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Immune-mediated β-cell death in type 1 diabetes: lessons from human β-cell lines

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Abstract

Type 1 diabetes (T1D) is a chronic, multifactorial disorder that results from a contretemps of genetic and environmental factors. Autoimmune attack and functional inhibition of the insulin-producing β cells in the pancreas lead to the inability of β cells to metabolize glucose, and thus results the hallmark clinical symptom of diabetes: abnormally high blood glucose levels. Treatment and protection from T1D require a detailed knowledge of the molecular effectors and the mechanism(s) of cell death leading to β-cell demise. Primary islets and surrogate β cells have been utilized *in vitro* to investigate in isolation-specific mechanisms associated with progression to T1D *in vivo*. This review focuses on the data obtained from these experiments. Studies using transformed β cells of human sources are described.

Keywords

Autoimmunity; human β-cell lines; islets; type 1 diabetes; β cells; β-cell killing

Type 1 diabetes

Type 1 diabetes (T1D) arises because of unchecked aberrant immune responses against an individual's own insulin-producing β cells, ultimately leading to exogenous insulin dependence. T1D accounts for 5–10% of reported cases of diabetes. While prevalence and incidence vary by region, global incidence of T1D is increasing globally. During the course of the disease, T cells become reactive to β-cell antigens and islet cell antibodies produced by B cells are also detected. High titres of autoantibodies correlate with T1D, making them valuable prognostic markers for disease risk. Subsequent to the measurement of autoantibodies against β-cell antigens, a decline in first phase insulin release (FPIR) in response to glucose challenge can be measured in those at risk for developing T1D, allowing further risk to be determined [1]. However, the development of the destructive pathological lesion, known as insulitis, and the steps leading to T1D in humans are not well understood. Identification and study of immune cell infiltration in patients with T1D has been problematic [2]. Consequently, most of our knowledge of the pathology of T1D stems from animal models that develop autoimmune T1D-like disease, either spontaneous autoimmune or experimentally induced, as well as from *in vitro* studies using primary islets and β-cell lines, from human and murine sources. The use of *in vitro* models has been particularly advantageous when assessing the specific contributions of individual effector molecules and

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Conflicts of interest and financial disclosures

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molecular pathways to β-cell destruction. A breakdown of self-tolerance renders β cells susceptible to an arsenal of immune cells and their killing mechanisms, each must then be analysed independently to formulate targeted treatment options. In addition, given the differences between human and rodent β cells, efforts should be redirected to focus on human β-cell surrogates as targets in cytotoxicity assays. This review describes the current information we have gained about β-cell death mechanisms in human T1D development from *in vitro* killing assays of primary human islets and human β-cell lines, highlighting the limitations of the models as well as potential uses of the systems outlined.

Cellular effectors in T1D development

Both B and T cells respond and gain effector function against β-cell antigens in patients with T1D. B cells, in addition to their antibody-secreting actions, are important antigen-presenting cells (APCs). Human studies have demonstrated a role of B cells as APCs in T1D [3]. In contrast, the presence of autoantibodies, while useful markers for T1D risk [4] as they indicate autoreactive T-cell activation, do not appear to be directly pathogenic to β cells [5]. Immunohistological examination of pancreatic tissues from patients with T1D has demonstrated that, in contrast to the animal models of spontaneous T1D, insulitis is a rare event in humans [2]; when present, the following cell types have been identified in the islets: lymphocytes that consisted mostly of $CD8^+$ T lymphocytes (CTL) but include B cells as well as $CD4^+$ T cells, macrophages and dendritic cells (DCs) [6–8]. Unfortunately, human samples with established T1D do not delineate the successive events that culminate in autoreactive lymphocyte activation and β-cell killing, and only recently has information emerged on the nature of insulitis in T1D-free autoantibody positive organ donors [2,9,10]. In one study, only two of 62 autoantibody-positive individuals' organ donors without a diagnosis of T1D showed signs of insulitis [9]. These two cases represented individuals who were positive for at least three autoantibodies. The infiltrating immune cells were mostly CTL and macrophages with minor representation of B cells and $CD4^+$ T cells; however, islets exhibiting insulitis represented a minority of the total islets (<10%). These results highlight that even when obtained from at-risk individuals, donor organs or biopsy samples rarely exhibit insulitis, creating difficulty for the study of cellular events leading to autoimmune-mediated β-cell death [2,9,10].

