characterized by immune cell-mediated β-cell destruction through signaling mechanisms that are still unclear [6-8]. Progression of β-cell death is variable in duration, and clinical symptoms generally begin appearing after the loss of approximately 70-80% of β-cells [9]. Typical

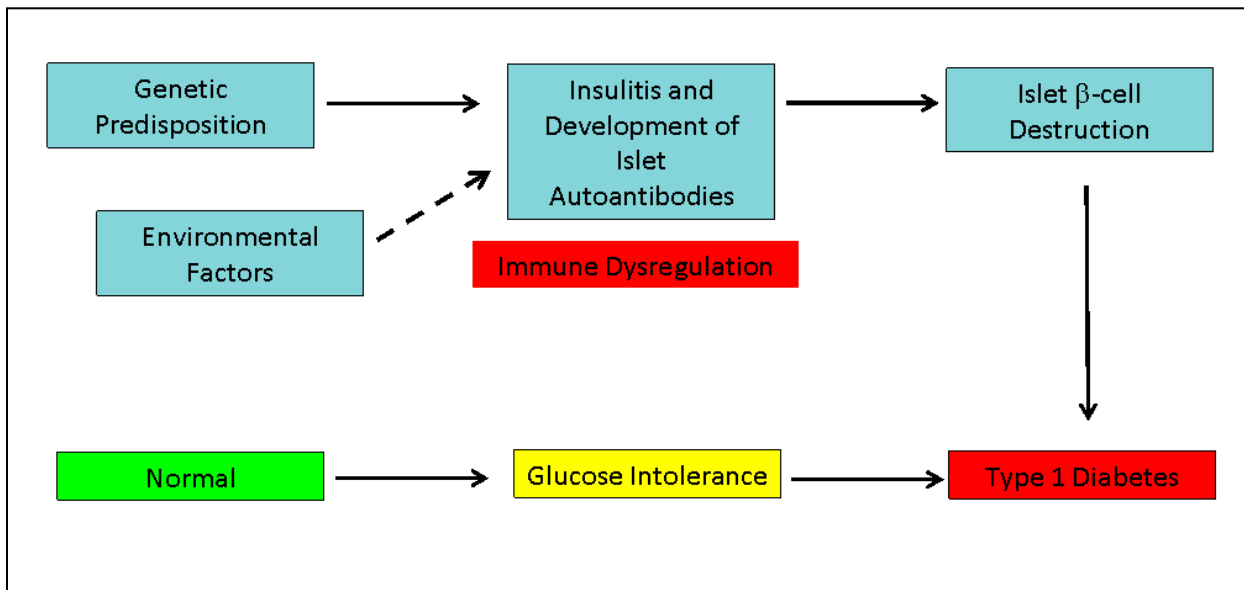

Figure 1. Schematic that diagrams progression of the islet β-cell to autoimmune-mediated death, and leads to development of overt Type 1 diabetes mellitus.

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signs of chronic hyperglycemia most commonly include polyphagia, polyuria, polydipsia, weight loss, and lethargy [10].

After diagnosis of T1DM, treatment involves achieving a critical balance between insulin administration and food intake; the ultimate goal is to minimize fluctuations in self-monitored blood glucose levels, that when uncontrolled, enhance the risk for long-term complications. Insulin was discovered as the secreted pancreatic factor required to cure the disease symptoms [11] and indeed, insulin administration is still the only therapy available to treat Type 1 diabetes. For a number of years, subcutaneous injection multiple times daily was the only method of insulin administration. However, alternative routes of insulin administration have recently become available and provide the capability for tighter regulation of blood glucose levels, in addition to other factors. For example, the insulin pump allows for a constant basal insulin drip and is connected subcutaneously, so lessens the invasiveness of the treatment conferred by insulin injection. Furthermore, the pump may offer a way to maintain glycosylated hemoglobin (HbA1c) in a narrower range; HbA1c is a good measure of long-term average blood glucose levels. Importantly, the ability to maintain blood glucose levels near the normal range (70-100 mg/dL) significantly lessens the risk for long-term complications [12]. Individuals with poorly controlled diabetes mellitus are at an enhanced risk for a plethora of long-term complications, including nephropathy, neuropathy, retinopathy, amputation, heart disease, and earlier than average mortality rates [13].

II. Predisposing Factors that Affect Risk for T1DM

Some genes have a significant association with T1DM, and can have a role in either protection or enhanced vulnerability for the disorder. The human leukocyte antigen (HLA) is a region on chromosome 6p21 that is also known as insulin-dependent diabetes mellitus locus (IDDMI). Susceptibility loci found on the HLA region account for nearly 45% of genetic vulnerability for T1DM, more than any other

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associated gene, although more than 30 non-HLA regions have also been shown to affect risk for the disease [14]. HLA genes can also be used as markers of risk for T1DM, in combination with the presence of autoantibodies. This genetic link associated with vulnerability for T1DM is exemplified by family members of a Type 1 diabetic who themselves are also at enhanced risk for diabetes [15].

External precipitating events are just as noteworthy as genetic background in regards to triggering an autoimmune response towards the pancreatic islet. However, a more complex model than one single environmental factor might be required to explain disease onset; indeed, T1DM progresses through multiple stages, each of which could be influenced by one or multiple environmental triggers [16]. A number of environmental agents have been associated with enhanced risk for the disease; moreover, the rising incidence of the disease suggests that there is a corresponding increase in exposure to such factors. Viral infection is one such factor, and has been heavily investigated for correlation with T1DM. Indeed, the seasonal peak of enterovirus infections parallels a seasonal rise in incidence of T1DM [9]. Specifically, antibodies against the Coxsackie B family of enteroviruses have been identified as being present in recent-onset T1DM patients. However, direct measurement of intra-islet viral presence is difficult to attain in a non-invasive fashion. Although viral infection is the most well-studied non-genetic factor associated with vulnerability for T1DM, numerous others have also been suggested to have the ability to modify disease risk. These include climate, vaccine administration, psychological stress, early infant diet, exposure to dietary or environmental toxins, sun exposure, and population hygiene [17].

III. Prevention/ Interventions against Overt T1DM

In recent years, a widespread effort has been focused on prevention of T1DM in pre-diabetic individuals, in addition to disease-abrogating therapies targeted towards overt diabetics. Recent-onset diabetes patients are the most frequent population that such therapies have been tested on. A number of interventions that have been successful in animal models of spontaneous diabetes have been further

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investigated in human clinical trials, but none have yet seen permanent remission from disease and such trials confer a number of limitations. There are currently no biomarkers that correlate with disease progression, and endpoints for clinical trials are often limited to analysis of β-cell function after a pre-defined period of time [16]. Furthermore, non-invasive techniques to analyze β-cell mass have not yet been developed, which prevents direct measurement of therapy effectiveness within the islet. Stem cell transplantation and islet transplantation are noteworthy potential therapies for T1DM, but outcomes with these have been overall unsuccessful; the transplanted islets end up susceptible to the same destructive autoimmune attack as the endogenous islets. Although overt diabetics transplanted with healthy islets or pancreata have remained insulin-free for a period of time, they often endure a rigorous regime of immunosuppressant drugs to prevent autoimmune attack on newly-transplanted cells [18].

Prevention trials are of interest in the diabetes community, especially among individuals identified as high-risk in regards to T1DM development. First-degree relatives of a Type 1 diabetic who test positive for one autoantibody have a less than 20% risk for disease development, whereas the presence of two or more circulating autoantibodies confers a 90% risk [16]. However, as in trials for overtly diabetic individuals, prevention trials have also presented with disappointing results. Participant recruitment requires screening of a large number of people, since autoantibody frequency among relatives of Type 1 diabetics is only about 3.5%. Moreover, individuals who do enroll in such trials must not have already undergone islet autoimmune attack, and experimental conditions must be exact in regards to dosing of the given experimental agent [16]. Before implementation of a successful prevention for pre-diabetics or therapy for overt diabetics, a number of barriers need to first be overcome. Centrally, the autoimmune trigger and mechanism of β-cell death are still unclear; without a comprehensive understanding of either of these factors, it will be extraordinarily difficult to devise an effective intervention.

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Elucidation of the β-cell death mechanism has proven challenging to investigators; although it still remains unclear, a number of proteins and pathways have been suggested to have a role in the process. Controversy resounds in both the proposed intracellular signaling pathway and whether this pathway should be classified as an apoptotic or non-apoptotic event [19-21]. Two signaling pathways in particular have been investigated for a role in β-cell death; Fas receptor and the perforin/granzyme pathway have critical function in maintenance of immune homeostasis and in mediation of target cell death. Although research into the role for perforin/granzyme in development of T1DM has been relatively consistent in that it likely mediates the effector phase of β-cell death, the role for Fas is more obscure. The signaling pathway initiated by interaction between Fas and Fas ligand (FasL) has been shown to be both dispensable and indispensable for β-cell death and development of T1DM, although a number of separate genetic backgrounds, mutations, and transgenic models have been used to derive these results. This is significant in that each of these model systems might confer separate mechanisms of β-cell death, and could explain the inconsistencies in Fas β-cell research. This literature review was undertaken with the intent of reconciling the current state of the Fas β-cell death field. Extensive searches were conducted to compile all relevant literature **(Appendix: Figure 3, Tables 3 & 4)**, which was subsequently organized and analyzed based on each group's choice of model system to test a similar hypothesis. Each mutation or transgene used in Fas β-cell literature was examined for reported findings, in addition to strengths and limitations in the context of usefulness for investigation of Fas-mediated β-cell death. To date, there is no single document that summarizes the research relevant to Fas signaling in β-cell death; this document will be innovative in that it will be the first to do so, and will provide a comprehensive reference for researchers interested in investigating the role for Fas-FasL interactions in β-cell death and development of T1DM.

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CHAPTER II

Death in the FAS Lane: A Role for Fas Receptor Signaling in Pancreatic β-Cell Death?

I. Introduction

Type I diabetes mellitus (T1DM) is a chronic autoimmune endocrine disorder that continues to increase in incidence, and confers an enhanced risk for a number of long-term health consequences. Although innumerable groups have continued for years to investigate its pathogenesis, the mechanism of how immune-mediated β-cell death occurs is still unclear. A number of proteins capable of mediating cell death have been implicated in this process [22], one of which is the Fas receptor (also known as CD95/APO-1). Interaction between Fas and its respective Fas ligand (FasL/CD95L/APO-1L) has already been established as critical for homeostasis among cellular populations of the immune system, as well as in maintenance of immune privilege in some tissues. Because FasL is expressed on the surface of activated lymphocytes, it is possible that FasL on autoreactive islet-infiltrating cells could mediate pancreatic β-cell death through interaction with β-cell surface Fas. The ability of Fas to be induced to the β-cell surface strongly supported this notion [23], and was further investigated with the demonstration that diabetes-prone mice with a systemic deficiency in Fas or FasL are protected from diabetes development [24, 25]. However, the absence of Fas-FasL interactions for maintenance of immune system equilibrium results in a number of physiological abnormalities that affect its natural response to an autoimmune trigger.

The problems inherent to a systemic Fas deficiency were circumvented by multiple groups that generated new methods for investigating Fas-FasL interactions in the islet β-cell, without depleting its function in hematopoietic tissues. However, this resulted in a field comprised of animals and cell lines from separate genetic backgrounds coupled with knockout mutations and transgenic overexpression, of which together form an extraordinarily convoluted role for Fas as an effector of β-cell death.

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If Fas is indeed critical for β-cell death, interruption of the interaction between β-cell Fas and immune cell FasL should confer a level of protection against β-cell death and ultimately development of T1DM. This review aims to characterize the role of Fas-FasL interactions in both physiological homeostasis and in β-cell pathology leading to T1DM, and will investigate the proposed links between its known homeostatic functions and its potential ability to mediate autoimmune pathogenesis leading to T1DM. Additionally, we will identify and relate the studies of Fas/FasL on β-cell death by presenting their findings, inconsistencies in relation to each other, and alternative approaches for therapeutic use of Fas-FasL in protection of the β-cell from autoimmune-mediated death. From these findings, future investigators interested in the role for Fas and FasL in β-cell death will be better equipped to choose an appropriate model system for derivation of findings that could ultimately translate to therapeutic interventions.

II. Characterization of Fas & FasL

General Mechanism of Action

Discovery of the Fas receptor was preceded by the synthesis of two monoclonal antibodies that recognized the same target receptor; both had cytolytic activity in a variety of human cell types, and were termed anti-APO-1 (apoptosis antigen 1) and anti-Fas [26, 27]. Isolation of cDNAs encoding the antigen to anti-Fas led to identification and characterization of the Fas receptor [28], whose gene lies on human chromosome 10q24.1 [29], and on its homologous mouse counterpart chromosome 19 [30]. Characterization of the APO-1 antigen and its nucleotide sequence revealed that it lay on chromosome 10q23 [31] and shares sequence identity with Fas antigen gene [26]; hence, the terms Fas and APO-1 are often used interchangeably.

