Extracellular Release of High Mobility Group Box1 Protein from Necrotic β-Cells in the Pathogenesis of Type 1 Diabetes Mellitus

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Abstract

Nonobese diabetic (NOD) mice, an animal model of human type 1 diabetes mellitus (T1DM), exhibit impaired phagocytosis of apoptotic cells. In addition to phagocytosis, degradation of apoptotic cells determines the level of dead cells in tissues. Therefore, the work examined the kinetics of apoptotic cell degradation *in vitro*. The work revealed that macrophages from NOD mice digested internalised apoptotic thymocytes at a reduced rate compared to macrophages from control mice.

How defective clearance leads to the development of T1DM is unclear. Necrosis is associated with inflammation, and high mobility group box 1 protein (HMGB1) released from necrotic cells induce inflammation. The relationship between β-cell death and HMGB1 release was investigated. The results showed that HMGB1 was released from necrotic β-cells in a dose-dependent manner. If impaired, clearance of apoptotic β-cells results in an increased population of necrotic β-cells. HMGB1 release could initiate or exacerbate an inflammatory response in NOD mice.

Keywords: type 1 diabetes mellitus; autoimmunity; degradation of apoptotic cells; high mobility group box 1; β-cells

Subject Terms: Inflammation; Autoimmune Diseases; Cell Death; Diabetes Research

日本の両親へ捧げます。

(To my parents)

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1.1 Background

1.1.1 Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is a disease in which the β-cells in the islets of Langerhans in the pancreas are selectively destroyed by immune cells that infiltrate the pancreatic islets. β-cells produce and secrete insulin, which is a critical hormone for proper regulation of blood glucose. The loss of these β-cells eventually leads to the inability to produce endogenous insulin, resulting in the need for administration of exogenous insulin. Devastating complications such as atherosclerosis, retinopathy, and neuropathy may develop because delivery of exogenous insulin cannot mimic the fine-tuning of insulin secretion by β-cells.

T1DM used to be known as juvenile-onset diabetes mellitus, as its onset was often observed in young children, which helped coin the term. It is devastating to see small children suffer from the disease. Unfortunately, the number of children who are currently suffering from T1DM is increasing worldwide although there is variability in the incidence of T1DM among ethnic groups (1). Onkamo and colleagues (1999) estimated that the incidence of T1DM among children will increase by 40% between 1998 and 2010. Currently, all T1DM patients rely on daily injections of exogenous insulin to control blood glucose levels. Islet transplantation could be another treatment option, but it is not yet feasible and available to every patient due to complicated issues such as insufficient donor islets. Additionally, immunosuppressive regimens have side effects, and are therefore currently unsuitable for children who would face lifelong challenges with immunosuppression.

Prior to the onset of T1DM, insulitis progresses slowly and asymptomatically. Insulitis refers to the event that the pancreatic islets of Langerhans are infiltrated by mononuclear cells such as macrophages, dendritic cells (DCs) and T lymphocytes (T cells). Following the infiltration by these cells, β-cell-specific T cells selectively destroy β-cells (2). β-cell specific CD8+ cytotoxic T cells (CD8+ T cells) have been identified as effector cells of β-cell killing (3, 4).

In spite of extensive studies investigating the pathogenesis of T1DM for the past 30 years, the exact mechanism(s) of autoreactive T cell activation is still a mystery. Hence, a cure for T1DM remains to be identified and patients must continue using treatments for supplying insulin exogenously. Science has not yet provided the answers to the questions that are necessary to prevent or reverse T1DM (5). Genetic factors alone make it difficult to predict diabetes development. As many as 18 loci have been identified as susceptible to T1DM in humans (6). To make the pursuit of a diabetes cure even more complicated, various environmental factors have been suggested as triggers for the disease. Trudeau *et al.* (7, 8) have recently provided some hope for prediction of disease onset using the nonobese diabetic (NOD) mouse, an animal model of T1DM, through detecting β-cell reactive T cells in circulation prior to disease onset. However, a practical and cost-effective means for the prediction of diabetes development is not yet available for humans.

1.1.2 Nonobese diabetic (NOD) mouse

The nonobese diabetic (NOD) mouse has been extensively used for research in organ-specific autoimmune disease (9) resulting in much information that contributes to our understanding of autoimmunity. The NOD mouse spontaneously develops T1DM with a disease process and pathology that resembles human T1DM (reviewed in (4, 10)). The autoimmune process in the NOD pancreas has two phases: insulitis and overt diabetes. Insulitis occurs when inflammatory mononuclear immune cells infiltrate the pancreatic islets, which begins as early as 3 weeks of age. In the second phase, overt diabetes is characterized by continuous hyperglycaemia, and its onset occurs asynchronously after 12 weeks of age.

The NOD mouse is considered the most useful model currently available for the study of human T1DM even though some disease features are different from human T1DM. For instance, there is a pronounced female gender bias in diabetes development in these mice (11). Female NOD mice develop a more severe insulitis and their incidence of T1DM is significantly higher than that of males (80-90% in females vs. 10-40% in males) (4). Furthermore, the time of disease development is also different between this mouse model and humans. Humans develop T1DM most often at or before puberty, whereas NOD mice present symptoms later in the life course.

1.1.3 β-cell apoptosis in type 1 diabetes mellitus

A neonatal wave of β-cell death, which refers to an increase in the rate of cell death during the developmental period in the pancreatic islets, occurs around

10 to 14 days of age in rodents (12) and two months before and after birth in humans (13). The neonatal wave of β-cell death occurs by apoptosis (14, 15), congruent with the fact that cells die by apoptosis in many situations during the developmental period (16). *In-situ* staining using terminal dUTP nick-end labelling (TUNEL), which detects apoptotic cells, demonstrates the incidence of the neonatal wave of β-cell apoptosis in both a mouse and a rat models of T1DM.

The exact role of the neonatal wave of β-cell apoptosis in initiation of β-cell-directed autoimmunity remains to be elucidated. There is some evidence that strongly suggests that apoptotic cells are one of the central players in regulation of immunity towards β-cells. Apoptosis is the major mechanism by which β-cells are destroyed in diabetes-susceptible animal models. It has been shown that the majority of β-cell death in chemical-induced mouse models of autoimmune diabetes (17) and spontaneous β-cell death in the NOD mouse (18) is by apoptosis. The juxtapositioning of the neonatal wave of β-cell apoptosis with the first appearance of immune cells into the islets is an interesting observation (18): even if the onset of diabetes is accelerated, the onset of insulitis begins just after the wave (17). Additionally, apoptotic cells are considered suppliers of autoantigens (19). Many proteins that are autoantigenic in autoimmune patients are uniquely modified and translocated to the surface of apoptotic blebs (20), which could be consistently taken up and processed for presentation to T cells by antigen presenting cells residing in their surroundings.