Molecular mechanisms of β-Cell death: *in vitro* killing of human pancreatic islets

$CD8^+$T lymphocytes, widely considered as final effectors for T1D, represent the largest population of cells within the insulitic infiltrates. However, little is known about the mechanisms involved in the killing of human islet β cells by autoreactive CTL, and direct evidence for the impact of T cells in T1D development only exists in animal models[11]. Nonetheless, autoreactive effector CTL that recognize β-cell-derived antigens can be detected in humans[10,12]. One of these epitopes, $IGRP_{265-273}$(islet-specific glucose 6 phosphatase catalytic subunit-related protein), elicits a T-cell response in NOD mice and in humans[13]. T-cell responses to proinsulin, aninsulin precursor, have also been detected in patients with T1D[14]. Preproinsulin-specific CTL required cell-to-cell contact to selectively lyse β cells in dispersed human islet preparations; however, the mechanism of killing was not investigated further [15].

Thus far, mechanistic studies involving CTL killing of human islets have been accomplished using viral-specific CTL clones and human islets pulsed with the appropriate viral peptide [16]. In the absence of cytokines, peptide-specific, HLA-restricted killing of human islets was found to be perforin dependent. Upregulation of surface Fas expression on the target cells required pretreatment of the islets with the proinflammatory cytokines interleukin 1

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beta (IL-1β) and interferon gamma (IFNγ). Further, blocking FasL expression on the CTL did not improve target cell viability. Interestingly, pan-caspase inhibition failed to protect human islets from CTL-mediated killing, indicating that perforin-induced killing of human islets by the virus-specific CTL occurs through caspase-independent pathways [16]. Moreover, although the islet cells were specifically lysed, in these experiments, peptide pulsing of the islet cells did not allow for β-cell specificity, as all the cells within the islets would have presented the exogenously added peptides. In addition, the high affinity of these virus-specific CTL for viral peptides does not represent the weak interaction between autoreactive T-cell receptors (TCRs) on CTL and presented β-cell antigens [17]. As it has been shown to be the case in animal models of T1D, killing of human β cells is expected to occur through a range of redundant and compensatory mechanisms; thus, combination interventions will likely be necessary to prevent human β-cell destruction.

β-Cell lines for *in vitro* CTL studies

Primary islets are composed of a mixture of hormone-producing cells; however, β cells are selectively destroyed in T1D. Therefore, β-cell lines represent valuable tools in understanding and preventing autoimmune β-cell destruction. While rodent β-cell lines have proven most useful in immunological studies because of the inherent phenotypic instability that limits their use in functional studies [18], significantly less work has occurred with human β-cell lines. The ideal *in vitro* human β-cell model should remain stable with the passages and maintain insulin secretion in response to glucose stimulation, as well as preserve the expression of other β cell-specific markers and autoantigens. Several human β-cell lines have been established to date; these cell lines are HP62 [19], CM [20], NES2Y [21], β Lox5 [22], NAKT-15 [23] and EndoC-β H1 [24] (Table 1), as well as the fusion cell lines 1·1B4, 1·1E7 and 1·4E7[25].

As discussed below, most of the human β-cell lines available do not serve as models of normal β-cell function but have been useful for specific *in vitro* assays (Table 2). The recently developed insulin-secreting EndoC-β H1 cell line is a promising tool for both immunological and functional studies, as these cells maintained glucose-stimulated insulin secretion (GSIS) after 75 passages [24]. EndoC-β H1 cells were generated from human foetal pancreas cells that were transduced to express the SV40 T antigen under the control of the rat insulin promoter; as a result, only insulin-producing cells became immortalized. The reversibly immortalized NAKT-15 cell line [23] was originally reported to be a functional human β-cell line that displayed molecular characteristics of pancreatic β cells, maintained insulin secretion and reversed chemically induced diabetes in mice. Unfortunately, since the initial report with NAKT-15 [23], no further studies have been published.