The Fas protein is a trimeric receptor that is a member of the death receptor (DR) subfamily and is part of the larger tumor necrosis factor receptor (TNF-R) superfamily [32]. Members of the DR

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subfamily share a cysteine-rich extracellular domain and a cytoplasmic region, which is referred to as a death domain (DD), and is required for apoptotic signal transduction upon receptor activation [33, 34]. Additionally, the receptor is characterized as a type I transmembrane protein due to the location of its extracellular N-terminal and intracellular C-terminus [28]. The cytolytic activity initiated by binding of anti-Fas to its receptor suggested that Fas might have a natural ligand which communicates an apoptotic signal. Indeed, the Fas ligand (FasL/CD95L/APO-1L) was first identified on the surface of a cytotoxic T cell hybridoma by use of soluble Fas antigen [35], and soon after confirmed to be the ligand specific for Fas receptor; activation of the receptor induced cytotoxic activity against Fas-expressing cytotoxic T lymphocyte (CTL) hybridomas but not against those lacking Fas activity [36].

FasL is a member of the TNF family whose gene resides on human and mouse chromosome 1 [37, 38], and is classified a type II transmembrane protein due to the presence of an internal hydrophobic domain and a COOH-terminal region that resides outside the cell [8]. Although it occurs naturally in a membrane-bound form (mFasL), mFasL cleaved by metalloproteinases results in a soluble form of the ligand (sFasL), which can exist as a trimer [39, 40]. Whereas sFasL can still bind Fas, mFasL is a much more potent stimulus for cytotoxic activity than sFasL. [41-43]. In fact, sFasL has been shown to promote autoimmunity, tumorigenesis, and protection from the apoptotic effects of mFasL [44, 45], although it can still function as a death effector [40].

Fas-FasL interactions occur in an autocrine or paracrine fashion through one of three types of interactions [8]:

1. *trans* interaction; Fas and mFasL, expressed on the surface of distinct cells, cross-link to mediate death of the Fas-expressing cell.

2. *cis* interaction; both Fas receptor and mFasL are expressed on the surface of the same target cell.

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3. sFasL binds to Fas expressed on the same cell sFasL is released from (*cis*), or sFasL binds to Fas on the surface of a neighboring cell (*trans*).

Binding of FasL to its receptor induces trimerization of Fas and initiates a conformational change within the cytoplasmic death domain that promotes association of an intracellular Fas-associated death domain (FADD), pro-caspase-8, and the caspase-8 regulator cellular FLICE-like inhibitory protein (cFLIP); these together comprises the death-inducing signaling complex (DISC). Although cFLIP is most commonly an anti-apoptotic molecule, the cFLIP long ($cFLIP_L$) isoform can support cell death [46]. The FADD promotes oligomerization of procaspase-8 and through autoproteolysis is subsequently released as active caspase-8 into the cytoplasm [46]. At this stage of the apoptotic pathway, the type of cell in question determines how the pathway will proceed; this can be either directly through an extrinsic pathway (type I cell) or indirectly through an intrinsic mitochondrial pathway (type II cell) [47]**(Figure 2)**.

Cell types such as thymocytes, activated T cells, and B lymphoma cells characterize the type I cell

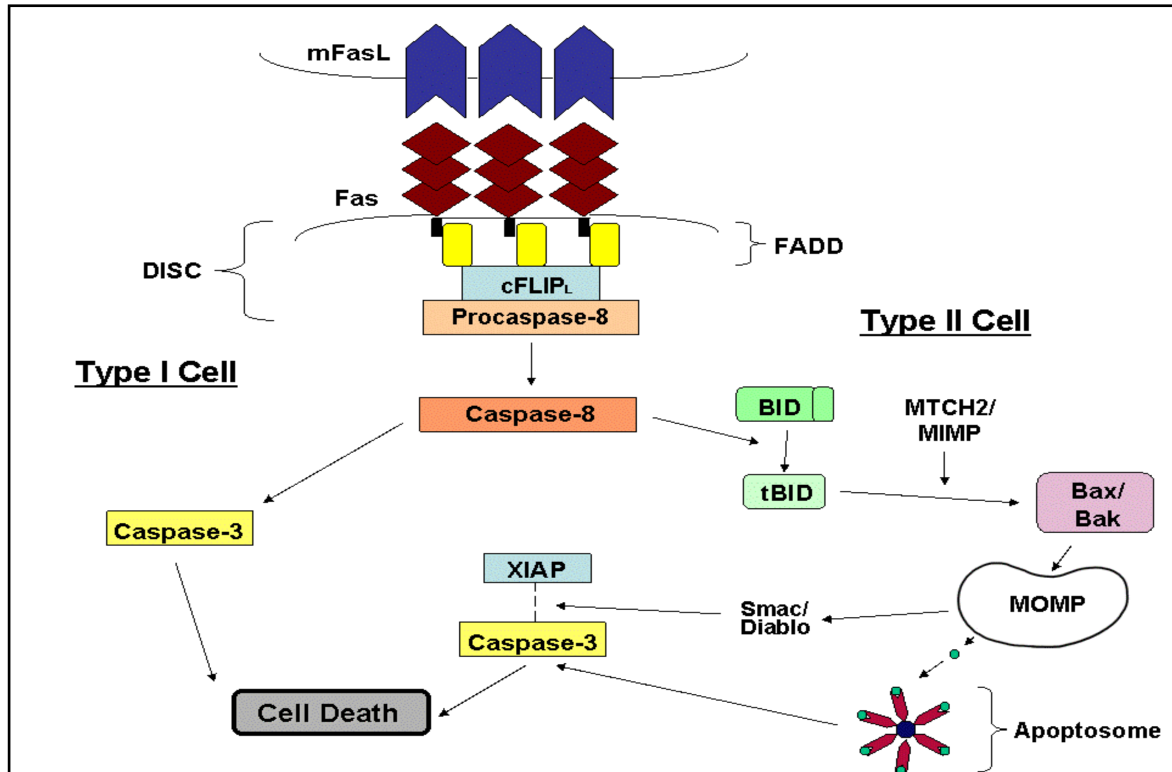

Figure 2. Schematic representation of the Fas-induced signaling pathway that leads to AICD and target cell death.

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[48], whereas hepatocytes and β-cells are classified as type II cells [49, 50]. X-linked inhibitor of apoptosis (XIAP) is the only member of the mammalian IAP protein family that directly inhibits caspases and acts on the effector caspases -3 and -7 [51]. This mechanism discriminates between type I and type II apoptosis, because the absence of XIAP causes hepatocytes to undergo apoptotic death independent of the mitochondrial pathway [52, 53]. In contrast with type I cells, type II cells have much lower levels of Fas-dependent DISC formation and subsequent caspase-8 activation [46]. The limited availability of active caspase-8 in type II cells prevents accumulation of adequate quantities of cleaved caspase-3 for facilitation of apoptosis; such quantities of caspase-8 are, however, sufficient to cleave Bid and allow type II cells to progress through the intrinsic mitochondrial pathway [54, 55].

Downstream of caspase-8 activation, type II cells proceed through a mitochondrial pathway initiated with caspase-8-mediated cleavage of the BH3-only protein Bid. Newly-cleaved tBid, facilitated by interaction with mitochondrial carrier homologue 2/Met-induced mitochondrial protein (MTCH2/MIMP), translocates to the mitochondrial outer membrane to activate the pro-apoptotic proteins Bax/Bak [56]. Bax/Bak mediate mitochondrial outer membrane permeabilization (MOMP), releasing a second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (Smac/DIABLO) [57], cytochrome c, and other apoptogenic factors from the mitochondria. In the presence of ATP, cytochrome c binds apoptotic protease activating factor-1 (APAF-1) to form the apoptosome; recruitment of inactive caspase-9 to the apoptosome leads to its autoactivation, allowing it to cleave caspase-3 [58]. Additionally, the XIAP antagonist Smac/DIABLO promotes caspase-3 cleavage by blocking XIAP-caspase-3 binding [51, 53]**(Figure 2)**.

By contrast, type I cells use caspase-8 to directly cleave and activate caspase-3 [53]. Active caspase-3 induces proteolysis of structural proteins and other essential cellular components, leading to the hallmark characteristics of apoptosis. Although Fas-FasL interactions are most well-characterized for

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their apoptotic activities, survival pathways may also be initiated; however, these concepts are beyond the scope of this article (see Ref. [59-62] for further review).

Constitutive Expression

The Fas receptor is constitutively expressed in a variety of tissues throughout the body, with abundant expression in the thymus, liver, ovary, and heart [30]. Fas mRNA expression has also been demonstrated in thymocytes, where activation of mature T and B cells promotes Fas cell surface induction [63, 64].

Because of its ability to induce death in Fas-expressing cells, FasL tissue expression is tightly controlled. FasL mRNA is constitutively expressed in a select range of tissues, centrally splenocytes and thymocytes, and its cell surface expression can be induced after activation of T lymphocytes and natural killer (NK) cells [35, 39]. FasL mRNA has also been demonstrated at low levels in mouse lungs, small intestine, kidney, and liver [65, 66], and the protein is constitutively expressed on the cell surface of immune-privileged tissues such as the eye, testes, and thymus [66, 67].

FasL assists in maintaining immune privilege in a number of tissues, where its constitutive expression at the surface of such sites can induce apoptosis in activated Fas^+ infiltrating lymphocytes [66-68]. Immune privilege is generally defined as a situation in which a tissue is protected from immune infiltration, even during foreign invasion, as the proinflammatory immune response would be more destructive than its foreign target [69]. The eye is the most well-characterized site of immune privilege and multiple studies support a role for FasL in prevention of immune infiltration in the eye [66, 67]. Additionally, the cornea has an 80-90% transplantation success rate with no immunosuppressant use [69], and FasL expression is required for corneal graft survival [70]. Studies in the testes have shown similar results; loss of FasL expression in testicular grafts transplanted under mouse kidney capsule causes them to undergo immune infiltration and rejection, yet transplants that express FasL can survive [68].

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The concept of immune privilege has also been extended to tumor cells that express FasL and are therefore resistant to lymphocyte invasion, supporting a role for FasL in tumorigenesis [71, 72]. Inhibition of FasL expression in murine colon tumor cells suppresses tumor growth and formation and increases lymphocyte infiltration [73], indicating that tumor FasL expression encompasses both defensive and offensive strategies for tumor survival. However, controversy surrounds the role of FasL in tumor immune escape, likely stemming from transplantation studies in which FasL overexpression can cause rejection of tissue transplants [72]; although beyond the scope of this article, the interested reader is directed to Ref. [74] for further review.

Role in Immune Homeostasis

Immune homeostasis is known as the capability of an organism to maintain equilibrium among cells of hematopoietic origin. One of the important roles for Fas-FasL interactions is in maintenance of immune homeostasis by preventing overgrowth in this cellular population, and is particularly significant for $CD4^+$ and $CD8^+$ T lymphocytes [75-77]. $CD4^+$ T cells are referred to as helper T (T_H) cells and shape adaptive immune responses by producing cytokines and enhancing expression of costimulatory factors. $CD8^+$ T cells, in contrast, are known as cytotoxic T (T_C) cells due to their direct role as cytotoxic effectors against target cells, but are also capable of cytokine production [78].

To mount an appropriate T cell-mediated response, the immune system first requires a method of antigen recognition. This is accomplished by a high-affinity interaction between the T-cell receptor (TCR)/CD3 complex with MHC-associated antigenic peptide [79]. Appropriately regulated lymphocyte growth and maintenance are critical for prevention of recognition of self antigens and initiation of autoimmunity.

Differentiation from a multipotent hematopoietic stem cell to mature T cell is a complex process that requires a coordinated effort by various transcription factors and regulatory mechanisms. Although they originate in the bone marrow, T cell precursors migrate to the thymus to undergo maturation as

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thymocytes and can eventually become self-restricted single positive (SP) $CD8^+$ or $CD4^+$ T lymphocytes [80].

On arrival to the thymus, T cell precursors do not express any of the characteristic cell surface markers of a mature T cell; additionally, approximately 95% of mouse thymocytes express the Fas protein on their cell surface [81]. A period of rapid proliferation continues through the first two of four double negative (DN) stages of development ($CD4^- CD8^-$), all of which are characterized by the coordinated expression of specific cell surface markers [82]. DN2 thymocytes begin the rearrangement of a T cell receptor TCR-β chain and conclude the process of somatic recombination upon progression to the DN3 stage [79, 83].

Progression to the DN3 stage indicates commitment to the T cell lineage [80], and the DN4 stage is a period of rapid proliferation preceding transition to double positive (DP) thymocyte which confers cell surface expression of both the CD4 and CD8 coreceptors [84]. TCR-α chain rearrangement, occurring at the conclusion of this final proliferative stage, marks TCR completion and movement to the rigorous process of thymic selection, from which only 5% of the original thymic population emerge as mature T cells [84]. Thymic selection comprises three checkpoints that must all be cleared for a thymocyte to become a mature T cell: death by neglect, positive selection, and negative selection.