Furthermore, some studies have illustrated the importance of apoptotic β-cells in the development of T1DM by manipulating the incidence of β-cell apoptosis. Limiting β-cell apoptosis during the neonatal period by a caspase inhibitor results in decreased priming of BDC2.5 T-cells (21). Caspase-3 knockout mice are protected from multiple-low-dose streptozocin-induced diabetes (22). Moreover, increasing the number of apoptotic β-cells enhances the priming of diabetogenic $CD8^+$ T-cells (23). Interestingly, a small increase in β-cell apoptosis has a protective effect on the development of T1DM in NOD mice (24). The results from these studies suggest that the relationship between β-cell apoptosis and autoimmunity is not simply a matter of more or less cell death, but is likely to comprise complex dynamic responses as has been implicated by others (25).

1.1.4 Phagocytosis of apoptotic cells in type 1 diabetes mellitus

Phagocytosis is another critical factor that determines the number of apoptotic β-cells *in situ*. Aberrant phagocytosis of apoptotic cells has been observed in animal models of T1DM. O'Brien *et al.* demonstrated defective clearance of apoptotic cells in both a rat (diabetes-prone BioBreeding rat) and a mouse (NOD mouse) model of T1DM (14, 26). Macrophages from diabetes-prone animals internalise UV-irradiated apoptotic thymocytes less efficiently than do phagocytes from diabetes-resistant strains *in vitro*. Both peritoneal and bone-marrow-derived macrophages from NOD mice exhibit the defect (26), suggesting the defect is intrinsic. Macrophages from the NOD mouse also display impaired

phagocytosis of apoptotic β-cells (26). O'Brien and her co-investigators have further shown *in vivo* the phagocytic defect of apoptotic thymocytes by macrophages from NOD mice (27). The phagocytic defect of NOD macrophages observed *in vivo* appears to be systemic: the defect is found in the peritoneum, skin, and thymus. Moreover, challenging the NOD mouse with apoptotic loads by UV irradiation to the skin leads to increased antibodies to DNA in circulation, which is typically observed in systemic autoimmune disease.

1.1.5 Degradation of apoptotic cells in autoimmunity

Phagocytosis is not the only important factor to consider in disease development. In addition to engulfment, degradation of apoptotic cells also determines the level of non-functional cellular material left in viable tissue that must endeavour to operate normally. After phagocytosis, phagocytes form a membrane-bound vacuole called a phagosome, in which phagocytosed cells remain (reviewed in (28)). Phagosomes traffic through cytoplasm, although it is not well understood how, and then fuse with endosomes and/or lysosomes. The fusion results in the formation of phagolysosomes, sometimes referred to as matured phagosomes. Like late endosomes, they possess a number of complementary degradative properties such as a very low pH, lysosomal enzymes to digest internalised materials, and machinery capable of generating reactive oxygen intermediates.

Defective degradation of apoptotic materials has been associated with initiation of autoimmunity. Macrophages were shown to play a role in the digestion of DNA from apoptotic cells (29, 30). Macrophages in mice lacking

DNase II are not able to digest chromosomal DNA from phagocytosed apoptotic cells (31). This leads to the development of polyarthritis in these mice, which is an autoimmune disease resembling human rheumatoid arthritis. One of the initiating events appears to be the release of a strong inflammatory cytokine, TNF-α, from activated macrophages that store many undigested DNAs in their lysosomes. Elevated antinuclear antibodies in circulation are one of the typical manifestations of systemic autoimmune disease. By the same rationale, defective degradation of apoptotic entities may also play a role in the initiation of T1DM.

1.1.6 Initiation of β-cell-directed autoimmunity – A working model

The mechanism for the initiation of T1DM is elusive. The goal of this section is to propose a model of initiation of this disease. The model is based on the hypothesis postulated by Finegood and others (12, 32) with that proposed model being the basis for this thesis. The model starts with a neonatal wave of apoptotic β-cells. Application of a mathematical model reveals that a similar rate of apoptosis exists both in diabetes-prone (*dp*) and in diabetes-resistant (*dr*) animals (14, 15). However, in the islets of *dp* as compared to *dr* mice, higher numbers of apoptotic β-cells were detected. The higher incidence of apoptotic β-cells in *dp* mice is due in part to impaired phagocytosis by professional phagocytes (14, 26, 27). As a result, during the neonatal period the number of apoptotic β-cells is inversely correlated with phagocytic ability of macrophages. Degradation of internalised apoptotic cells could also be impaired. We hypothesised that as a result of defective clearance (i.e., phagocytosis and

degradation) of apoptotic cells following the neonatal wave of β-cell apoptosis increased loads of apoptotic β-cells develop in the pancreatic islets.

Another factor involved in the development of diabetes is the immune response to apoptotic β-cells, which should be an otherwise normal quiescent process. Macrophages exert an anti-inflammatory effect upon phagocytosis of apoptotic cells (33, 34). Impaired phagocytosis of apoptotic cells could potentially affect the ability of macrophages to suppress the inflammatory response through a process such as cytokine dysregulation. The clearance defect could provide antigen presenting cells (APCs) more material to present to autoreactive immune cells. Inappropriately degraded peptides could be autoreactive by themselves. Moreover, macrophages that are carrying undigested apoptotic β-cell entities could be activated and release inflammatory cytokines in the same manner as macrophages carrying undigested DNA particles (31). These mechanisms all help to describe how the defective clearance of apoptotic cells may result in an inflammatory milieu within the pancreatic islets.

Apoptotic cells could be another factor that exacerbates inflammation in the development of diabetes. Apoptotic β-cells may remain in the islets for a prolonged period if not taken up and cleared. Those apoptotic cells could become necrotic, which is a process known as secondary necrosis. Secondary necrotic cells experience further breakdown of cell constituents, followed by release of their debris (35-37). Additionally, inflammatory cytokines released from activated immune cells can kill β-cells exclusively by necrosis (38).

Intracellular contents released from necrotic cells are inflammatory, and activate the immune system. Such inflammatory mediators are known as danger signals. Several proteins have recently been identified as danger signals, including uric acid, heat shock proteins, and high mobility group box 1 (HMGB1) protein (reviewed in (39)). Among those, HMGB1 has recently drawn much attention as a key player in the process (40). HMGB1 acts as an inflammatory cytokine by activating APCs (41), and it is released from secondary necrotic Jurkat cells (37). The following chapter of this thesis work investigates whether HMGB1 is also released from secondary necrotic β-cells, which may help further elucidate the aetiology of diabetes development.