A different approach was used to develop the 1·1B4, 1·1E7 and 1·4E7 [25] cell lines. Glucose responsiveness was achieved after electrofusion of human islets with the exocrine tumour-derived PANC-1 cell line [26]. Of the three fusion cell lines, 1·1B4 cells demonstrated the best secretory response to glucose challenge, with a stimulation index of approximately 2. Pseudo islet formation with 1·1B4 cells did not enhance insulin content of the cell line but did improve GSIS [27], emphasizing the importance of islet structure integrity for optimum secretory function. The usefulness of the 1·1B4 cell line for *in vitro* immunological assays has not yet been tested.

Created two decades ago, the HP62 cell line was obtained from the transfection of human islet cells with a plasmid vector encoding SV40 viral DNA [19]. Early passages of these cells secreted glucagon and somatostatin (passage 3); insulin was later detected, but secretion, or synthesis alone, was short lived (passages 6 and 7, respectively). Interferon gamma (IFNγ) priming of HP62 led to an increase in the expression of HLA Class II

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molecules [19], suggesting a possible application of this cell line in immunological studies. Consequently, the HP62 cell line has since been used to study the cytotoxicity and modulatory effects of cytokines on the expression of adhesion molecules that facilitate contact between CTL and target cells [28,29]. Because of its endocrine lineage, HP62 cells have also been useful in confirming the expression and functionality of endotoxin receptors measured in isolated human islets [30]. Previously, sera from patients with T1D containing islet cell antibodies (ICA) failed to react with this cell line [31], casting doubt on its antigenicity and β-cell likeness. Still, along with the human insulinoma cell line, CM [20], HP62 cells have continued to be tested as β cells in cytotoxicity assays, with responses similar to those of primary islet cells [32,33].

The CM cell line was generated from a patient with a malignant pancreatic insulinoma [20]. Although CM cells lose insulin secretion with long-term passage, the cell line retains many β-cell-specific characteristics [34]. CM cells grown in medium containing high glucose-stimulated proliferative responses of T cells isolated from patients with T1D to a greater extent when compared to control subjects; the same pattern was not observed in low glucose conditions [35], but the findings further suggested their usefulness in cell-mediated lymphocytotoxicity (CML) assays. Indeed, glutamic acid decarboxylase (GAD)-specific $CD8^+$ CTL expanded from patients with T1D lysed both CM and HP62 in ^{51}Cr release assays [33]. The mechanism of CTL killing was not elucidated; however, transfection of the cell lines with B7-H4, an inhibitory co-signal molecule expressed on the cell surface, decreased the percent specific lysis measured, indicating that the GAD-reactive T cells killed the β cells by a mechanism requiring direct cell contact. The CM insulinoma cell line has been valuable as a β-cell model in a wide range of *in vitro* assays [32,33,35–39], including studies aiming to understand the effect of genetic splice variants on β-cell function [40], but not without criticism [41,42]. In addition to poor GSIS, several chromosomal abnormalities were noted in CM cells. While genetic defects in the CM cell line are not surprising because of their tumourigenic source [43], these cells are likely most beneficial when analysing killing mechanisms utilized by autoreactive immune effectors to destroy human β cells, rather than in functional assays.

Another pancreatic cell line, NES2Y, was derived from the islets of a patient with persistent hyperinsulinemic hypoglycaemia of infancy (PHHI). Similar to the β cells of PHHI patients, NES2Y cells constitutively secrete insulin due to loss-of-function mutations resulting in defective ATP-sensitive potassium (K_{ATP}) channel activity [21]. NES2Y cells also lack expression of the homeodomain transcription factor, PDX-1 [21]. These cells have been useful in determining the contribution of normal calcium signalling and PDX-1 expression / function within the β cells [44–46]. Transfection studies with genes encoding the K_{ATP} channel subunits [sulfonylurea receptor (SUR) 1 and Kir6·2] and PDX-1 not only rescued GSIS and glucose-regulated insulin promoter activity [47], but also highlighted the requirement of both SUR1 and Kir6·2 for K_{ATP} channel function [45], as well as the role of PDX-1 in the control of insulin gene transcription upon glucose stimulation [44]. The functional outcomes of mutations in the PDX-1 gene, associated with maturity-onset diabetes of the young (MODY), have also been studied in NES2Y cells transfected to express normal or mutant forms of this gene, where the disease-associated variants resulted in reduced insulin gene transcription compared to wild-type PDX-1 [48]. Despite the aforementioned dysfunctions, NES2Y cells have been utilized as human β cells in cell death assays relevant to both type 1 and type 2 diabetes (T2D).