Death by neglect occurs alongside positive selection, and is a passive form of cell death in which a cortical thymocyte fails to receive necessary signals for survival, as its TCR has not ligated with MHC-self peptide. The vast majority of cortical thymocytes, approximately 90%, die during positive selection, specifically by failing to receive survival signals that are transmitted upon cross-linking of TCR with MHC-self peptide [84].

Characterized by MHC restriction, positive selection ensures that the mature T cell can recognize self-MHC complexes, a necessary quality for eliciting an immune response against a foreign antigen. TCR expression enables low-affinity binding with MHC Class-I or Class-II bound with self peptide, which must

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be recognized by the DP thymocyte. Thymocyte TCR cross-linking with MHC-self antigen promotes thymocyte survival and movement to the next stage of thymic selection [82].

In contrast to positive selection, negative selection only clears approximately 5% of the initial thymocyte pool [84] but is fundamental to prevention of an autoimmune response. Negative selection is characterized by high-affinity engagement of the thymocyte TCR with self-MHC from dendritic cells (DC) or medullary epithelial cells, resulting in apoptotic death; only moderate-affinity interactions between TCR and MHC-self peptide allow for survival during the negative selection process.

Although not critical for thymic negative selection [85-87], Fas-FasL interactions are indispensable for peripheral selection, or post-maturation hematopoietic cell deletion, and maintenance of immune homeostasis. This was illustrated by loss-of-function mutations in both Fas receptor and Fas ligand. Lymphoproliferation (*lpr*) is an autosomal recessive mutation in the Fas receptor that was first identified in MRL/MpJ mice and has since been localized to mouse chromosome 19 [88, 89]. Generalized lymphoproliferative disorder (*gld*) is a mutation in FasL that originated in the C3H/HeJ mouse strain [90] on mouse chromosome 1 [38], and although nonallelic to *lpr,* produces nearly identical serological and immunopathological markers as *lpr* on the same mouse strain. These mutations were originally postulated to be involved in metabolic cycling until bone marrow transfer experiments conducted by Allen et al. suggested that they might be mutations in a pair of interacting molecules, such as that of receptor and ligand [91]. Indeed, genetic mapping analysis showed that *lpr* is located in close chromosomal proximity to the Fas receptor [92], and *gld* to that of the Fas ligand [38]. *lpr* is thus a mutation in Fas receptor and *gld* a mutation in Fas ligand, discoveries made through comparison of wild-type (wt) and mutant cells for Fas and FasL gene expression [38, 92].

The *lpr* mutation occurs as a result of premature splicing due to insertion of a early transposable element (ETn) containing two poly-A adenylation sites at the long terminal repeat (LTR) region within intron 2 of the Fas antigen gene [93]. Although initiated normally, Fas mRNA transcription is terminated

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before exon 3, resulting in limited expression and function of the Fas receptor in both MRL/MpJ and CH3/HeJ mice [92]. However, *lpr* is a leaky mutation; Fas receptor can be expressed in *lpr/lpr* mice, at approximately 1-2% of the wild-type level [94]. FasL is constitutively expressed and fully functional in *lpr/lpr* mice [95]. lpr^{cg}, a dominant-negative mutation that is allelic to *lpr*, was originally detected in mutant mice from the CBA/K1 mouse strain [96] and is known as '*lpr* complementing *gld*' due to its ability to induce weaker lymphadenopathy than *lpr/lpr* when the heterozygous *gld/+* mutation is also present [97]. lpr^{cg} also blocks functional Fas expression, but through a point mutation in the cytoplasmic apoptotic signal-transducing region of the Fas gene. Although lpr^{cg} Fas is expressed at similar levels as wild-type, the receptor cannot transmit a death signal and is thus nonfunctional [81, 92].

gld, the loss-of-function mutation in FasL, is characterized by a point mutation within the extracellular region of the FasL [38]. Unlike *lpr*, which limits Fas expression, *gld* still confers FasL expression. And although it has a low level of function, FasL in *gld/gld* mice displays the same constitutive expression pattern as in *lpr/lpr* mice [95, 98].

The physiological and etiological implications of homozygosity for *lpr/lpr* and *gld/gld* are nearly the same. Both mutations incur massive lymphoproliferation and progressive infiltration of the spleen and lymph nodes with nonmalignant double negative (DN) $CD4^-$, $CD8^-$, and $Thy1^+$ T cells, as well as increased cell surface expression of B220, which is normally expressed on B cells, and other abnormal T cell-surface markers [99], all of which manifest in an age-dependent manner [99]. Enhanced production of autoantibodies such as anti-ssDNA, IgG, and IgM [100] in *lpr/lpr* and *gld/gld* mice, links these murine mutations to their closest human syndrome, an autoimmune disease known as systemic lupus erythematosus (SLE) [101]. Another such disorder, autoimmune lymphoproliferative syndrome (ALPS), often confers a mutation in the Fas gene and results in symptoms similar to that seen in *lpr/lpr* mice [102]. Finally, the *lpr* gene is fittingly expressed in bone marrow hematopoietic stem cells [99], the site of origin for the global immune T cell population [8].

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The immunological implications of *lpr/lpr* and *gld/gld*, coupled with the demonstration of Fas receptor expression on the cell surface of activated B and T cells [64, 103], led to the postulation that Fas-FasL interactions play a critical role in immune homeostasis [87, 92, 104]. Studies conducted with both *lpr/lpr* and Fas^{-}/$^{-}$ mice indicate an obligatory role for Fas in peripheral clonal deletion, as evidenced by lymphocyte accumulation in the spleen and lymph nodes of the mutated mice [86, 87, 105]. Additionally, when the Fas transgene is expressed in *lpr/lpr* mice the lymphoproliferation phenotype is abolished, reinforcing the indispensable role of Fas-FasL interactions in peripheral deletion [106].

Another critical role for Fas-FasL interaction in the immune system is the ability to mediate target cell death by signaling from CTLs and NK cells; this is in combination with other mechanisms, namely the perforin/granzyme pathway, in which exocytosis of perforin granules and granzyme B from the CTL causes target cell death [8, 22, 107, 108]. Although their method of target cell recognition are different, CTLs and NK cells kill by the same lytic mechanism [109]. Naïve lymphocytes store FasL in intracellular lytic granules [110], and TCR/CD3 lymphocyte stimulation causes FasL localization to the immunological synapse [111]. This event is controlled by a proline-rich domain in the cytoplasmic tail of FasL, and is critical for prevention of constitutive FasL on the surface of activated cytotoxic cells. Ligation of cell surface FasL to target cell Fas then initiates the signaling cascade as described above **(Figure 2)**. Conclusion of this cytotoxic activity and the expansion phase mark the start of a contraction phase, whereby immune cells begin undergoing activation-induced cell death (AICD) to bring the cell numbers down to normal levels [112] and prevent overgrowth that is seen in homozygous *lpr/lpr* and *gld/gld* mice.

Antigen-presenting cells (APC) are another type of immune cell, which present antigen on their cell-surface MHC complex for interaction with T_H and T_C cells and have the ability to secrete cytokines; professional APCs, which express an MHC class II complex, are characterized by B lymphocytes, DCs, and

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macrophages. B lymphocytes are most easily distinguished from T lymphocytes by the presence of the CD40 B cell receptor (BCR), a membrane-bound immunoglobulin that is heavily involved in activation and determination of cell fate [113]. In addition to their function as APCs, B cells can produce antibodies to enable cytotoxic activity. Fas expression has been detected on B cells, albeit to a lesser extent than T cells [64], with a marked increase in cell surface expression upon activation and corresponding increase in sensitivity to Fas-mediated lysis [114]. Fas-mediated B cell elimination is critical for homeostasis, where its absence leads to autoimmune manifestations such as hyperimmunoglobulinemia and splenomegaly [115]. In conclusion, these data indicate an essential role for Fas-FasL interactions in APC elimination and B cell-mediated target cell death; however, Fas-FasL probably acts in concert with other known death effectors.

III. Role of Fas Signaling in β-Cell Death

Failure to appropriately regulate the immune system's autoreactive T cell repertoire can promote autoimmune disease. A classic example of this occurs in type 1 diabetes mellitus (T1DM), a common endocrine disease that is characterized by selective immune-mediated destruction of insulin-secreting pancreatic β-cells [116]. T1DM development is initiated with insulitis, a term describing immune cell accumulation in both the periphery as well as within the islet of Langerhans. This immune cell invasion is followed by an effector phase whereby leukocytes recognize exposed β-cell antigens presented by MHC complexes on the surface of APCs within the islet and instigate β-cell death [117]. Both $CD4^+$ and $CD8^+$ CTLs are critical for autoimmune-mediated β-cell death [118, 119]. In support of this, immunohistological analysis has shown leukocytic islet infiltrates to be mostly composed of T cells [116]; $CD4^+$ lymphocytes are the central effector in this process [120, 121]. DCs and macrophages, also present in the islet infiltrate, are the first invaders that trigger the adaptive immune response, typically through release of the pro-inflammatory cytokine interleukin-1β (IL-1β) [122-124]. Invading T

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lymphocytes and resident macrophages secrete IL-1β and by virtue of signaling through the IL-1 receptor (IL-1R) on the islet β-cell surface, produce key intracellular events in the development of insulitis and β-cell death. Moreover, when primary islets or insulinoma cell lines are exposed to IL-1β alone or IL-1β combined with interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), there is a loss of cellular function as measured through GSIS and eventual progression to cell death [19-21, 123, 125, 126]. Finally, IL-1β-stimulated increases in the inducible form of nitric oxide synthase (iNOS), which catalyzes production of nitric oxide (NO), likely acts as a direct mediator of β-cell death [21, 127-129]. However, the molecular mechanism underlying cytokine-mediated β-cell destruction is still not well understood. Although MHC class II expression on autoreactive $CD4^+$ lymphocytes is normally required for autoantigen recognition, its expression has not been observed on islet-invading $CD4^+$ lymphocytes [130]. Additionally, it is unlikely that there is one single effector mechanism of β-cell death, and likely varies amongst species and even individuals. Multiple proteins capable of instigating cytotoxic intracellular signaling might have a role in the process, with evidence implicating perforin/granzyme, TNF/TNFR1, and Fas-FasL [6, 7, 22, 24, 131-133].

The majority of studies investigating the mechanism of β-cell death and development of T1DM have focused on a potential role for Fas-FasL interactions. Although Fas and FasL do not contribute to genetic susceptibility for T1DM [134-136], their interaction was initially implicated in β-cell death when autoimmune diabetes-prone non-obese diabetic (NOD) mice carrying the *lpr/lpr* mutation failed to develop diabetes [24, 25]. Adoptive transfer of diabetogenic $CD8^+$ islet-specific T cells or total splenocytes also failed to induce diabetes in Fas-deficient *lpr/lpr* mice [24, 25]. Moreover, IL-1β-induced β-cell-surface Fas expression could provide a mode of β-cell death by Fas receptor activation [23]. These findings led to the commencement of a span of studies that explored the role for Fas-FasL interactions in β-cell death, yet the complexities involved with islet physiology have made it difficult to conduct *in vivo* Fas β-cell studies. Accordingly, multiple systems have been designed to simulate a human diabetes

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patient **(Tables 1 & 2)**, and have majorly contributed to the inconclusive and conflicting results of subsequent Fas β-cell death studies. This review summarizes the progression of the Fas β-cell field by reporting the *ex vivo* genetic manipulations and transgenic models, and whether or not these confirm a dependence on Fas-FasL interactions for β-cell death and development of T1DM.