A high occurrence of apoptotic β-cells followed by defective clearance of apoptotic cells could lead to an increase in necrotic β-cells, setting up an inflammatory environment in the islets of the pancreas. From here, the proposed model of diabetes development ends with the activation of autoreactive T lymphocytes. APCs residing in the islets may take up β-cell-associated proteins (β-cell antigens) from unphagocytosed or undigested apoptotic β-cells. β-cell antigens could then be transported into the draining pancreatic lymph nodes, in which naïve autoreactive T lymphocytes encounter the β-cell antigens. During transit from the islets to the pancreatic lymph nodes, the APCs process the antigens and undergo maturation. The putative maturation of APCs may be driven by exposure to danger signals such as HMGB1. Mature APCs are competent in the presentation of processed antigens with major histocompatibility complex (MHC) proteins to other immune cells. Consequently, naïve T cells

become activated and are able to migrate into the islets, recognise the cognate β-cell antigens, and destroy β-cells. A summary of the model is depicted in Figure 1.

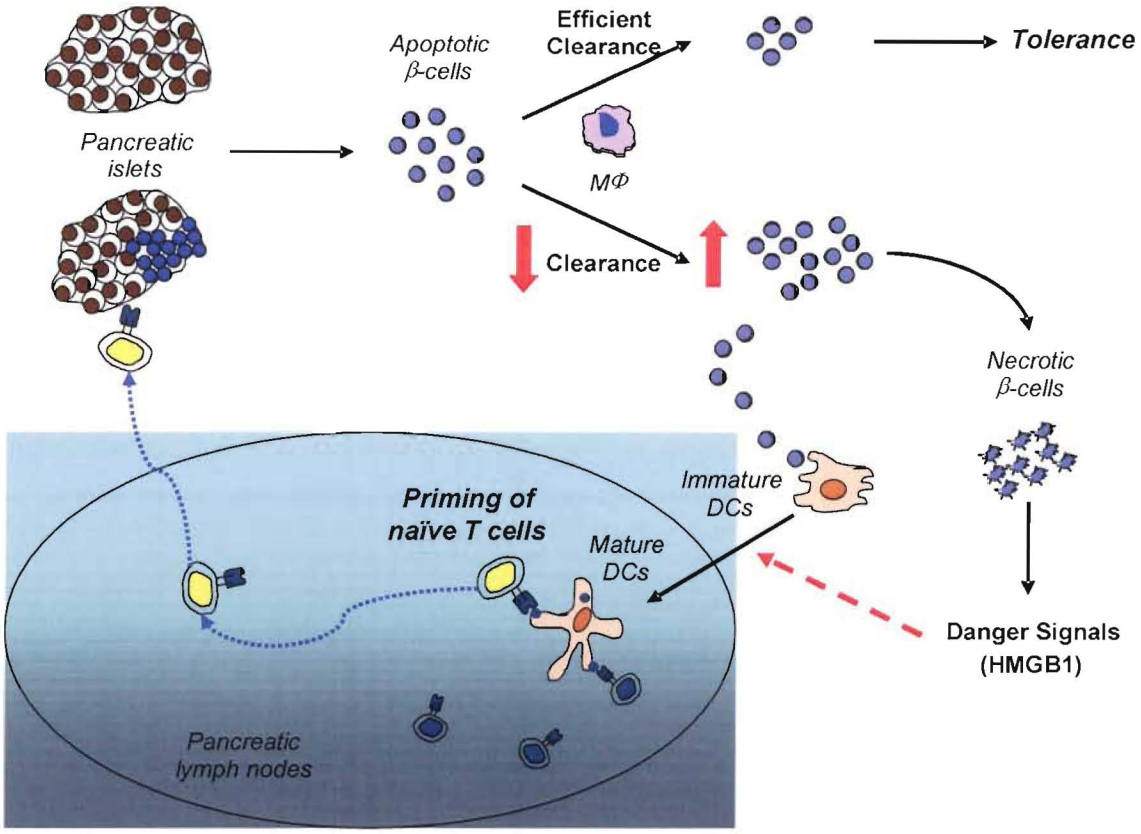


Figure 1. Pathogenesis of type 1 diabetes mellitus – A working model

In healthy individuals, tolerance is induced in the peripheral β-cell-reactive T cells. In diabetes-susceptible individuals with decreased phagocytosis and degradation by macrophages, apoptotic β-cells are not efficiently cleared, leading to increasing levels of β-cells materials that may be taken up by the tissue residing antigen presenting cells. The tissue remaining apoptotic β–cells may become secondary necrotic β-cells, and necrotic β-cells can release danger signals (i.e., HMGB1). These danger signals induce maturation of dendritic cells which in turn activate naïve β-cell-specific T cells. Once activated, the T cells are able to migrate into the tissue and recognise β-cell-derived antigens to start the process of killing β-cells, which leads to overt diabetes.

1.1.7 Basis for thesis design

The experiments influencing the direction of my research date back to the work done by O'Brien *et al.* (2002) (26). O'Brien and her colleague investigated the *in vitro* phagocytosis of apoptotic cells in young, pre-diabetic NOD mice and demonstrated impaired phagocytosis of apoptotic cells by macrophages from these mice. They also showed that the defect exists *in vivo* as well (27). A similar defect has also been observed in animal models of other autoimmune diseases (42) and in human autoimmune patients (43). In addition, experimentally induced decreases in *in vivo* phagocytosis of apoptotic cells are associated with the development of autoantibodies (44, 45), suggesting a link between a phagocytic defect and the development of autoimmune symptoms.

While many studies suggest that impaired phagocytosis of apoptotic cells even from pre-inflammatory foci contributes to autoimmune disease, it is important to understand that the clearance of apoptotic cells depends upon multiple processes including binding, engulfment, and degradation of apoptotic cells. Macrophages from NOD mice demonstrate defective uptake of apoptotic cells not only *in vitro* but also *in vivo*, yet display normal binding of apoptotic cells. However, there is not a published study investigating the competency of degrading apoptotic debris after phagocytosis in an animal model of T1DM. Therefore, the purpose of this part of the thesis to further elucidate a macrophage defect in TIDM by investigating *in vitro* degradation of apoptotic cells by peritoneal macrophages from diabetes-prone NOD and diabetes-resistant BALB/c mice.

1.1.8 Hypothesis

Macrophages from diabetes-prone NOD mice digest internalised apoptotic cells at a reduced rate compared to those from diabetes-resistant BALB/c mice *in vitro*.