Mechanisms of fatty acid-induced toxicity, unrelated to immune-mediated cytotoxicity in T1D but linked to β-cell death in T2D, were previously tested in these cells [49,50]. T2D-associated insults led to activation of the caspase cascade and endoplasmic reticulum (ER) stress induction in NES2Y cells. In the context of T1D, NES2Y membranes successfully

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induced proliferation of autoreactive T cells isolated from new onset patients with T1D who were selected for granule membrane reactivity [51], making this cell line an attractive target of autoreactive CTL. NES2Y cells, like CM cells, have also been used to study regulatory components that protect against apoptotic stimuli, namely tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) [38,39]. TRAIL signalled for cell death through Fas-associated death domain (FADD) to activate caspases in NES2Y and CM cells, and also induced NFκB activation and mitochondrial damage. NES2Y and CM cells responded similar to primary human islets in inhibition studies whereby the overexpression of the anti-apoptotic molecules Bcl-2 and the X-linked inhibitor of apoptosis (XIAP) prevented TRAIL-mediated cell death [38,39]. Efforts are still underway to create a physiologically relevant β-cell line from NES2Y cells by fusing the cell line with primary human islet β cells [52]. These β-cell hybrids represent a potential source of glucose-responsive β cells that are capable of promoting an immune response [51].

Transformation with oncogenes and enhanced telomerase activity (SV40 T antigen, Ras^{val12}, and hTRT) of adult pancreatic islets enriched for β cells led to the development of the β Lox5 cell line [22]. β Lox5 cells are not responsive to high glucose challenge with increasing passage number; however, this cell line can be manipulated to regain insulin secretory function through the combination of promoting cell–cell contact, inducing PDX-1 expression, and supplementation of exendin-4 [a homologue of the insulin secretagogue glucagon-like peptide (GLP-1)] in the growth media [53].

βLox5 cells express Fas receptors on the cell surface, adding to its worth as a CTL target [54]. To mimic Fas-mediated CTL killing of human β cells, β Lox5 cells were incubated with IFNγ (to increase surface Fas) and CH-11, a human anti-Fas antibody, and it was found that these cells died by caspase-dependent apoptosis after Fas stimulation [54]. IFNγ was also required for Tumour Necrosis Factor alpha (TNFα) killing of β Lox5 cells [54]. Therefore, direct CTL killing mechanisms can be tested further with the β Lox5 cell line.

Soluble mediators in T1D onset

In addition to the direct β-cell cytotoxicity of effector cells, islet-infiltrating immune cells are also sources of reactive oxygen species (ROS) and proinflammatory cytokines. When compared to other tissues, islets are more sensitive to oxidative stress [55], therefore, it has been proposed that ROS may act as soluble mediators of β-cell death in T1D [56]. Additional cytotoxic free radicals in the prediabetic state are likely generated within the β cells themselves in response to mixtures of monokines and lymphokines [57–59]. Post-mortem pancreatic tissue samples from patients with T1D demonstrate increased interferon alpha (IFNα) expression, and IFNγ-secreting lymphocytes have been identified in the islets [60]. TNFα- and IL-1β-producing macrophages and DCs have been detected in patients with recent-onset T1D [61]. Cytokines are not only able to control the type of immune response mounted, but also prime β cells for heightened immune surveillance and clearance, and in combination are β-cytotoxic [56]. Because cytokines exhibit immunomodulatory effects *in vivo*, their direct cytotoxicity to β cells has been studied *in vitro* with isolated islets and β-cell lines.

In vitro cytokine-induced β-cell killing

Of the cytokines identified *in vivo*, *in vitro* cytokine killing experiments have focused on the individual and combined actions of IFNγ, IL-1β and TNFα. While *in vitro* cytokine-mediated destruction of mouse and rat islets occurs through nitric oxide (NO)-dependent necrosis with almost no contribution from apoptosis [62], preventing iNOS function does not consistently prevent human β-cell death [63]. Furthermore, the responses of primary human islets to cytokines are inconsistent from donor to donor, resulting in the publication