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Table 1. Summary of mutations and transgenes that comprise spontaneous and pharmacologically-induced models used for investigation of Fas-mediated β-cell death.
*Mice of non-NOD genetic background

Method of diabetes induction	Mutation/Transgene	Reference
Spontaneous	*lpr/lpr*	[24, 25, 137]
	gld/gld	[138]
	RIP-lpr^{cg}	[139]
	lpr/+	[24, 138, 140]
	gld/+	[139, 141]
	IL-10R-neutralized-*gld/+*	[142]
	IL-10-transgenic-*lpr/lpr*	[143]
	IL-1$\beta^{-}/^{-}$, ICE$^{-}/^{-}$	[144]
	4.1-NOD.*lpr/lpr*	[145]
	Ad-IL-1R$^{-}/^{-}$	[146]
	4.1-NOD/RIP.SOCS-1	[147]
	RIP-dnFADD	[148]
	BID$^{-}/^{-}$	[149]
	RIP-Cre^{+}Fas$^{fl/fl}$	[150]
	Neutralizing anti-Fas Ab at 2-4 weeks old	[151]
Cyclophosphamide	FasL-neutralizing Ab	[152]
	TCR-HA^{+}Ins-HA$^{+/-}$Fas$^{fl/fl}$, RIP-Cre$^{+/-}$*	[153]
	BDC2.5/NOD-*lpr/lpr*	[154]
MLDS	RIP-Cre^{+}Fas$^{fl/fl}$*	[155]
	AdCTLA4-FasL*	[156]
CD8+ T cell-mediated	RIP-SOCS-1.NOD8.3	[157]

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Table 2. Summary of adoptive transfer models from *in vivo* studies that investigated the role of Fas-FasL interactions in β-cell death.
*Treated with a neutralizing/antagonist anti-FasL antibody
**Pharmacologically induced to diabetes

Cell Type	Splenocytes			Pancreatic Islets		
Genetic Background	**Donor**	**Recipient**	**Reference**	**Donor**	**Recipient**	**Reference**
	NOD-wt	NOD-*lpr/lpr*	[24, 139, 140]	NOD-wt	diabetic NOD-wt	[137]
	NOD-wt	NOD-*scid/scid*	[141]	NOD-wt	diabetic NOD-wt*	[158]
	NOD-wt	NOD-*scid/scid-gld/gld*	[141]	NOD-wt	male NOD-wt**	[137]
	diabetic NOD-wt	NOD-wt*	[151, 152]	C3H/HeJ-wt	NOD-wt**	[159]
	diabetic NOD-wt	NOD-*scid/scid-lpr/lpr*	[138, 139]	NOD-*lpr/lpr*	NOD-wt	[152]
	SA-FasL from diabetic NOD	NOD-*wt*	[160]	NOD-*lpr/lpr*	male NOD-wt**	[137]
	IL-10-transgenic NOD-*lpr/lpr*	NOD-*scid/scid*	[143]	NOD-*lpr/lpr*	diabetic NOD-wt	[137]
	NOD-*lpr/lpr*	NOD-*scid/scid*	[143]	C3H/HeJ-*lpr/lpr*	NOD-wt	[159]
	NOD-gld/+	NOD-scid/scid	[141]			
Cell Type	**CD8+ islet-specific T cells**			**CD4+ islet-specific T cells**		
Genetic Background	**Donor**	**Recipient**	**Reference**	**Donor**	**Recipient**	**Reference**
	NOD-wt	NOD-*lpr/lpr*	[25]	NOD-wt	NOD-wt	[151]
	NOD-wt	NOD-wt*	[151]	YNK7.3 clones, no donor	NOD-*lpr/lpr*	[151]
	HA-specific from Balb/c clone-4 TCR transgenic	Balb/c Ins-HA-*lpr/lpr*	[161]	HA-specific from TCR-HA transgenic RAG-2^{-}/$^{-}$	INS-HA-RAG-2^{-}/$^{-}$	[153]
	OVA-specific from OT-1 TCR transgenic	B6.MRL-*lpr/lpr*	[162]	HA-specific from TCR-HA transgenic RAG-2^{-}/$^{-}$	INS-HA-RAG-2^{-}/$^{-}$-Fas^{-}/$^{-}$	[153]
	NOD-8.3 from NOD-*lpr/lpr*	NOD-8.3	[163]			

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lpr & gld, Loss-of-Function Mutations

Although the original *lpr/lpr* mouse was identified in the MRL/MpJ mouse strain, the mutation is often transferred to the non-obese diabetic (NOD) mouse for β-cell studies investigating the contribution of the Fas receptor. The NOD mouse is the most frequently used murine model for diabetes studies because it develops the disease in a T cell-dependent manner that is reminiscent of a human T1DM patient [164, 165]. There is a difference in rate of diabetes onset between male and female NOD mice. Females have decreased insulin content at 12 weeks of age and a 90-100% diabetes incidence by 30 weeks old; males have a 40-60% incidence by the same age. Female NOD mice are thus more appropriate for spontaneous diabetes studies, whereas males are well-suited for studies of accelerated disease transfer (Jackson Labs website). Fas expression is elevated in female NOD mice at 15 weeks of age relative to their male counterparts and correlates with the overall gender difference observed in NOD mice [166]. Similar to the MRL-*lpr/lpr* model, NOD-*lpr/lpr* mice develop lymphadenopathy and massive infiltration of nonmalignant DN $CD4^-$ and $CD8^-$ T cells [24, 138].

The initial studies implicating Fas-FasL interactions in β-cell death showed that NOD-*lpr/lpr* mice failed to develop spontaneous diabetes as compared with their wild-type littermates [24, 25]. FasL-deficient NOD-*gld/gld* mice were also protected from diabetes development [138]. Further, adoptive transfer of $CD8^+$ islet-specific T cells or diabetogenic splenocytes from NOD-wt donor mice to NOD-*lpr/lpr* mice failed to initiate diabetes, and together these studies indicated a significant role for Fas-FasL interactions in development of autoimmune diabetes [24, 25]. The lpr^{cg} mutation is less commonly used in Fas β-cell studies, but one group showed some protection from development of spontaneous diabetes amongst NOD mice carrying the lpr^{cg} mutation, which was driven by the rat insulin promoter (RIP) and thus confers β-cell-specific expression; the extent of diabetes protection was dependent on the level of transgene expression [139]. This is a significantly different observation from the *lpr/lpr* and *gld/gld* studies because the systemic Fas deficiency could confer altered immune function that results in

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prevention of autoimmune-mediated β-cell destruction, as opposed to diabetes prevention by loss of Fas specifically in the β-cell.

The validity of the *lpr/lpr* mouse as a reliable model for Fas β-cell studies has been subsequently questioned [137, 140]. The systemic Fas deficiency introduced by a homozygous *lpr/lpr* mutation may prevent diabetes, not due to absence of Fas from β-cells, but as a consequence of an inappropriately functioning immune system that cannot generate a normal inflammatory response [137, 138, 140, 152]; abnormal $CD4^{-}$ $CD8^{-}$ $B220^{+}$ cells of NOD-*lpr/lpr* mice are also more resistant to sublethal irradiation relative to their NOD-wt counterparts [167]. Additionally, failure of adoptively transferred splenocytes to initiate diabetes in recipient NOD-*lpr/lpr* mice occurs as a result of constitutive FasL expression on endogenous lymphocytes, which arises as one of multiple abnormalities resulting from the *lpr/lpr* mutation. FasL overexpression on NOD-*lpr/lpr* lymphocytes kills diabetogenic Fas-expressing cells through cell surface Fas-FasL interactions before diabetes can be transferred to the recipient mouse [137, 140]. Furthermore, localization of the *gld/gld* mutation to hematopoietic tissues by adoptive transfer of *gld/gld* bone chimeras into NOD-wt mice results in DN lymphoproliferation, demonstrating that FasL deficiency on immune cells is the cause of the abnormal phenotype that occurs in *gld/gld* mice [141]. In order to circumvent the confounding immunological effects of the NOD-*lpr/lpr* and NOD-*gld/gld* mouse, alternative genetic alterations and methods of diabetes induction such as islet transplantation and heterozygosity for *lpr* and *gld* have been developed and will be described below.

Interestingly, the abnormal phenotype associated with homozygosity for *lpr* and *gld* is not evident in mice that are heterozygous for either mutation. Heterozygotes behave in a comparable fashion to wild-type mice; whereas NOD-*lpr/lpr* mice fail to develop diabetes, NOD-*lpr/+* develop diabetes at a similar rate to wild-type mice [24, 140]. Further, although NOD-*gld/+* mice have a 0% incidence of spontaneous diabetes, they have normal-sized lymphoid organs and don't develop lymphadenopathy or splenomegaly [138]. The absence of lymphoproliferative effects in *gld/+* mice

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indicates that these mice have a sufficient decrease in FasL expression to prevent β-cell death, but there is adequate FasL expression to prevent the immune implications that result from a complete loss of FasL [141].

IL-10 is an anti-inflammatory cytokine that has a significant role in growth and differentiation of various hematopoietic cells and can prevent onset of some inflammatory conditions [168]. Although its involvement in T1DM development is ambiguous, IL-10 might be protective against diabetes development in NOD-*gld/+* mice. Neutralization of pancreatic B cell-surface IL-10 receptor (IL-10R) causes a mostly $CD4^+$ T cell-comprised islet cell infiltration, but not transition to diabetes [142]. However, this protective effect is likely specific to NOD-*gld/+* mice that do not naturally develop diabetes, as blocking of the IL-10R in NOD-wt mice has little consequence on insulitis or diabetes incidence. Additionally, β-cell-specific gene transfer using recombinant adeno-associated virus vector (AAV) for IL-10 in NOD-wt mice has little protective effect against diabetes development [169]. These recent findings corroborate the above results in NOD-wt mice yet dispute the IL-10-mediated diabetes protection observed in NOD-*gld/+* mice. Moreover, another group actually reported an acceleration of NOD diabetes when made transgenic for IL-10. They showed that this acceleration was independent of Fas, in that NOD-*lpr/lpr* mice were not protected from IL-10-induced diabetes. Splenocytes from these mice were able to transfer diabetes to NOD mice deficient in T- and B-lymphocytes by the severe combined immunodeficiency (*scid*) mutation (NOD-*scid/scid*), in contrast to their non-IL-10-transgenic NOD-*lpr/lpr* counterparts that could not cause this induction [143]. Yet lymphocytes from NOD-*gld/+* mice still carry diabetogenic potential, as NOD-*scid/scid* mice reconstituted with splenocytes from NOD-*gld/+* or NOD-wt mice develop diabetes at similar rates to each other [141].

A novel approach of analyzing the NOD-*gld/gld* mouse was taken by separating FasL expression in the hematopoietic and nonhematopoietic compartments of NOD-*gld/gld* mice and showed that blocking Fas expression in either compartment could prevent diabetes incidence [141]. However,

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experiments in which wild-type splenocytes failed to cause diabetes in NOD-*scid/scid-gld/gld* mice with a systemic deficiency in FasL and the T- and B-lymphocyte population (but are Fas-sufficient) demonstrated that protection from diabetes development in NOD-*gld/gld* and NOD-*gld/+* is not a result of abolished β-cell Fas-FasL signaling and thus favors a Fas-independent mechanism of β-cell death [141].

Sublethally irradiated NOD-*lpr/lpr* mice did not develop spontaneous diabetes upon adoptive transfer of diabetogenic cells [24, 25, 137] because abundant FasL in NOD mice homozygous for the *lpr* mutation was likely killing the adoptively transferred splenocytes [137, 140]. Therefore, the destructive role of Fas-FasL interactions in murine autoimmune β-cell death was investigated by grafting islets from murine *lpr/lpr* or wild-type mice into non-diabetic NOD-wt mice. Adoptive transfer of pancreatic islets from *lpr/lpr* donor mice into wild-type recipients should confer some level of islet survival if Fas were to play a necessary role in β-cell death, in that absence of functional Fas on the donor islet would prevent initiation of the intracellular death cascade via interaction with endogenous recipient FasL. However, after adoptive transfer of diabetogenic splenocytes to male NOD mice that had been grafted prior with fetal pancreatic islets from NOD-*lpr/lpr* or NOD-wt mice, the NOD-*lpr/lpr* β-cells fared better than the NOD-wt islets although both were eventually destroyed [137]. While modest, these findings suggest a role for Fas in autoimmune β-cell destruction. Another group also reported effective destruction of *lpr/lpr* islets transplanted into NOD-wt mice, and concluded a dispensable role for Fas-FasL in β-cell death but did not transplant wild-type islets for comparison [152]. Injection of a neutralizing anti-FasL antibody (Ab) did not affect diabetes incidence after adoptive transfer of diabetogenic splenocytes or diabetes induction with cyclophosphamide (CY), indicating Fas-independent β-cell death. In contrast, treatment with a neutralizing anti-FasL Ab after syngeneic islet transplantation to diabetic NOD mice restored normoglycemia [158]. Additionally, CH3/HeJ-*lpr/lpr* islets survived as indicated by normal blood glucose for up to 2 months in their wild-type recipient after diabetes induction with streptozotocin

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treatment. Although the wild-type islets were destroyed, there was evidence of $CD4^+$ and $CD8^+$ lymphocyte migration to the kidney capsule in both models, which indicates Fas-independent insulitis but an effector phase of β-cell death that may be Fas-dependent [159]. Furthermore, NOD-*lpr/lpr* fetal pancreas grafts could not reverse diabetes in diabetic female NOD mice; although the NOD-*lpr/lpr* islets fared better relative to wild-type islets, they were eventually destroyed [137].

Another way to reduce peripheral lymphoid populations and constitutively-expressed FasL conferred by the *lpr/lpr* mutation is to delete the potentially abnormal T and B cells altogether. This model requires use of the *scid* mutation [170]; studies investigating diabetes incidence in *scid/scid-lpr/lpr* mice used mouse models of similar genetic background and diabetes induction, and were consistent in their reported results. Reconstitution of sublethally irradiated NOD-*scid/scid-lpr/lpr* mice with diabetogenic spleen cells conferred a significant reduction in diabetes incidence relative to NOD-*scid/scid* mice that carry functional Fas [138, 139]. This could infer both Fas-dependent and –independent mechanisms; the primary justification is that the reduction in diabetes incidence could now be directly attributed to absence of Fas expression. An alternative explanation is that the *lpr/lpr* mutation allows for a sufficient level of functional Fas receptor activity or that autoimmune disease in the adoptive transfer model can also be initiated by Fas-independent mechanisms. This would potentially allow for a low level of diabetes incidence.