1.2 Materials and Methods

1.2.1 Animals

Five-week-old female nonobese diabetic (NOD) mice and BALB/c mice were purchased from Taconic (Germantown, NY), and housed in the Animal Care Facility at Simon Fraser University. Animals were maintained in a pathogen free condition and had free access to standard laboratory chow and water. Control strain BALB/c mice exhibit the same major histocompatibility complex (MHC) class I molecule H-2^d as NOD mice (46). Animals were handled in accordance with the guidelines and regulations of the Canadian Council on Animal Care, and all experimental manipulations of the animals were approved by the Animal Care Committee at Simon Fraser University.

1.2.2 Animal procedure

All surgical procedures were carried out after mice were sacrificed by CO_2 asphyxiation as described in the approved protocol.

1.2.3 Isolation and culture of macrophages

Macrophages were collected from mice by peritoneal lavage without elicitation. The peritoneum was exposed after retraction of the skin. Roughly 8 ml of ice cold "complete RPMI," consisting of RPMI1640, 100µg/ml streptomycin 100 unit/ml penicillin, and 10% v/v heat inactivated fetal calf serum (FCS) (all reagents from GIBCO, Burlington, Ontario, Canada), was injected into the peritoneal cavity. The peritoneum was gently massaged with a cotton-swab, and the peritoneal lavage fluid was collected into a syringe. Peritoneal exudates from

multiple mice of a single strain were pooled together and washed once with ice cold sterile Phosphate Buffer Saline (PBS) (GIBCO). Peritoneal cells were counted using a haemacytometer (VWR International, Mississauga, ON, Canada), and equal volumes of aliquots were seeded into eight-well chamber slides (Nalge Nunc, Rochester, NY). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 hours, allowing macrophages to adhere on the slides. At the completion of incubation, unbound cells were washed three times with sterile PBS before use.

1.2.4 Induction of apoptosis

Thymuses were harvested from BALB/c mice and rinsed in cold sterile PBS. A single cell suspension of thymocytes was prepared by gently mincing thymuses through a sieve under sterile conditions. Thymocytes were washed with sterile PBS twice and resuspended in RPMI1640 medium with 100unit/ml of penicillin and 100μg/ml of streptomycin (FCS-free RPMI). Thymocytes were then counted and diluted with FCS-free RPMI. To induce apoptosis, a suspension of thymocytes was irradiated by exposure to 254nm ultraviolet (UV) light (Philips UV-C TUV30W G30T8) for 20 minutes. The cells were then cultured for 2 hours at 37°C, 5% CO_2 in a humidified atmosphere to allow apoptosis to progress. FCS was added (10% v/v) to a suspension of UV-exposed apoptotic thymocytes before use.

1.2.5 *In vitro* degradation assay

The suspension of apoptotic thymocytes was seeded into a well containing macrophages at a ratio of 5:1; apoptotic thymocytes to macrophages. This mixture of cells was cultured at 37°C, 5% CO_2 in a humidified atmosphere for two hours to allow phagocytosis of apoptotic bodies. At the end of 2-hour co-culture of macrophages and apoptotic thymocytes, the complete RPMI containing unengulfed apoptotic thymocytes was discarded. The macrophages underwent a series of vigorous washes with sterile PBS to remove unengulfed apoptotic thymocytes. Fresh complete RPMI was added, and adherent cells were further cultured for 1, 2, or 3 hours at 37°C, 5% CO_2 in a humidified atmosphere. At the end of each period, cells were washed with PBS and fixed in 10% neutral buffered formalin (Sigma-Aldrich, Oakville, ON, Canada) for 5 minutes. Fixed cells were then stained with Mayer's haematoxylin and Eosin (both from Sigma) for identification of apoptotic bodies and macrophages. Cells were observed using light microscopy at 1,000-fold magnification under oil immersion.

1.2.6 Evaluation of degradation of engulfed apoptotic thymocytes

Engulfed apoptotic thymocytes were quantified by counting and classifying approximately 1,000 macrophages. Each macrophage was assigned to a class (i=0, 1, 2 ..., 7+) according to the number of internalised apoptotic bodies it contained. Only apoptotic bodies clearly visible within the perimeter of the macrophage were counted. Results were expressed as Phagocytic Index (PI), the total number of apoptotic bodies per 100 macrophages. The rate of degradation of engulfed apoptotic thymocytes was determined by the slope of a

regression line connected through PI plots at four different time-points in each mouse strain.

1.2.7 Statistical analysis

All statistical analyses were performed using SigmaStat 3.1. Results are expressed as means ± the standard error (SE). A two-way ANOVA on repeated-measurements was performed for the animal strains (BALB/c and NOD) and time of incubation (0, 60, 120, and 180 min.) as independent variables. Post hoc analysis was done using Tukey's test. Regression analysis was performed to test the slope of each regression line. Differences were deemed statistically significant at $p<0.05$.

Coincidence of two regression lines was also tested. When two regression lines are coincident, the lines have the same slope and intercept. Coincidence was tested by assessing whether fitting the two sets of data with discrete regression lines (BALB/c or NOD) in which the slopes and intercepts were different produced smaller residuals than fitting the whole set of data consisting of both BALB/c and NOD with a single regression line using a single slope and intercept.

1.3 Results

The ability of macrophages to degrade engulfed apoptotic materials may be compromised in T1DM. To explore this possibility, apoptotic bodies were quantified within non-elicited peritoneal macrophages from diabetes-prone NOD and diabetes-resistant BALB/c mice after macrophages were fed with UV-irradiated apoptotic thymocytes for 2 hours *in vitro* (Figure 2). Figure 2 shows macrophages from diabetes-prone NOD mice exhibit reduced amounts of internalised apoptotic thymocytes, compared to macrophages from diabetes-resistant BALB/c mice.