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of a variety of mechanisms as essential for human beta cell death. Some of the variation may result from variability in the expression of heat shock protein 70 (HSP70) in human islets [64], as heat shock alone can prevent NO-mediated cytokine-induced dysfunction and cytotoxicity of rat islets[65]. Human islets are most sensitive to the combination of IL-1β, TNFα and IFNγ [57]. As noted above, variability may further result from the doses of cytokines used as well as the length of time islets are exposed to these soluble mediators. For example, addition of only TNFα to human islets inhibited function and, when combined with IFNγ, reduced islet cell viability with long-term incubation [28]. Of note, when cultured with whole islets, cytokines are not specifically cytostatic or cytotoxic towards β cells [66]. Although this might dispute the cytotoxic role of cytokines *in vivo*, it is possible that *in vivo* autoreactive T cells in very close proximity to their intended target, the β cells, could produce significant damage by cytokine release.

Collectively, cytokine killing assays with isolated human islets suggest that β cells die by necrosis as well as by apoptosis. To understand the cytotoxicity of cytokines specifically on β cells, the described studies have also been performed with β-cell lines. We have used the β Lox5 human β-cell line to study the cell death pathways activated after cytokine (TNFα and IFNγ) incubation. In addition to NO-independent necrotic cell death, mitochondrial-mediated, caspase-dependent and caspase-independent apoptosis was detected [54].

The human islet cell line, HP62, and the human insulinoma cell line, CM, were treated with the cytokines TNFα or IFNγ alone, but not in combination. Only TNFα was cytotoxic to HP62 (~15% cell death) but not to CM, and the mechanism of killing remains unresolved [32]. Other groups have reported that treatment for primary islets with cytokines can trigger different apoptotic pathways. For instance, in one study, cytokine-treated primary human islets died via the intrinsic apoptotic pathway [67], which involves the activation of caspases downstream of mitochondria damage, while another group demonstrated that caspase-independent apoptosis took place (mouse) when the necrotic component was eliminated with iNOS inhibition [68]. Therefore, *in vitro*, cytokines are capable of activating a multitude of cell death pathways that, although not the same in all the models used, ultimately lead to the common outcome of β-cell demise. The common denominator in all *in vitro* models outlined, however, is necrotic cell death. Thus, necrosis, a potent promoter of inflammation, might be the most relevant cell death pathway triggered by cytokines in the context of T1D.

Mitochondria and the mechanisms of cell death

In β cells, mitochondria are essential for several cellular processes. Functionally and structurally sound mitochondria are essential for GSIS. However, these organelles are also important as regulators of cell death. Apoptotic stimuli lead to pro-apoptotic Bcl family member activation, mitochondrial membrane permeabilization, as well as the release of proapoptotic proteins like cytochrome c, apoptosis-inducing factor (AIF) and endonuclease G (Endo G). Pro-apoptotic Bcl family members include Bax, Bak, Bid, Bim, Bad and PUMA. Anti-apoptotic members such as Bcl-2 and Bcl-XL hold these proteins in check. Consistent with this, human islets overexpressing Bcl-2 show reduced susceptibility to the combined actions of IFNγ, IL-1β and TNFα, as measured by decreased DNA fragmentation, cell death and β-cell dysfunction after a 5-day incubation period [69]. Similarly, knockdown of the pro-apoptotic member Bim with siRNA technology decreased IFNγ- and TNFα-induced cell death of dispersed human islets [70]. Primary human islets were incubated with IFNγ and TNFα for 48 h and resulted in nearly a 35% reduction in viability; Bim knockdown rescued viability by 7%. As mentioned earlier, the cytokine combination of TNFα and IFNγ kills the human β-cell line, β Lox5, via the activation of different pro-death pathways. All forms of cell death induced by TNFα and IFNγ appeared to be dependent on functional mitochondria, as mitochondrial DNA (mtDNA)-deficient cells

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were resistant to cytokine killing [54]. Together, these studies support an important role for the mitochondria in β-cell death through the differential activation of pro- and anti-apoptotic Bcl family members by distinct stimuli.