Studies using the *lpr* and *gld* mutations, either alone or paired with other mutations, genetic models, or methods of diabetes induction, comprise the majority of the Fas β-cell field **(Tables 1 & 2)**. Although the homozygous mutations alone confer a dysfunctional immune system and abnormal phenotype that make it difficult to directly address the role of Fas-FasL interactions in β-cell death, a number of groups have found ways to circumvent these abnormalities and manipulate the mutations in combination with other genetic modifications to investigate the mechanism responsible for autoimmune-mediated β-cell death.

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Induction of β-Cell Surface Fas Expression

Although the *lpr/lpr* mutation sparked initial interest in Fas-dependent β-cell death, support for Fas involvement was built on the *ex vivo* demonstration that isolated islets from both diabetic rodents and humans have abundant Fas β-cell surface expression [25, 131, 171-173], whereas β-cells isolated from disease-free organisms have negligible quantities of cell-surface Fas expression [174-176]. To corroborate these findings, syngeneic islets from non-diabetic donors transplanted into diabetic NOD mice had abundant levels of Fas expression, whereas their normoglycemic counterparts had a significantly lower level of Fas expression [177]. In addition, adoptive transfer of spleen cells from diabetic NOD donor mice conferred Fas expression in β-cells of recipient NOD-*scid/scid* mice [178]. Some murine studies, however, reported low levels of Fas expression on the β-cell surface of diabetic NOD and mice induced to diabetes with CY [179-181], which may reflect a species-specific preference for mechanism of β-cell death and/or a disparity between modes of diabetes induction. One of these groups later found that the low level of Fas expression in islet tissue originally reported [179] was actually Fas expression on leukocytes, as indicated by dual staining with anti-CD45 and Fas [178]. Additionally, invading mononuclear and activated T cells stain positive for Fas activity in immunohistochemistry studies and become apoptotic after treatment with an anti-Fas Ab, illustrating that autoreactive cells can also be deleted by AICD during islet infiltration [171, 176, 180-182]. An *in vivo* study in which IL-1β-deficient female NOD mice were used noted that spontaneous diabetes was not significantly decreased in comparison with NOD-wt [144]. In a separate experiment, the same group blocked IL-1β expression by creating NOD mice deficient in IL-1β-converting enzyme (ICE), which converts the inactive IL-1β precursor to its active form. Spontaneous diabetes was again unaffected; if Fas, whose expression is induced by IL-1β, were indeed the primary β-cell death effector, β-cell death and diabetes induction would at least be reduced in this circumstance.

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Cell surface FasL expression *ex vivo* is perhaps more perplexing in that the findings are not generally in agreement. FasL expression has been demonstrated in mice with insulitis [176, 180] but not in the pancreas of NOD-*scid/scid* mice after adoptive transfer of splenocytes (data not shown) [183]. FasL is also reported to have constitutive expression in islet α-cells [181], and β-cells of Type 2 diabetic patients [184] and non-diabetic human donors [175, 184]. However, other extant studies show FasL expression to be present on infiltrating mononuclear cells and activated $CD4^+$ and $CD8^+$ lymphocytes but not on human or mouse islet endocrine cells [131, 171, 179, 185]. One study did note the presence of FasL transcript from diabetic pancreata preparations, but it wasn't clear if this was β-cell specific or from another pancreatic cell type [131].

The most commonly cited source of the general discrepancy in detection of FasL expression is that the anti-FasL antibodies used in these studies range in their specificities for FasL, which can confer false positive staining [171, 180, 186]. Additionally, detection of FasL could be impacted by methodological problems such as failure to account for FasL that has been cleaved from the cell surface or destruction of cell-surface FasL during the process of islet isolation and purification. To avoid such problems, studies that intend to analyze β-cell FasL expression should rigorously test anti-FasL antibodies for specificity before use and employ dual staining for FasL and insulin to localize its expression to pancreatic β-cells.

Although *ex vivo* FasL studies do not conclusively establish ability for the β-cell to express FasL mRNA or protein, it is well-established that FasL expression occurs on the surface of activated resident and invading mononuclear cells to the islet during insulitis, as described above. This makes inducible β-cell-surface Fas expression the limiting factor for potential occurrence of β-cell-immune cell Fas-FasL interactions, and is a critical item to address for *in vitro* studies that require a method of cell surface Fas induction. Incubation of primary islets or transformed insulinoma cell lines with pro-inflammatory cytokines (e.g. IL-1β) simulates the autoimmune-mediated attack that is postulated to occur *in vivo*,

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since IL-1β is released from immune cells during islet infiltration. *In vitro* incubation with IL-1β also appears to be a reliable method of Fas β-cell surface induction. Treatment of primary human, mouse, and rat islets with IL-1β alone or IL-1β in combination with IFN-γ and TNF-α increases Fas mRNA production and promotes cell surface localization in a dose-dependent manner [23, 131, 173, 177, 187-189]. Expression of Fas after IL-1β treatment may depend on dose and time of exposure [179]. The combination of IL-1β, IFN-γ, and TNF-α synergizes to enhance Fas expression, but IL-1β is likely the dominant signal in human and murine islets [131, 179]. A similarly strong induction of cell surface Fas expression can be obtained through treatment of non-diabetes-prone Balb/c mouse islets with IL-1α [190]. Some studies then confirmed functionality of cytokine-induced cell surface Fas by demonstrating an increase in apoptosis after treatment with an agonist-Fas Ab or sFasL [179, 187, 190, 191]. One group also noted a dose-dependent increase in Fas expression after treatment of human β-cells with glucose concentrations of up to 33.3 mmol/L [184] yet Fas expression on isolated rat β-cells was only observed on β-cells of older (7-8 mos.) rats [192]. Furthermore, glucose-induced Fas expression on rat islets is not enhanced by treatment with cytokines or streptozotocin treatment [193]. Interestingly, one human islet study noted an increase in Fas transcript expression after 96 hours amongst islet cells in culture without treatment, at comparable levels to islets treated with IL-1β, but incubation of the untreated islets with an agonist anti-Fas Ab failed to induce apoptosis [194]. This suggests that cytokines promote Fas accumulation at the cellular membrane leading to activation of downstream intracellular cell death machinery [195]. Thus, isolation of islets or conditions present in culture media can potentially impact baseline Fas expression.

Unlike that reported in primary murine islets, investigation of Fas mRNA and cell surface expression in insulinoma cells exposed to cytokines revealed contrasting findings. RIN-5F insulinoma cells express Fas mRNA after treatment with IL-1β, IFN-γ, and TNF-α, as well as after exposure to streptozotocin [196]. Exposure of NOD-derived NIT-1 cells to IL-1β and IFN-γ in combination caused an

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induction of Fas cell surface expression [173, 191, 197] and total protein levels were nearly unchanged between untreated and cytokine-treated cells, which indicates that pre-transcribed Fas protein resides within the cell [191]; this intracellular store of Fas protein was also demonstrated in isolated islets from diabetes-prone BB/OK rats [189]. Silencing of Fas expression by small interfering RNA (siRNA) transfection in NIT-1 cells reduced Fas mRNA transcription by nearly 90%, and although the siRNA also conferred a significant decrease in cell surface Fas expression, it only reduced total protein expression after prolonged treatment [198]. This could indicate a slow turnover rate of β-cell Fas protein. C57BL/6 mouse-derived β-TC1 cells exposed to Fas siRNA demonstrated a similar but less severe phenotype than in NIT-1 cells, in that β-TC1 cells had only a 70% decrease in Fas mRNA abundance [198]. This difference in mechanism thus appears to not only occur within groups, but between groups as well. Whereas NIT-1 insulinoma cells can down-regulate cytokine-induced cell surface Fas after exposure to agonistic FasL, primary NOD islets and BALB/c islets do not show this response and maintain the same level of cell surface Fas expression with or without exposure to FasL [199].

In contrast to the above, another group treated the same NIT-1 cell line with IL-1α and IFN-γ, which induced Fas mRNA expression but did not confer cell surface expression [190]. Further, NOD mouse-derived MIN6N8 insulinoma cells expressed Fas mRNA in both the unstimulated and cytokine-stimulated state, but Fas cell surface expression was not detected after treatment with IL-1β or IFN-γ [185]. Additionally, treatment of the MIN6N8 cells with an agonist anti-Fas Ab did not result in significant apoptosis, which suggests either non-functional Fas or a very low level of Fas expression.

Studies investigating β-cell-specific Fas and FasL expression have had difficulty coming to a consensus on these criteria, in both the healthy and pathological state, for a number of reasons. Experimental problems such as β-cell specificity in immunostaining techniques and specificity of anti-FasL antibodies for FasL present easily avoidable false-positives or –negatives. But these problems primarily surfaced because of use of separate genetic models, with this variation extending within and

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between primary islets and transformed cell lines. However, the ultimate ability of multiple groups to demonstrate Fas cell surface expression during the natural progression of diabetes in humans and mice was taken to mean that Fas-FasL interaction is the major mechanism of β-cell deletion.

Because NO production is required for IL-1β-mediated cytotoxicity, it could serve as a link between cytokine exposure and β-cell surface Fas expression. However, a consensus on the requirement of NO for induction of Fas mRNA and cell surface expression has not been reached. Exposure of iNOS-deficient murine islets to cytokines had no effect on Fas expression relative to Fas-sufficient islets [187, 200]; and although the data was not shown, one of these groups also reported that exposure of human islets to the NOS inhibitor N-Methylarginine (NMMA) in addition to cytokines did not prevent induction of Fas mRNA [200]. This lack of response was also replicated in NOD islet cells treated with the NMMA and cytokines, with no significant change in Fas expression [179]. However, one of these studies reported only a small induction of Fas expression after cytokine treatment and was already at odds with other studies that report abundant cytokine-induced Fas expression [179]. In contrast to the above studies, treatment of non-human primate (NHP) islet β-cells with NMMA after incubation with cytokines resulted in attenuation of Fas expression. Additionally, treatment of NHP cells with the NO donor sodium nitroprusside (SNP) alone induced abundant Fas expression, and exposure of primary rat islets to cytokines after silencing iNOS expression by siRNA transfection abrogated Fas mRNA expression [188], and together indicate that IL-1β-induced Fas expression might at least be partly dependent on the presence of NO [131].

This controversial involvement of NO in induction of β-cell Fas expression also appears to extend to its role in Fas-mediated β-cell death. There was little to no change in cell death among islets from wild-type as compared with iNOS-deficient mice [187], as well as amongst mouse islets treated with NMMA [179, 190]. One study reported an essential role for Fas in β-cell death by showing that treatment of human islets with an anti-Fas antagonist in addition to either IL-1β or the NO donor SNP

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had no effect upon cell death [131]. However, although SNP alone was able to induce Fas expression in NHP cells, cell death was analyzed in human islets and the ability of SNP to induce Fas expression in this cell type had not been established. Furthermore, the link between Fas and NO would have been strengthened had the human islets been treated with the combination of IL-1β, SNP, and anti-Fas antagonist. Taken together, a majority of these studies indicate that NO is not required for induction of Fas expression or Fas-mediated β-cell death. Although IL-1β has been shown by multiple groups to be separately required for both NO and Fas expression, there appears to be no dependence for NO on Fas expression independent of IL-1β.

Transgenic Models of T Cell-Mediated Diabetes

The TCR transgenic mouse with a rearranged TCR specific to either the $CD4^+$ or $CD8^+$ T cell population is an attractive experimental model system for study of Fas-FasL interactions in β-cell death for two fundamental reasons: 1) Although both leukocyte populations are already known to be required, the mechanisms of $CD4^+$ and $CD8^+$ T cell-induced diabetes can be uncoupled from each other and separately analyzed, with a primary focus on $CD8^+$ CTLs due to their known role as effectors of β-cell death [116], and 2) Transgenic mice provide a model of diabetes that is accelerated and simplified in comparison to spontaneous diabetes, so that the T cell repertoire and direct β-cell death mediators can be studied efficiently and without the complexities that are inherent to spontaneous diabetes [154, 163, 201, 202]. Transgenic mice can thus be used to address the autoimmune requirement and mechanism of cell death required for T cell-mediated disease induction, neither of which has been fully elucidated.

TCR transgenic β-cell death studies are unique in that most do not solely focus on Fas-FasL interactions as the primary death effector. As mentioned above, T cell homeostasis and CTL-mediated target cell death are dependent on Fas-FasL interactions in addition to other death effectors, centrally the perforin/granzyme pathway [22, 107, 108], and to a much lesser extent, the TNF/TNFR1 pathway [203, 204]. Thus, a role for perforin in β-cell pathology leading to T1DM has also been investigated. Yet

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unlike the ambiguous role for Fas-FasL interactions, perforin/granzyme appears to have a critical role in the effector phase of β-cell death [6, 205]. Additionally, in contrast to the initial Fas β-cell death studies that solely addressed Fas as a β-cell death mediator, much of Fas-relevant TCR transgenic diabetes research examines the role for Fas in addition to other relevant pathways in β-cell death and generally acknowledges that there is more than one direct mechanism involved in β-cell death.