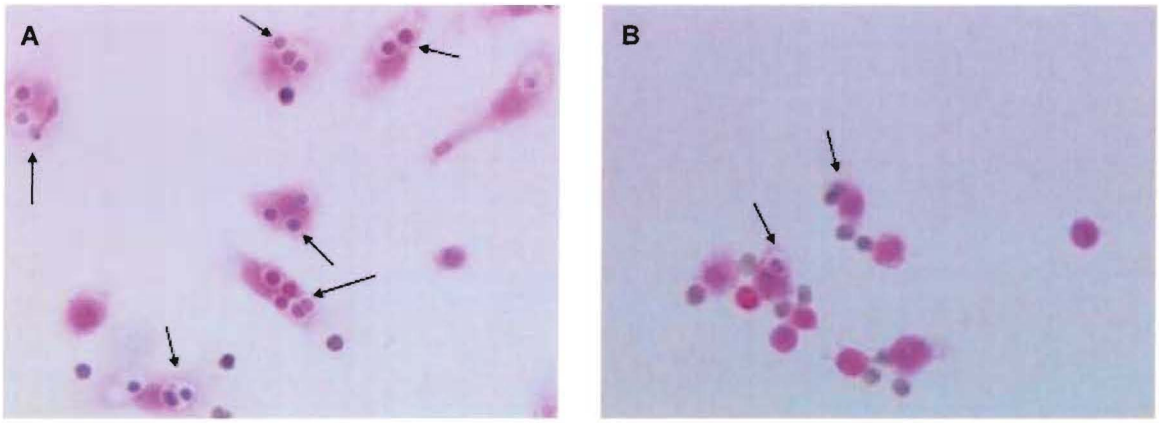


Figure 2. Haematoxylin and Eosin staining of peritoneal macrophages

Peritoneal macrophages were incubated with UV-induced apoptotic thymocytes for 2 hours. The figure is microphotographs of macrophages from diabetes-resistant BALB/c mice (A) and from diabetes-prone NOD mice (B) with apoptotic thymocytes. An arrow points to macrophages that have engulfed apoptotic thymocytes. Magnification: 1000x

To determine the rate of degradation of apoptotic thymocytes by macrophages, apoptotic cells were removed at a specific time point, and apoptotic cells within macrophages were quantified. Analysis of the kinetics of degradation revealed that macrophages from NOD mice showed a pattern of

relatively constant phagocytic index (PI) while macrophages from BALB/c displayed a statistically significant reduction in PI over time.

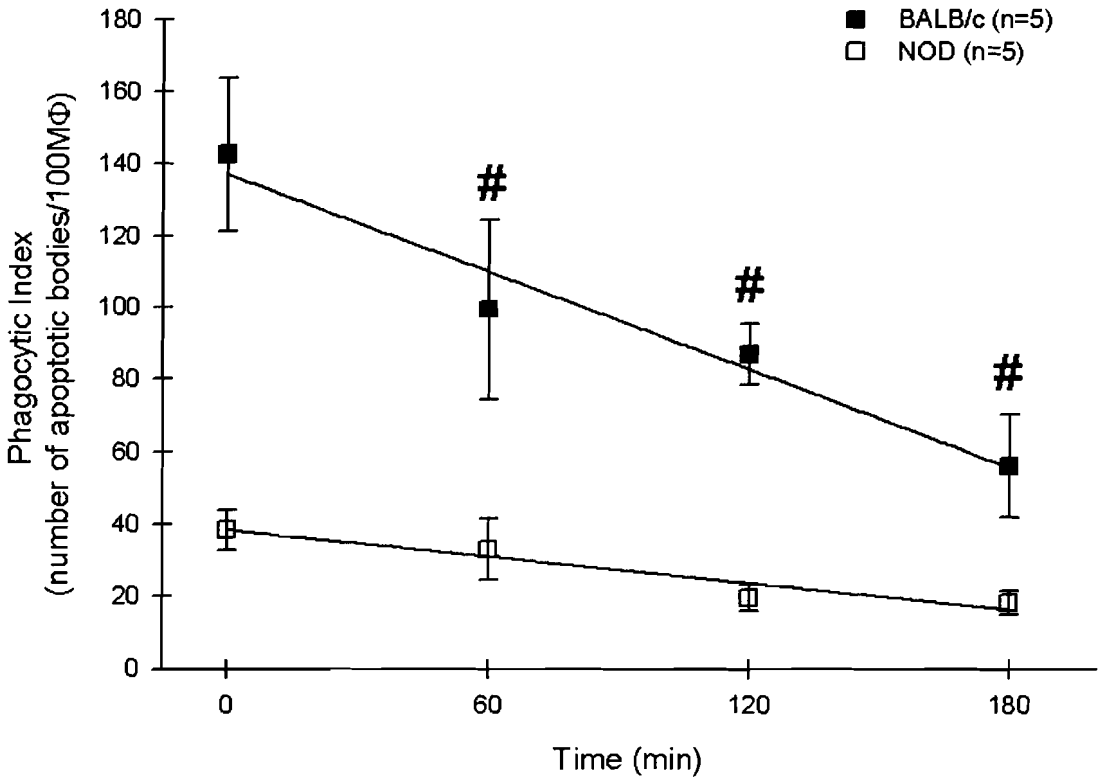


Figure 3. Kinetics of degradation of engulfed apoptotic thymocytes in macrophages from diabetes-resistant (BALB/c) and diabetes-prone (NOD) mice

Macrophages from each strain were incubated with UV-induced apoptotic thymocytes for 2 hours. All unbound/unengulfed apoptotic cells were washed away at which point the time was designated as 0. Macrophages were cultured in fresh media for additional 3 hours. At each hour, cells were washed, fixed, stained, and then apoptotic bodies inside macrophages were counted. Results are shown as mean ± SE. #: $P<0.05$ against time 0 MΦ: macrophages

There were no significant differences in PIs of NOD macrophages at any time point. In contrast, PIs of macrophages from BALB/c mice displayed a statistically significant decrease at time 60, 120, and 180 from at time 0 ($p<0.03$: time 0 vs. time 60, $p<0.005$: time 0 vs. time 120, and $p<0.001$: time 0 vs. time 180).

To describe the changes in phagocytic index across four time points, regression lines were computed for each of the two groups. Regression analysis showed that there was a slight decrease (-7.4 PIs per hour) in a slope of a regression line in PIs of macrophages from NOD mice and a greater decrease (-27.2 PIs per hour) in PIs of macrophages from BALB/c mice. Both slopes are significantly different from zero ($p<0.01$ for NOD mice and $p<0.005$ for BALB/c mice), suggesting that the rate of degradation of internalised apoptotic cells by macrophages from NOD mice is reduced compared to those from BALB/c mice.

A test of coincidence of the two regression lines revealed that the slopes of the two regression lines are different from that of each other ($p<0.01$, F=31.0). The observed value of F exceeds the critical value of F, which means that a significantly better fit to the data was obtained by fitting the two sets of data with separate regression lines than by fitting all the data with a single line. Thus, the two sets of data were drawn from populations with different lines of means. The interpretation is that the relationship between PI and time is different for BALB/c and NOD macrophages, and that the NOD macrophages displayed reduced degradation of apoptotic thymocytes compared to control BALB/c macrophages. To further delineate where the difference arose, the intercepts and slopes of two regression equations were compared by Student's *t*-test. Both the intercepts and slopes showed a statistically significant difference ($p<0.001$ and $p< 0.05$ for the intercepts and the slopes, respectively). Hence, it was concluded that the intercepts of the two lines were significantly different, and that the slopes of the two lines were significantly different. The decrease in PI over an hour was

smaller for macrophages from NOD mice than for those from BALB/c mice. Additionally, the initial PI was smaller in NOD macrophages than in BALB/c macrophages.