Also known as programmed cell death, apoptosis is an energy-requiring process. Consequently, mitochondria, being the powerhouses of the cell, can regulate the form of cell death through supply of ATP and NADH. Endogenous inhibitors of mitochondrial respiration and production of ATP via oxidative phosphorylation, such as NO, lead to modest decreases in cellular ATP concentrations that may result in a switch from apoptosis to necrosis in metabolically suppressed cells (i.e. β cells in pre-T1D) that have already been signalled for apoptotic cell death. Mitochondria are also major sources of cellular ROS, and mitochondrial respiratory chain inhibition induces ROS production from the mitochondrial electron transport chain complexes I and III. Cellular ROS, regardless of where they are produced, can lead to caspase-dependent apoptosis in β cells [71]. However, although not tested in β cells, high levels of hydrogen peroxide (H_2O_2) have been shown to inhibit caspases [72] and promote necrosis. Therefore, excessive or continued mitochondrial ROS production represents another mechanism by which mitochondria may determine the fate of the cell and the choice of death mechanism.

Concluding remarks

Insulin-producing β cells are specifically destroyed in autoimmune T1D. Resolving the critical molecular and cell death mechanism(s) in T1D will allow the development of targeted therapies toward the prevention of T1D and protection of pancreatic islet grafts. Whereas the cellular effectors important for T1D development have been identified, the molecular mechanisms and the cell death pathway(s) activated by these effectors are controversial and remain under intensive investigation. *In vivo* as well as *in vitro* studies support a role for perforin / granzyme, Fas / FasL, TRAIL / TRAIL Receptors, proinflammatory cytokines and free radicals in β-cell killing. Conducive to the advancement in our understanding of human T1D is further research using primary human β cells as well as the available human β-cell lines. In comparison with primary islets, the human β-cell lines do offer some advantages such as: constant availability, ease of genetic manipulation, and the expression and presentation of autoantigens by known Class I HLA alleles [33,51]. Recognition of the β-cell lines by activated, autoreactive CTL promotes the use of human β-cell lines in cytotoxicity and screening assays that may lead to not only a better understanding of the mechanism(s) of human β-cell death during the progression to T1D, but also to the identification of molecular targets that, when blocked, inhibit CTL-mediated killing. However, these human β-cell lines must be used in parallel with primary human islets so that the mechanisms or molecular targets identified can be confirmed, and thus ensure both the biological and translational significance of the findings.

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Table 1

Human β-cell lines

Cell line	Cell origin	Insulin	GSIS	Method	Ref.
HP62	Pancreatic islets	Early	Early	SV40 T antigen	[19]
CM	Insulinoma	Yes	Early	Malignant insulinoma subculture	[20]
NES2Y	PHHI islets	Yes	No	Continual islet cell subculture	[21]
NISK9	PHHI islets	Yes	Yes	NES2Y transfected with Kir6.2, SUR1 and PDX-1	[47]
βLox5	Adult β cells	Yes	Early	Floxed SV40 T antigen, Ras^{val12} and hTERT	[22]
NAKT-15	Pancreatic islets	Yes	Yes	Floxed SV40 T antigen, hTERT and EGFP	[23]
EndoC-βH1	Foetal Pancreas	Yes	Yes	RIP-SV40 T antigen and hTERT	[24]

PHHI, persistent hyperinsulinemic hypoglycemia of infancy; GSIS, glucose stimulated insulin secretion

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Table 2

Utility of human β-cell lines for *in vitro* cytotoxicity assays

Cell line	T-Cell assays / direct killing	Killing mechanism	Cytokine sensitivity	Killing mechanism	Ref.
HP62	CD56+ NKT cell target; TRAIL	N / T	IFNγ + TNFα	N / T	[28,32]
CM	T1D T-cell stimulation; CD56^{+} NKT cell target; TRAIL	TRAIL death inhibited by Bcl-2 & XIAP	TNFα	ROS	[32,35,36,38,39]
NES2Y	Granule-specific T-cell stimulation; TRAIL	TRAIL death inhibited by Bcl-2 & XIAP	N / T	N / T	[38,39,51]
NISK9	N / T	N / T	N / T	N / T	
βLox5	Fas-FasL	Caspase dependent	IFNγ+TNFα	Caspase-dependent / independent; ROS	[54]
NAKT-15	N / T	N / T	N / T	N / T	
EndoC-βH1	N / T	N / T	N / T	N / T	

TRAIL, Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand; N / T, Not Tested; ROS, Reactive Oxygen Species; XIAP, X-linked Inhibitor of Apoptosis Protein.