One of the first studies that took this approach used transgenic mice that express β-cell-surface influenza virus hemagglutinin (HA), so the β-cells can be deleted by HA-specific $CD8^+$ T cells extracted from TCR-transgenic mice [161]. Both the perforin and Fas pathway were separately blocked in these transgenic mice by use of a perforin inhibitor or by crossing the transgenic mice with the *lpr/lpr* mutation, then adoptively transferring Ins-HA recipient mice with these donor CTLs. Interestingly, loss of perforin alone caused a reduced incidence of diabetes, and loss of both pathways conferred a complete loss of diabetes induction. However, loss of Fas expression alone had a minimal impact on diabetes induction. Additionally, much larger numbers of perforin-deficient CTLs were required to adoptively transfer diabetes than Fas-deficient CTLs, and mice that received perforin-deficient CTLs still stained positive for small amounts of insulin amidst less islet infiltration than the mice that received Fas-deficient CTLs. These together indicated that perforin is the major method of β-cell cytotoxicity, but in the absence of perforin expression, Fas-FasL interactions can cause significant cytotoxicity [161]. Two studies published in 2006 by the same group but using separate TCR transgenic models corroborated these results. Ovalbumin (OVA)-specific CTLs from OT-1 TCR transgenic mice can rapidly induce diabetes upon adoptive transfer to recipients that have β-cell expression of OVA and in the absence of perforin, β-cell killing is severely reduced. Yet absence of Fas alone has minimal effect on antigen-specific β-cell killing, whereas perforin- and Fas-deficiency cause complete loss of antigen-specific β-cell killing [162]. Islets from NOD-*lpr/lpr* donors were also not protected *in vivo* when grafted under the kidney capsule of mice with a rearranged TCR that is representative of the islet-specific TCR recognized by diabetogenic

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cytotoxic CTLs (NOD8.3 mice) [163]. Additionally, NOD-wt islets were killed after treatment with CTLs from perforin-competent NOD8.3 mice, and this killing was severely reduced when the CTLS were from perforin-deficient NOD8.3 mice and completely abolished when NOD-*lpr/lpr* islets were exposed to the perforin-deficient CTLs [163]. Finally, treatment of 7-day-old NOD mice with an antagonist anti-FasL Ab after adoptive transfer of islet-specific CD8$^+$ T lymphocytes did not inhibit diabetes or insulitis. However, treatment with the anti-FasL Ab after adoptive transfer of total splenocytes from diabetic mice conferred insulitis but not overt diabetes [151]. These results reiterate the essential role for perforin in CD8$^+$ T cell-mediated β-cell death, where Fas-mediated β-cell death is still possible but likely responsible for insulitis and not the effector phase of β-cell death.

On the other hand, a separate study that also used NOD8.3 mice suggested a Fas-dependent mechanism of β-cell death after perforin-deficient mice actually demonstrated accelerated diabetes, and NOD8.3-CD8$^+$ CTLs (either perforin-sufficient or -deficient) could kill Fas-sufficient but not Fas-deficient *lpr/lpr* NOD β-cells *in vitro* [206]. A third study that also used the NOD8.3 experimental mouse model determined that perforin is required for the later stages of insulitis, whereas other perforin-independent effector mechanisms are used in the early stages of insulitis [207]. Interestingly, although they used the same genetic mouse model, each of the NOD8.3 studies used CD8$^+$ T cells that recognize separate β-cell peptides, making it difficult to draw conclusions from the combined study results since each might confer a different mechanism of action. To further complicate matters, a CD8-mediated transgenic model of diabetes in which mice express RIP-driven transgenes for TNF and CD80, both of which are required for diabetes induction, indicated a possible requirement for TNF in β-cell death, yet little to no role for perforin or Fas [202].

Studies in which CD4$^+$ T lymphocytes carry a transgenic TCR that recognizes a specific β-cell peptide, or CD4$^+$-induced diabetes, are less frequently investigated than CD8$^+$ TCR transgenic studies. Like CD8$^+$ CTLs, CD4$^+$ T cells are capable of β-cell cytotoxicity, as evidenced by their ability to kill Jurkat

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cells *in vitro*, and can also express FasL upon activation [151]. Although these activated FasL^{+} T cells can kill NOD-*scid/scid* islets after IL-1α treatment to induce islet Fas expression, exposure to an anti-FasL Ab can't decrease this death; additionally, treatment of NOD-*lpr/lpr* islets with the same T cell clones can be killed as efficiently as wild-type mice [151]. Cre-loxP is the term for a method of site-specific deletion of a gene of interest (in this case Fas receptor) [208], and can be driven by the RIP to confer β-cell specific Fas deletion (RIP-Cre^{+}Fas$^{fl/fl}$). This abrogates the lymphoid abnormalities associated with the systemic *lpr/lpr* mutation and makes it an ideal model system for generating tissue-specific deletion of a given molecule. Adoptive transfer of TCR-HA-CD4^{+} T cells into mice with a Cre-loxP Fas deletion had little difference in diabetes development relative to their Fas-sufficient counterparts. Furthermore, there was actually an accelerated rate of diabetes development in these Fas-deficient mice when induced to diabetes with CY [153]. Additionally, in a transgenic model of diabetes in which the T cell repertoire is composed of rearranged TCR genes from a CD4^{+} T cell clone (BDC2.5/NOD), Fas-deficient mice carrying the *lpr/lpr* mutation still developed insulitis and only a small proportion developed diabetes after treatment with CY [154]. Although this could be interpreted as an essential role for Fas in the BDC2.5/NOD mouse model, T cells transferred from BDC2.5/NOD-*lpr/lpr* mice to BDC2.5/NOD-wt mice had difficulty with proliferation; thus, like non-transgenic NOD-*lpr/lpr* mice, the decreased incidence of diabetes in BDC2.5/NOD-*lpr/lpr* is likely due to alterations in the T cell repertoire and not directly due to a Fas deficiency in the target tissue.

In stark contrast to the above, incubation of IL-1α- and IFN-ϒ-treated NOD β-cells with a CD4^{+} clone that can differentiate into a CTL and expresses a highly diabetogenic TCR (4.1-CD4^{+} CTL) results in killing that is likely Fas-mediated, since this killing was abrogated when the islets were made Fas-deficient by the NOD-*lpr/lpr* mutation [145]. *In vivo*, the 4.1-NOD.*lpr/lpr* mouse developed insulitis but not diabetes, unlike the wild-type NOD-*lpr/lpr* mouse, which develops neither insulitis nor diabetes; these results indicate that in this transgenic model Fas may be necessary for the effector phase but not

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the initiation phase, whereas in the spontaneous model Fas may be responsible for the initiation phase of β-cell death [145]. These findings were corroborated when adoptive transfer of islet-specific $CD4^+$ T cell clones (YNK7.3) into NOD mice, along with administration of an anti-FasL Ab, still conferred insulitis but significantly decreased diabetes incidence, yet when the recipient mice carried the *lpr/lpr* mutation insulitis was also significantly decreased [151]. In the same 4.1-NOD mouse model, another group showed that an IL-1R antagonist did not block induction of β-cell Fas expression, and indicates that IL-1β does not participate in upregulation of Fas expression in this particular diabetes model. These mice also appear to be capable of Fas-independent β-cell death, as demonstrated when NOD-*lpr/lpr* islets grafted into NOD4.1 mice were destroyed [147]. In contrast to these results, NOD mice infected with a replication-deficient adenovirus for an IL-1R antagonist protein protected the islets from activation of a protein known to be stimulated during Fas-mediated apoptosis after treatment of the islets with IL-1β and an agonist Ab for Fas. However, the possibility exists that this marker is not stimulated during inflammation-mediated cell death and other measures of protection from Fas-mediated cell death would have verified these results [146].

Interestingly, one group that found little to no Fas expression on islet endocrine cells of spontaneously diabetic NOD mice after cytokine stimulation [179] did note Fas expression on β-cells of TCR transgenic models of diabetes [178]. They theorized that this contrast might be due to β-cell expression of certain endogenous inhibitors of Fas, such as suppressor of cytokine signaling-1 (SOCS-1) or an IL-1R antagonist. SOCS-1 has also received attention in some TCR transgenic mouse models due to this characteristic and its ability to provide a level of protection against diabetes induction by preventing the action of multiple cytokines via blocking the Jak-STAT signaling pathway [209] and hence, Fas cell-surface induction. Indeed, SOCS-1 was expressed in NOD mouse islets deficient in Fas expression beginning at an age when they would be developing diabetes [178], and β-cell death and diabetes onset were prevented when SOCS-1 was overexpressed in β-cells of the NOD8.3 mouse model (RIP-SOCS1-

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.NOD8.3) of $CD8^+$-mediated diabetes [157, 163]. But this protection was perforin-dependent and Fas-independent, as seen when NOD-*lpr/lpr* islets overexpressing SOCS-1 were killed by 8.3 CTLS but perforin-deficient islets were not [163], and when NOD4.1 mice overexpressing SOCS-1 did not show an induction of β-cell Fas expression yet still developed diabetes [147]. Additionally, OVA-pulsed islets from B6.RIP-SOCS-1 were killed by OT-1 CTLs at a comparable level to wild-type B6 islets, but perforin-deficient OT-1 CTLs couldn't kill the SOCS-1-protected islets [162]. These findings also corroborate the results from some transgenic models of $CD8^+$-mediated diabetes, and together indicate a majorly perforin-dependent mechanism of β-cell death.

Intracellular Pathway of Fas-Mediated β-Cell Death

Under the assumption that β-cell death is dependent upon the presence of Fas-FasL signaling, some groups moved to begin deducing the intracellular components required for occurrence of Fas-mediated β-cell death. Although the intracellular pathway of Fas-mediated death has been largely defined in some tissues, centrally those of hematopoietic origin, available data in the context of the pancreatic β-cell is severely limited. Furthermore, the processes of cytokine-mediated β-cell death and Fas-mediated β-cell death are often uncoupled from each other under the premise that cytokines can mediate β-cell death directly by binding to cytokine receptors on the β-cell surface or indirectly, by inducing cell surface expression of death effectors such as Fas or perforin. Some studies have indicated separate intracellular mechanisms of death for cytokine-mediated and Fas-mediated β-cell death [210], which seems to be paradoxical since induction of cell-surface Fas requires β-cell exposure to cytokines. For the purposes of this review, however, the discussion will be limited to the intracellular components required for Fas-mediated β-cell death.

Within the rat Fas promoter, an IL-1β-responsive region was determined to be between nucleotides -223 and -54 [211]. The transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and CCAAT-enhancer-binding protein (C/EBP) are found within the IL-1β-

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responsive sequence at nucleotide -142 and -130 respectively, and both were determined to be critical to the Fas IL-1β response, as mutation of either alone causes a loss in expression normally induced by IL-1β [211].

The responsiveness of the Fas promoter to IL-1β ultimately results in cell surface induction of Fas receptor. As reviewed above **(Figure 2)**, subsequent association of the FADD adaptor protein precedes oligomerization and activation of caspase-8. Indeed, this progression also occurs in the β-cell, where a β-cell-specific dominant-negative FADD (RIP-dnFADD) lacks a death effector domain and competitively inhibits binding of endogenous FADD to the death domain of Fas, which would ultimately prevent activation of the downstream components in this death pathway [148]. *In vitro* treatment of NOD-RIP-dnFADD islet cells with IL-1β + IFN-ϒ + FasL conferred resistance to cell death, whereas wild-type controls were sensitive to cell death after these treatments. Additionally, diabetes incidence of homozygous RIP-dnFADD mice was slightly reduced as compared with NOD-wt control mice, and indicates a role, albeit minor, for Fas-FasL signaling in β-cell death [148]. These results also were corroborated *in vitro*, whereby blocking FADD activity in NIT-1 cells leads to a significant but not complete protection from cell lysis [212].

Just downstream of the FADD, initiator caspase-8 activity also appears to be required; however, evidence for the critical role of caspase-8 in Fas-mediated β-cell death is derived from islets of human Type 2 diabetic islets, so should be cautiously translated to the Type 1 diabetic circumstance. As indicated above, *in vitro* islet exposure to glucose can induce Fas cell surface expression [184]. FLICE-like inhibitory protein (FLIP), an endogenous protein that competitively inhibits caspase-8 by its structural similarity and lack of proteolytic activity, has decreased protein expression levels within islets from Type 2 diabetics relative to healthy controls. This decrease in FLIP after *in vitro* glucose exposure also has an inverse correlation with cell surface Fas expression, where Fas expression increases as FLIP expression

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decreases [213]. Although not directly measured in the study, these data and correlations could indicate an essential role for caspase-8 in Fas β-cell signaling.