1.4 Discussion

In this study, we observed a reduced rate of degradation of internalised apoptotic thymocytes by macrophages from diabetes-prone NOD mice *in vitro* compared with macrophages from diabetes-resistant BALB/c mice. The degradative capacity of macrophages from diabetes-prone NOD mice has not been studied, and the results presented here reveal a further defect of macrophages possibly involved in the aetiology of T1DM. Defective degradation of apoptotic cells may be significant in setting up conditions that lead to initial stages of β-cell-directed autoimmunity following the neonatal wave of β-cell apoptosis. The novel finding presented here provides further evidence for aberrant clearance of apoptotic cells in NOD mice namely that the degradation of apoptotic cells is reduced in NOD mice in addition to phagocytosis which has been described previously (26, 27). A more extensive quantitative analysis of degradation and phagocytosis of apoptotic thymocytes in the pathogenesis of T1DM was conducted on these data by our collaborators using a nonlinear mathematical model (47). The mathematical model analysis produced the same conclusion as the one presented here, that the degradation capacity of NOD macrophages is reduced compared to that of BALB/c macrophages.

One could postulate that the phagocytosis and/or degradation defect that is presented here affects the process of phagosome maturation. Professional antigen presenting cells such as dendritic cells (DCs) exhibit a way to arrest phagosome maturation and thus can control degradation of phagocytosed entities (48). This maximises the generation and half-life of peptides suitable for

a proper antigen presentation. Macrophages also display an antigen-presenting capacity (49). Additionally, macrophages play a role in the digestion of DNA from apoptotic cells (29, 30). Macrophages from NOD mice presumably have impaired digestion of apoptotic-β-cell-associated proteins. The undigested β-cell-derived proteins may be antigenic and could be presented to β-cell-reactive T cells, which may augment the destruction of β-cells. Interestingly, healthy individuals do not generate auto-immunity against β-cell antigens in spite of detectable levels of circulating antibodies to the β-cell (50), suggesting that β-cell-derived antigens alone are not sufficient to induce autoimmunity.

Macrophages carrying undigested apoptotic β-cells potentially release inflammatory cytokines in the pancreatic islets, which may attract more immune cells to the islets or activate immune cells that are already in the islets. Mice with macrophages that lack DNase II in lysosomes exhibit an autoimmune phenotype (31). Kawane *et al.* showed that macrophages produce an inflammatory cytokine, TNF-α, when they cannot digest DNA from apoptotic cells. The release of TNF-α stimulates other cytokine production and results in the eventual development of chronic polyarthritis. In addition, the phagocytic function of macrophages is reduced even further after exposure to an inflammatory cytokine, interferon-γ, demonstrated using rat alveolar macrophages (51). In contrast to the inflammatory properties already discussed, macrophages have mechanisms to resolve possible tissue inflammation by secreting anti-inflammatory cytokines upon phagocytosis of apoptotic cells (52). Macrophages can also suppress the secretion of inflammatory mediators (33). Although the exact mechanism by

which clearance of apoptotic cells suppresses a potential inflammatory response, defective phagocytosis or defective degradation could lead to dysregulation of signal transduction, resulting in a pro-inflammatory immune response instead of an anti-inflammatory one. Pro-inflammatory immune response could lead to further reduction in clearance of dead β-cells. Therefore, β-cell-directed immunity could be exacerbated in the islet environment.

The swift clearance of apoptotic cells has immunological importance. Fast clearance can decrease the number of apoptotic and potentially necrotic cells available for APCs, preventing an immune response due to the inappropriate release of intracellular materials. The present finding emphasises defective clearance of apoptotic cells in a diabetes-prone animal, and there is accumulating evidence that links defective clearance of apoptotic cells to autoimmunity (reviewed in (52)). Animal models characterised by macrophages that lack one of the receptors for apoptotic cell detection or in which phagocytosis of apoptotic cells is blocked display a systemic autoimmune-like syndrome. Lack of C1q, one of the complement proteins, that opsonises apoptotic cells for phagocytosis, has been associated with systemic lupus erythmatosus (SLE) (53). Mer-receptor-deficient mice, whose macrophages lack the intracellular domain of the c-mer receptor for apoptotic cell recognition, also exhibit spontaneous development of SLE-like manifestations (45). Blocking *in vivo* phosphatidylserine on the surface of apoptotic cells with mutant milk fat globule-EGF-factor 8 protein in healthy mice leads to a decrease in apoptotic cell engulfment and to systemic autoimmune symptoms, such as IgG deposition in the kidney and increases in

antinuclear antibodies in circulation (44). Furthermore, systemic exposure to apoptotic cells intravenously induces autoantibodies normal mice (54). These animal models represent the various mechanisms that may be involved in defective clearance of apoptotic cells leading to autoimmune responses. Whether the defective phagocytosis and digestion of apoptotic β-cells, which was demonstrated in this thesis and elsewhere (26, 27), initiates β-cell-directed immunity in NOD mice remains to be clarified.

2: Extracellular Release of High Mobility Group Box 1 Protein from Necrotic β-Cells

2.1 Introduction

Macrophages from diabetes-prone rodents are deficient in phagocytosis of apoptotic cells (14, 26, 27). In the preceding section, we have demonstrated that macrophages from diabetes-prone rodents are also defective in the intracellular disposal of apoptotic cellular debris. Both defective phagocytosis and impaired degradation of apoptotic material have been associated with initiation of autoimmunity (31, 44, 45). However, it remains elusive how deficient clearance of apoptotic material leads to autoimmunity. In the following section, we explored a potential consequence of the persistent presence of dead and dying cells.