The intrinsic mitochondrial pathway appears not to be necessary in inflammation-mediated β-cell death; although required *in vitro* for Fas-mediated mouse islet cell death [50], NOD mice deficient in Bid were not protected from development of spontaneous diabetes *in vivo* [149]. Finally, the effector caspase-3 has been investigated by multiple groups for its potential role in Fas-dependent β-cell death. βTC1 insulinoma cells that were transfected with human Fas cDNA (hFas/βTC1) and exposed to an agonist anti-Fas Ab had a marked increase in caspase-3-like activity. hFas/βTC1 cells transfected with an anti-sense knockout of caspase-3 and then treated with agonist anti-Fas also had increased survival over control hFas/βTC1 cells that lacked the caspase-3 knockout [214]. Additionally, NIT-1 insulinoma cells treated with IL-1β + IFN-γ + FasL showed an increase in caspase-3 like activity, as compared with NIT-1 cells treated with cytokines alone [197, 215]. Finally, βLox5 is one of few available cell lines that is derived from adult human β-cells. When these IL-1β-resistant cells are made deficient in mitochondrial DNA, they are still sensitive to Fas-mediated killing, which is blocked upon addition of a pan-caspase inhibitor to the treatment. Although there was no negative control for comparison, these data indicate that in the absence of the mitochondrial pathway, Fas-mediated β-cell death can proceed in a caspase-dependent manner [210]. Although the above-mentioned elements appear to be critical for the effector function of β-cell Fas-FasL interactions, it is still unclear if Fas plays a role in any stage of β-cell death.

IV. Effect of Fas Signaling on Healthy β-Cell Function

A large proportion of Fas literature investigates its role in AICD and a growing body of studies analyzes its role in pathogenic β-cell death, which together and in addition to others characterizes Fas-FasL signaling as a mostly pro-death event. However, Fas-FasL interactions can promote survival, regeneration, and proliferation in some tissues [216]. This idea was extended to the β-cell, where two

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groups in particular analyzed the role of Fas in both healthy and pathological β-cell function by use of the Cre-loxP Fas deletion model [150, 155]. Schumann et al. crossed the RIP-Cre^{+}Fas$^{fl/fl}$ deletion into mice of the NOD.C57BL/6j background and reported a surprising finding in that absence of Fas signaling impaired glucose tolerance and lowered insulin levels. They speculated on a role for Fas in β-cell secretory function [150]. This investigation was conducted by the same group that earlier published a study characterizing a dual role for Fas signaling in the β-cell; Fas β-cell expression was previously shown to be induced by glucose [184], which also directs expression of the caspase-8 inhibitor FLIP [213]. A dual role for Fas was proposed in the latter study, where increased FLIP expression promoted Fas-mediated proliferation and decreased FLIP expression caused Fas signaling to promote cell death, all of which were dependent on islet exposure to high concentrations of glucose [213]; aging was also correlated with enhanced death in cells exposed to high glucose concentration [192]. These were followed up by Schumann et al., who showed that transfection of INS-1E insulinoma cells or mouse islets with FLIP promoted mRNA expression of pro-survival factors, such as PDX-1, insulin, and NF-κB; while Fas separately caused similar effects in this study, the dependence on Fas for the FLIP phenotype was not directly measured [150].

Another group, using the RIP-Cre^{+}Fas$^{fl/fl}$ mice of the 129J-C57BL/6 genetic background, reported opposing findings in that these mice had higher glucose tolerance and GSIS than their wild-type counterparts, although both wild-type and RIP-Cre^{+}Fas$^{fl/fl}$ mice had similar insulin content and total pancreatic content [155]. Absence of Fas signaling promoted β-cell function and Fas was not required for islet β-cell development, as has also been suggested by other groups [148]. Moreover, there was no change in incidence of multiple low-dose streptozotocin (MLDS)-induced diabetes between wild-type and RIP-Cre^{+}Fas$^{fl/fl}$ mice, indicating that Fas-FasL signaling is not critical to MLDS-induced autoimmune diabetes [155]. Choi et al. theorized that this disparity might result from the separate genetic backgrounds of the mice used in both studies, which is a likely possibility. Furthermore, the method of

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diabetes induction was different in each of these studies, where Schumann et al. used mice of the NOD.C57BL/6j background and Choi et al. induced T1DM with MLDS; however, the separate methods of diabetes induction still wouldn't explain the disparity between the studies in secretory function.

V. Transgenic FasL as a Means of β-Cell Protection

Whereas some groups have attempted to characterize a role for Fas-FasL interactions in β-cell pathology, others have taken a different approach by attempting to determine if Fas-FasL can confer β-cell protection by deletion of autoreactive Fas-expressing immune cells. Although the ability of islet β-cells to express FasL remains controversial [131, 171, 176, 179, 180, 185], its well-characterized role in maintaining immune privilege of some tissues [67, 68] encouraged experimentation with ectopically expressed β-cell FasL to render the β-cell an immune-privileged site and protect it from autoimmune destruction. Its requirement for corneal graft survival [70] also indicated FasL as a therapeutic target for successful transplantation of organ and islet allografts. Furthermore, the possibility that animals transgenic for FasL could essentially have an endogenous means of immunosuppression at sites that do not naturally confer immune privilege was extraordinarily appealing [217]. However, FasL overexpression also presents the risk of inducing cell death in any Fas-expressing cell located within proximity of ectopic FasL.

Indeed, experimentation with mice transgenic for FasL, however, did not yield the expected protective outcome. NOD mice that express FasL under control of either the human insulin promoter (HIP) or RIP, conferring β-cell-specific gene expression, developed diabetes at an accelerated rate as compared with their nontransgenic littermates [25, 139, 218, 219], and the rate of acceleration between transgenic strains was dependent upon the baseline level of FasL expression [25, 220]. Corroborating well with these findings, C57BL/6 mice transgenic for human FasL (hFasL) and induced to diabetes with MLDS developed the disease at an accelerated rate relative to their non-transgenic littermates [196].

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Ectopic FasL-driven accelerated disease development was particularly evident amongst transgenic NOD males, which develop spontaneous diabetes at a more delayed rate than female NOD mice, but develop accelerated diabetes at a similar rate as transgenic females. This diabetes acceleration was abolished, however, with addition of the dominant-negative *lpr^cg^* transgene. RIP-FasL- *lpr^cg^* mice developed spontaneous diabetes at a slightly decreased rate relative to NOD-wt mice, but after adoptive transfer of diabetogenic splenocytes into NOD-RIP-FasL mice, introduction of the *lpr^cg^* mutation was only able to reduce diabetes incidence to levels comparable to that of wild-type mice [139], and also indicates that Fas-independent mechanisms are employed in the adoptive transfer model of diabetes induction. Additionally, HIP-FasL mice of a non-diabetes-prone genetic background did not develop the accelerated spontaneous diabetes seen in NOD mice [218]. This indicates that the NOD, or diabetes-prone-background, is required for the accelerated diabetes caused by ectopic FasL expression; without chemically-induced or immune-mediated islet inflammation, Fas has no way of being induced to the β-cell surface and thus ligating FasL to initiate cell death. Finally, mice virally induced to develop T1DM by β-cell-specific expression of the nucleoprotein of lymphocytic choriomeningitis virus (RIP-LCMV) and carrying ectopic FasL (RIP-FasL) had a significant reduction in diabetes as compared to their non-RIP-FasL littermates [221]. Although this turned out to be due to FasL-mediated killing of autoreactive CD8+ lymphocytes, RIP-FasL mice infected with LCMV but lacking the nucleoprotein still developed diabetes. Significantly, the LCMV virus induced β-cell Fas expression; thus, this diabetes was caused by β-cell fratricide and exemplifies the central problem inherent to ectopically expressed β-cell FasL.

Accelerated rejection was also demonstrated amongst islets that were grafted from transgenic donor mice and transplanted under the renal capsule of recipient mice, an outcome that could be attained with either mFasL or sFasL [222]. In this scenario, FasL islet expression was driven by either the rat insulin promoter or a replication-deficient adenoviral vector containing FasL cDNA (AdFasL). In comparison with non-transgenic littermates or littermates transduced with an adenoviral

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vector lacking FasL cDNA, RIP-FasL or AdFasL islets were rapidly infiltrated and destroyed [220, 223, 224]. To corroborate these findings, diabetic NOD recipient mice transplanted under the kidney capsule with syngeneic islets maintained normoglycemia for 7-14 days, whereas treatment with a neutralizing anti-FasL Ab extended this period to 30 days [158]. In addition, administration of a neutralizing anti-Fas Ab to female NOD mice at 2-4 weeks of age almost completely prevented spontaneous diabetes onset and delayed its incidence when administered at 5-15 weeks old [151]. However, protection from accelerated islet rejection could be attained when the islets were co-transplanted with FasL-expressing myoblasts or testicular allografts, which may allow for continued interest in FasL as means of protection for islet transplantation [225, 226].

Interestingly, AdFasL islets from MRL-*lpr/lpr* mice and their Fas-sufficient counterparts were rejected in the same amount of time, which would suggest that FasL-expressing islets are not killed by autocrine interaction between islet Fas and FasL [224]. *In vitro*, however, treatment of RIP-FasL islets with IL-1β and IFN-γ resulted in an almost three-fold increase in cell death as compared with nontransgenic islets and was prevented by treatment with an antagonist anti-FasL Ab, which would suggest that cytokines upregulate β-cell Fas, hence making the cell susceptible to autocrine Fas-FasL interaction [219, 223]. These discrepant results could be rationalized by a different mechanism of action between RIP-FasL and AdFasL islets, but has not been conclusively determined. Finally, the genetic background of both donor and recipient mouse appears to plays a critical role in determination of whether an islet transplant will be successful or rejected. Whereas syngeneic grafts from nontransgenic C57BL/6 mouse donors had very little islet graft infiltration, grafts from RIP-FasL mice had undergone infiltration and some had already been rejected. In contrast, allogeneic grafts from both nontransgenic and transgenic hosts were rejected [220], and indicates that the donor and recipient must be genetically identical for successful graft transplantation but that, contrary to contributing to transplant success, islet FasL expression promotes rejection.

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The findings in transgenic mice and FasL-expressing islet grafts bring into question the difference in mechanism between naturally expressed ectopic FasL and transgenic FasL and why they confer such different outcomes. FasL islet expression appears to not only provoke acceleration of diabetes, but also is not an appropriate therapeutic target for islet grafting. Based on the herein reported results, mice transgenic for β-cell FasL may be susceptible to autocrine Fas-FasL interaction after FasL-mediated neutrophil recruitment and subsequent Fas β-cell-surface activity.

In opposition to transgenic β-cell Fas expression, silencing of Fas expression by siRNA technology may be a potential route to therapy. NOD mice were significantly protected from CY-induced diabetes when a systemic non-viral Fas siRNA was administered intravenously at the time of CY administration, but it is not known if these mice also carried the abnormal phenotype conferred by the *lpr/lpr* Fas deficiency mutation. The potential role for Fas in insulitis was reiterated when Fas siRNA was administered to these mice during the β-cell death phase of CY-induced diabetes, where there was no protection from diabetes [227]. This also indicates that therapies against Fas-FasL interactions might be most effective when targeting the insulitic phase of β-cell death.

VI. Conclusion

Fas-FasL signaling is fundamental to maintenance of homeostasis in multiple tissues. Binding of FasL to its receptor is characterized by autocrine or paracrine interaction, of which is largely determined by the ability of the tissue in question to express the receptor, ligand, or both [8]. Any tissue that can express cell surface Fas is vulnerable to death by ligation of FasL, and FasL expression is therefore strictly limited to activated T and NK cells, in addition to its constitutive expression on tissues of immune privilege. However, this concept of FasL-mediated immune privilege appears to be limited to specific tissues, in that ectopic FasL expression on pancreatic islets actually results in accelerated diabetes

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development, in stark contrast to the intended purpose of protection from autoimmune invasion [25, 139, 218, 219].

Perhaps the most critical role for Fas signaling is in peripheral selection and maintenance of immune homeostasis, as was revealed by the abnormal hematopoietic cell repertoire in mice with a systemic deficiency of Fas (*lpr/lpr*) or FasL (*gld/gld*). These mice exhibited a number of abnormal phenotypes, such as massive lymphoproliferation and accumulation of non-malignant double-negative T cells in the spleen and lymph nodes. Following these findings, investigation into the role for Fas signaling in β-cell death was sparked with the demonstration that mice carrying the *lpr/lpr* and *gld/gld* mutations were completely protected from autoimmune diabetes development [24, 25, 138] and NOD-*lpr/lpr* mice adoptively transferred with diabetogenic cells failed to develop diabetes, which together cast a critical role for Fas signaling in β-cell death. However, it was soon realized that this protection from diabetes did not result from absence of Fas signaling on the β-cell, but rather as a result of the lymphoid abnormalities conferred by absence of Fas on the hematopoietic cell surface, and prevented the autoimmune response required for T1DM initiation. NOD-*lpr/lpr* mice also express high levels of FasL, which was likely killing adoptively transferred splenocytes before they could initiate diabetes in the recipient mouse [137, 140]. Although Fas β-cell studies using only the systemic *lpr/lpr* or *gld/gld* mutation are not applicable to determining its role in β-cell pathology, interest in Fas as a β-cell death effector lingered with the demonstration that pro-inflammatory cytokines, namely IL-1β, induced mRNA and cell-surface β-cell Fas expression [23] and that β-cells isolated from newly-diagnosed diabetes patients had abundant β-cell-surface Fas expression [131, 171]. However, the multiple genetic backgrounds comprising the *in vivo* and *in vitro* Fas β-cell death research, in combination with numerous mutations and manipulations, have resulted in a convoluted and unclear role for Fas signaling in β-cell death.