2.2 Background

2.2.1 MIN6 cells

MIN6 cells are a commonly used mouse β-cell line established from β-cell tumour (insulinoma) tissue. The line was obtained by using targeted expression of the siman virus 40 T antigen gene connected to the insulin promoter in transgenic mice (55). The fusion gene was microinjected into fertilized eggs of C57BL/6 mice, and transgenic mice developed pancreatic insulinomas. Single tumours were excised and grown in culture. MIN6 cells exhibit homogeneous morphology. The cell line exhibits a marked increase in insulin secretion upon stimulation by glucose in a dose-dependent fashion as a physiological feature of pancreatic β-cells (55, 56). They do not express MHCs constitutively on the cell surface. However, upon stimulation with interferon (INF)-γ, MHC class I ($H\text{-}2K^b$) expression is greatly increased on the surface of MIN6 cells. Treatment with a combination of INF-γ and tumour necrosis factor-α induces both MHC class I and class II (A^b) expression on the cell surface (55).

2.2.2 Streptozocin

Streptozocin (STZ) is a toxin widely used experimentally to induce both type 1 and type 2 diabetes mellitus by destroying β-cells in the islets of the pancreas. STZ damages β-cells by means such as DNA alkylation and generation of reactive oxygen species that induce DNA fragmentation (57, 58). STZ also activates poly (ADP-ribose) polymerase (PARP). Activation of PARP synthesises great amounts of the (ADP-ribose) polymer using nicotinamide

adenine dinucleotide (NAD^+) as a substrate (59). This event can cause the concentration of NAD^+ within a cell to decrease to very low levels. This considerable decrease in NAD^+ is thought to abolish the ability of the cell to generate sufficient energy and, ultimately, to lead to cell death. In fact, animals lacking the PARP gene are protected from β-cell destruction and diabetes development induced by STZ (60). STZ is transported into the β-cell through binding of a glucose transporter, GLUT2, and is shown to have an increased transport and cytotoxicity in rat insulinoma cells overexpressing the GLUT2 transporter (61, 62).

STZ induces cell death in a rat insulinoma β-cell line (INS-1 cells) (63). Interestingly, the dosage of STZ is what dictates whether cells undergo the process of apoptosis or immediately progress to necrosis. Significant incidence of apoptosis starts at only 10 mM STZ and increases up to 30 mM STZ as determined by light microscopy. On the contrary, necrosis is not observed until 20 mM STZ, and it becomes the predominant mode of cell death at 30 mM and above. Cell death was also assayed in the same study by the expression of DNA ladder and morphological observation by electron microscopy. INS-1 cells treated with a low dose STZ exhibited a typical DNA ladder, which is a feature of apoptosis. In contrast, cells treated with a high STZ dose did not show a similar DNA ladder. Morphological analysis revealed a comparable result.

This system of differential dosing of STZ to induce apoptosis and/or necrosis was used in this thesis work as a tool to compare HMGB1 release from increasing amounts of necrotic cells. Although it is presumable that MIN6 cells

response to STZ in a similar manner since both INS-1 and MIN6 cells are murine insulinoma cell lines, it has not previously been shown whether the system is applicable to MIN6 cells. Therefore, a dose-dependent response of STZ in MIN6 cells was first re-examined in this work before using this paradigm to compare HMGB1 release from apoptotic and necrotic cells

Interestingly, the method of administration of STZ determines the type of diabetes development *in vivo*. For instance, treatment with multiple-low-dose (20-40 mg/kg body weight (BW)) of STZ results in the development of autoimmune diabetes characterised with the development of insulitis (64). Splenocytes from multiple-low-dose STZ-induced diabetes in the mouse are able to kill a β-cell line *ex vivo* at 10 days after the initial injection of STZ (65). Transfer of mononuclear spleen cells from multiple-low-dose STZ-treated mice into normal syngeneic recipients diminishes insulin secretion in the recipient mice (66). β-cells of mice treated with multiple-low-dose STZ die by apoptosis, and a wave of apoptosis occurs prior to an inflammatory reaction in the islets (17). Furthermore, deletion of macrophages by silica in multiple-low-dose STZ-treated mice suppresses the development of hyperglycaemia, suggesting a pathogenic role of macrophages in islet destruction (67). On the other hand, a single injection of high dose STZ (typically 200-250 mg/kg BW) administered to mice leads to hyperglycaemia within 48 hours after the injection, and β-cells presumably die by necrosis (68).

2.2.3 Danger model of immune response

How the immune system determines whether or not it will respond to a potential threat has been a key question of immunology, and the issue is far from reaching a definitive conclusion. A danger hypothesis was most recently proposed as a model of an immune response (69). In this theory, Matzinger challenges the traditional notion that the immune system distinguishes between self and non-self and responds directly to the latter. This theory also contradicts the previous model that the immune system responds only upon recognising pathogen-associated molecular patterns arising from something very foreign such as microbes. Instead, Matzinger argues that the immune system reacts to microbial constituents only when danger signals have been released by infected or injured cells. Danger signals could also originate from damaged self-tissue without infection. Whether they originate from exogenous (pathogen-specific) or endogenous (necrotic cells) sources, danger signals are patterns of protein or acid that are hidden or that are not normally exposed out of healthy living cells. These molecules can thus act as signs of damage and death and illicit an immune response.

The danger model is somewhat better able to fully explain how autoimmune response may occur than any previous models. Danger signals induce maturation of dendritic cells (DCs), professional antigen presenting cells (APCs) (70, 71). Danger signals from pathogen-infected cells work favourably to the host. The maturation of DCs is required for the initiation of protective immune responses against pathogens, since it allows the immune system to

focus on the antigens that are presented by DCs. However, stressed or damaged self-cells, such as secondary necrotic cells, can also express and release danger signals. In association with auto-antigens, the danger signals provide DCs with the ability to induce autoimmunity. In this scenario, instead of recognising microbial constituents, immune cells recognise signs of tissue distress, or molecules that are normally found only inside cells. Supporting this notion is the fact that necrotic cells have been shown to induce maturation of DCs, unlike apoptotic cells (72). When there is no release of danger signals and DC maturation is not induced, immature DCs that sample apoptotic cells instead induce tolerance to self-antigens. Some of the danger signals identified thus far include high mobility group box 1 proteins (HMGB1), S100 proteins, and heat shock proteins (HSPs) (39, 73). Such danger signals appear to override preventative mechanisms against autoimmunity by phagocytes. Thus, the immune system responds to the danger signals which leads to inflammation even in non-infectious situations (74).

2.2.4 High mobility group box 1 protein (HMGB1)

High mobility group box 1 protein (HMGB1) is a nuclear protein that is highly conserved across species. It was originally described as an intranuclear factor with an important structural function in chromatin organisation (reviewed in (39, 75, 76)). Nuclear HMGB1 is thought to be indispensable for survival because *Hmgb1*$^{-/-}$ mice die soon after birth due to hypoglycaemia (77). This protein has recently been shown to possess dual functions; it also acts like a

cytokine by inducing an inflammatory response. In the late 1990's, HMGB1 was identified as a late mediator of endotoxaemia and sepsis (78).