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There exists a broad repertoire of studies that have concluded some role for Fas signaling in β-cell death. Although the extent of its role varies between studies, there seems to be a general consensus that more than one death effector participate in β-cell death, and confers both Fas-dependent and Fas-independent mechanisms of death. For example, sublethally irradiated NOD-*scid/scid-lpr/lpr* mice reconstituted with diabetogenic spleen cells had a significant reduction in diabetes incidence relative to NOD-*scid/scid* counterparts that were sufficient in Fas; this suggests a Fas-dependent mechanism of disease development. However, a level of disease development still occurred in the Fas-deficient mice, which also suggests Fas-independent T1DM development [138, 139]. TCR transgenic mouse with a rearranged TCR specific to the $CD8^+$ T cell population, conferring $CD8^+$-mediated diabetes, found that the perforin/granzyme signaling pathway comprises the major effector in β-cell death, but in the absence of perforin, Fas signaling is also capable of executing β-cell death [161-163]. Also in $CD8^+$ and one model of $CD4^+$ transgenic mice, there were reports that the Fas signaling pathway is responsible for the initiation phase of β-cell death, whereas the perforin/granzyme pathway is responsible for the effector phase [145, 151]. Furthermore, one of the first studies that analyzed the contribution of perforin/granzyme to β-cell death found that perforin-deficient mice developed insulitis but not overt diabetes, supporting this role for Fas in the initiation phase of diabetes development [6]. These findings were also maintained by a study in which female NOD mice administered with a neutralizing anti-Fas Ab at 2-4 weeks old were almost completely protected from spontaneous diabetes, and when administered at 5-15 weeks they had a delayed incidence. In contrast, when the neutralizing Ab was administered at a later time when β-cell death was occurring, diabetes incidence was unaffected [151]. Together, these findings strongly suggest that the perforin/granzyme signaling pathway comprises the effector phase of β-cell death, and that Fas signaling is likely responsible for the initiation phase of this process.

Contrary to the above, however, a significant proportion of Fas β-cell research has indicated that β-cell death and development of T1DM are completely independent of Fas signaling. NOD-*scid/scid*-

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gld/gld mice reconstituted with wild-type splenocytes did not develop diabetes even though the recipients were Fas-sufficient and the adoptively transferred splenocytes were FasL-sufficient [141]. NOD mice deficient in IL-1β and thus unable to induce Fas to the β-cell surface also did not have decreased diabetes incidence relative to NOD-wt littermates [144]. Further, an *in vitro* model of transgenic $CD4^+$-induced diabetes revealed that treatment of NOD-*lpr/lpr* islets with the $CD4^+$ T cell clones elicited a similar killing efficiency as NOD-wt islets [151]. β-cell-specific deletion of Fas gene (RIP-$Cre^+Fas^{fl/fl}$) on the 129J-C57BL/6 mouse genetic background did not confer any protection from diabetes induced with MLDS relative to their Fas-sufficient counterparts [155]. These more recent results from a highly tissue-specific model diminish the likelihood of a critical role for Fas signaling during autoimmune-mediated β-cell death. Moreover, diabetes incidence was not affected upon injection of a neutralizing anti-FasL Ab after disease induction with either CY or adoptive transfer of diabetogenic splenocytes [152]. It does, however, bear mention that blocking the Fas pathway in the early stages of CY-triggered diabetes prevents diabetes incidence [143], not by blocking Fas β-cell signaling, but likely by preventing the T cell deletion that CY should confer [152, 228].

Nevertheless, Fas might still play a critical role in development of T1DM, not through its signaling in β-cells but from its altered signaling in the hematopoietic cellular population, which could result in dysfunctional AICD of autoreactive T lymphocytes localized to the pancreatic islet [229]. Indeed, newly-diagnosed diabetes patients have significantly decreased Fas expression on the surface of T and B cells relative to healthy controls [230] and T lymphocytes from NOD mice have demonstrated a resistance to AICD [231]. Also in support of this idea, treatment of NOD mice with an agonist anti-Fas Ab conferred resistance to diabetes; immunohistochemical analysis of pancreatic samples from these animals showed that infiltrating cells and not insulin-positive cells were being targeted by the Ab (data not shown)[182], although how the antibody selectively targeted hematopoietic cells and not endocrine cells is unclear. Furthermore, adoptive transfer of diabetogenic splenocytes that were pre-treated with a

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fusion protein of FasL bound to streptavidin (conferring an effective ability to induce cell death in diabetogenic cells) into NOD female mice resulted in a significantly delayed incidence of diabetes [160]. Similar results were also demonstrated in mice injected with an AdCTLA4-FasL fusion protein, when the mice were induced with MLDS and failed to develop diabetes; additionally, splenocytes from these mice could not induce diabetes in syngeneic recipients [156]. Taken together, targeting the autoreactive T cell repertoire by administration of an agonist Ab for Fas or soluble Fas ligand might be an effective therapeutic intervention. However, the treatment would have to be specific for the target population, as any cell that constitutively expresses Fas would be otherwise vulnerable to death, potentially resulting in severe side effects and even death [40, 81].

Islet β-cell death studies are conducted in a wide variety of *in vivo* and *in vitro* models that together comprise a number of genetic backgrounds, some of which are described above for investigation of Fas-FasL signaling in β-cell death and development of T1DM. This range of model systems can confer separate mechanisms of general function and death, as has been illustrated in the numerous Fas β-cell death studies that are unable to come to a consensus on the role for Fas signaling in β-cell death and T1DM development. Ultimately, the use of one single and appropriate model system would likely diminish this disparity in reported findings. A possibility of such is the Cre-loxP recombination system, whereby Fas is the target gene and Cre recombinase expression is driven by the RIP to confer β-cell-specific gene deletion; this would present a simple and effective model system for use in Fas β-cell research. Two groups have already used Cre-loxP gene targeting to analyze the role for Fas in insulin secretion (NOD genetic background) [150] and β-cell pathology (non-NOD genetic background) [155], but reported contrasting findings. Investigators have yet to explore β-cell pathology using Cre-loxP gene targeting on the NOD mouse background, which would confer β-cell-specific Fas deletion in a mouse model of spontaneous diabetes. This type of study design holds promise for the future of Fas β-cell research.

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Finally, although the NOD mouse is an extraordinarily useful model for the study of T1DM, it likely has a separate mechanism of β-cell death relative to humans; this is exemplified by the numerous cures identified in the NOD mouse, of which are yet to be successfully translated to a human T1DM patient [232]. Use of human islets for such studies may be the preferred genetic model for two fundamental reasons: 1) Use of one genetic model for all T1DM studies might eliminate the disparity between studies, and 2) Results from human islet studies are more likely to translate to a living human. However, human pancreatic biopsy sampling is exceptionally difficult and costly due to the inaccessible physiology of the pancreas. Although research into the mechanism underlying β-cell death has progressed significantly in recent years, an ideal model system has yet to be developed that can accurately translate experimental findings to a safe method of T1DM prevention.

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APPENDIX

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Classification of Literature Review Style

This literature review is categorized as a narrative review (vs. systematic) for the following reasons:

1. Critically analyzes and discusses the current state of a specific science topic.
2. Does not use a methodological approach in which readers could reproduce the data reported solely by reading the review document.
3. Narrative approach summarizes relevant primary literature. See **Figure 3** for method of article selection.
4. Qualitative vs. quantitative approach.

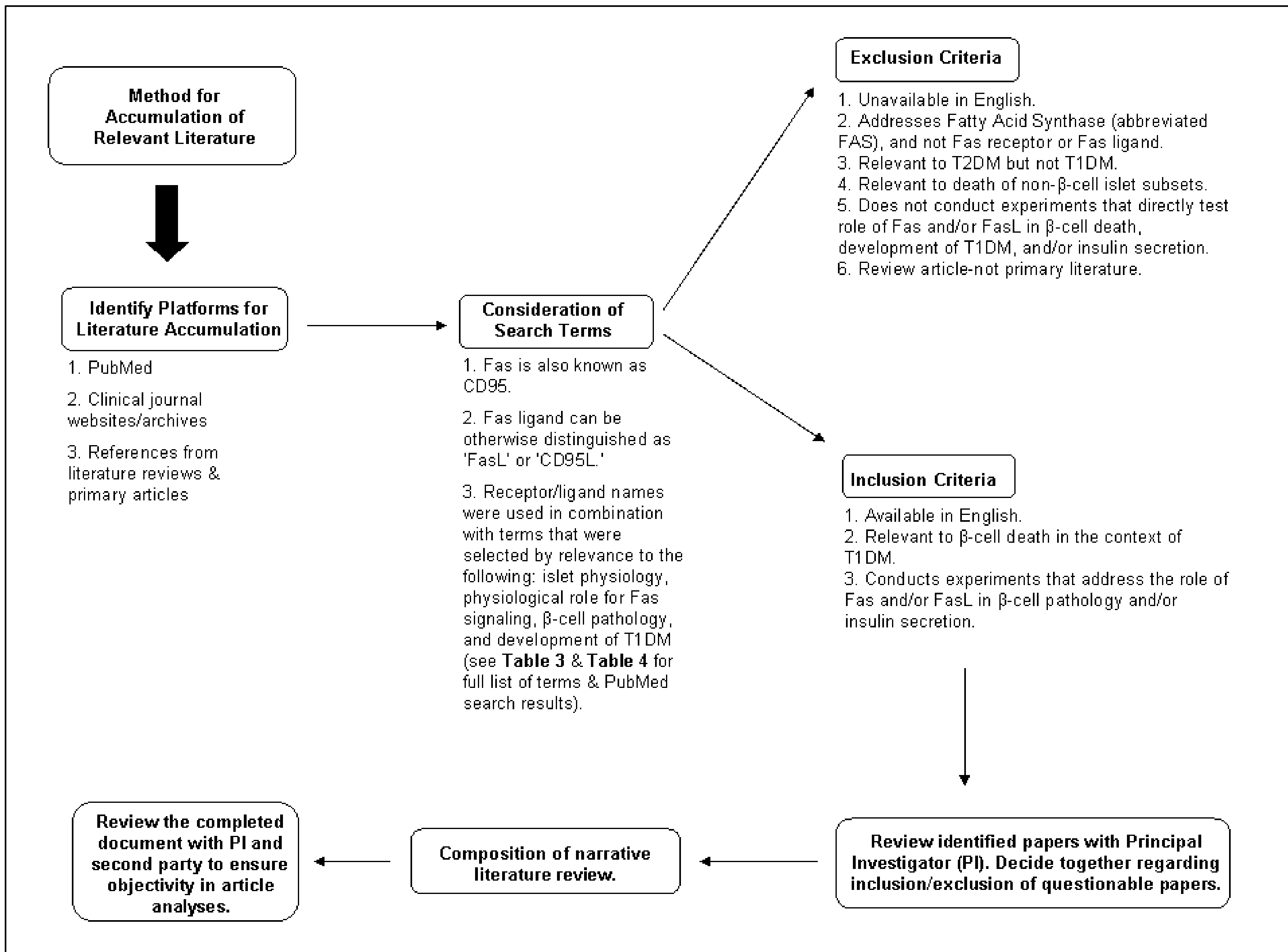

Figure 3. Method for accumulation of articles to be investigated in literature review.

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Table 3. Compilation of Fas β-cell death-relevant search terms and PubMed results for both primary literature and reviews.

	All Articles	Review
Fas + Islet	207	11
FasL + Islet	95	5
Fas ligand + Islet	121	9
Fas + β cell	1630	182
FasL + β cell	417	45
Fas ligand + beta cell	729	83
Fas + diabetes	662	84
FasL + diabetes	155	23
Fas ligand + diabetes	241	38
Fas + Type 1 diabetes	215	30
FasL + Type 1 diabetes	94	15
Fas ligand + Type 1 diabetes	124	21
Fas + T cell + diabetes	151	25
FasL + T cell + diabetes	72	12
Fas ligand + T cell + diabetes	102	20
Fas + T cell + T1D	103	17
FasL + T cell + T1D	51	9
Fas ligand + T cell + T1D	68	13

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Table 4. Compilation of CD95 β-cell death-relevant search terms and PubMed results for both primary literature and reviews.

	All Articles	Review
CD95 + Islet	89	6
CD95L + Islet	101	5
CD95 Ligand + Islet	112	7
CD95 + β cell	808	67
CD95L + β cell	596	55
CD95 Ligand + β cell	668	66
CD95 + diabetes	221	29
CD95L + diabetes	206	31
CD95 Ligand + diabetes	226	35
CD95 + Type 1 diabetes	121	17
CD95L + Type 1 diabetes	106	17
CD95 Ligand + Type 1 diabetes	119	20
CD95 + T cell + diabetes	86	15
CD95L + T cell + diabetes	87	17
CD95 Ligand + T cell + diabetes	97	20
CD95 + T cell + T1D	60	11
CD95L + T cell + T1D	58	11
CD95 Ligand + T cell + T1D	65	13

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VITA

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