HMGB1 must be transported to the extracellular space to act as an inflammatory mediator, and it is actively secreted by some immune cells under conditions of stress. In monocytes/macrophages, HMGB1 is detected in cytoplasm after stimulation with LPS, TNF-α, or Interleukin (IL)-1β while it is localised within the nucleus in the absence of the stimulation (78, 79). Mature dendritic cells (DCs) derived from human PBMCs also actively release HMGB1 (80).

HMGB1 is not only released actively from mononuclear cells. It was shown that HMGB1 was also released passively from necrotic cells (81). Freeze-thaw-induced necrotic HeLa cells release the nuclear HMGB1 into the supernatant while HMGB1 is not released from apoptotic cells in which the protein is sequestered in the nucleus. HMGB1 is retained and bound to DNA in cells that undergo TNF-α and cycloheximide-induced apoptosis, which explains why the secretion of the protein into cytoplasm is not observed.

Cell death induced HMGB1 is principally released from necrotic cells; therefore, it has been suggested to function as a "necrotic marker." Flow cytometric detection of HMGB1 release was shown to be comparable to other cell viability assays such as LDH release assay or ^{51}Cr release assay (82). Moreover, necrotic β-cells treated with IL-1β *in vitro* release significant amounts of HMGB1 in the extracellular surroundings (38).

The view that only necrotic cells release HMGB1 has been challenged by recent studies. Using a Jurkat human T-cell line, Bell *et al.* demonstrated that the induction of apoptosis by various stimuli leads to the extracellular release of HMGB1 (37). The process is either blocked or delayed by a broad-spectrum caspase inhibitor, and significant release of HMGB1 is observed at 24 hours after apoptosis induction. Another study showed that tumour cells cultured with cytolytic natural killer and CD8+ T cells released HMGB1 into the extracellular space. This release is also partially blocked by a broad-spectrum inhibitor of caspase (82). These findings suggest that HMGB1 release may not be a distinct feature of necrosis, and that its release can occur as apoptosis proceeds. Whether necrotic cells, apoptotic cells, or both release HMGB1 into the extracellular milieu needs to be clarified.

No matter what the origin is, extracellular HMGB1 acts as a potent inflammatory factor. It induces TNF and IL-1 synthesis and secretion from monocytes/macrophages, and administration of purified HMGB1 to mice results in an increase in serum TNF level (83). HMGB1 can activate macrophages, resulting in the release of more inflammatory mediators such as TNF-α (81). Extracellular HMGB1 promotes DC maturation. Necrotic *Hmgb1*$^{-/-}$ cells have a reduced capacity to activate DC *in vitro*, and inhibitors and antibodies against HMGB1 decrease DC activation by wild-type necrotic cells (41). Extracellular HMGB1 also induces the maturation of human DC (84). Several receptors have been suggested for HMGB1, and those include the receptor for advanced glycation end product (RAGE) (85) and Toll-like receptors (TLR) (86).

Interestingly, it was shown that exposure of apoptotic cells to macrophage culture can also stimulate the release of HMGB1 and other inflammatory cytokines in a dose-dependent manner (87). HMGB1 release can be reduced by caspase inhibitor, indicating that accumulation of apoptotic cells can activate macrophages to release HMGB1 and other pro-inflammatory cytokines. While the occurrence and subsequent efficient clearance of apoptotic cells does not set off an alarm for tissue damage to the immune system, the presence of a large number of apoptotic cells could trigger an immune response.

HMGB1 has been associated with some autoimmune diseases. In animal models of arthritis, in which TNF and IL-1 play key pathogenic roles, HMGB1 is expressed in the cytoplasm of mononuclear cells within inflamed synovial tissues (88). A similar pattern of expression of HMGB1 is detected in mononuclear, macrophage-like cells in biopsy specimens from human rheumatoid arthritis patients (88).

2.2.5 Basis for thesis design

There has been considerable interest in identifying danger signals and their role in the pathogenesis of various diseases. HMGB1 has recently drawn much attention as one danger signal associated with many diseases (40). One of the focused areas of HMGB1 research is the extracellular release from dying or dead cells. Scaffidi *et al.* demonstrated that HMGB1 is released exclusively from necrotic cells (81), but later that notion was challenged by others (37). There is still controversy as to whether or not apoptotic cells release HMGB1 into the extracellular milieu. In addition, the extracellular release of HMGB1 from

apoptotic β-cells is an unexplored area. Therefore, the purpose of the first half of this section of the thesis is to investigate the time-dependent HMGB1 release from apoptotic β-cells.

In T1DM, defective clearance of apoptotic cells following a neonatal wave of β-cell apoptosis presumably leads to the increased incidence of necrotic β-cells. Necrosis has been associated with inflammation, and recently HMGB1 has been identified as a strong inflammatory mediator released from necrotic cells (41). Using RINm5F β-cell lines and isolated rat islets, Steer *et al.* illustrated that IL-1-induced necrotic β-cells to release HMGB1 (38). However, the dose-dependent release of HMGB1 from necrotic β-cells has yet to be investigated. Streptozocin (STZ) induces a different mode of cell death (apoptosis versus necrosis) depending upon its dose (63), and is used routinely to induce diabetes experimentally. Therefore, the purpose of the second half of this section of the thesis is to explore the pattern of HMGB1 extracellular release by necrotic β-cells treated with increasing dosages of STZ.

2.2.6 Hypotheses

Apoptotic MIN6 β-cells release the danger signal HMGB1 into the extracellular milieu in a time-dependent manner. Necrotic MIN6 β-cells induced by increasing concentrations of streptozocin release the danger signal HMGB1 in a dose-dependent fashion.

2.3 Materials and Methods

2.3.1 Antibodies

A rabbit polyclonal antibody for HMGB1 was purchased from abcam (Cambridge, MA) and used in a 1:1000 dilution. A rabbit polyclonal antibody to cleaved capase-3 and a rabbit monoclonal antibody to full-length caspase-3 were obtained from Cell Signaling Technology (Danvers, MA) and used in a 1:1000 dilution. All secondary antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used in a 1:20,000 dilution.

2.3.2 Cell culture

MIN6 cells (mouse β-cell line) were a gift from the David Vocadlo's Laboratory at Simon Fraser University. The cells were maintained in medium consisting of Dulbecco's Modified Eagle Media (DMEM) (GIBCO, Burlington, ON, Canada) supplemented with 15% v/v heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 100 µg/ml streptomycin and 100 unit/ml penicillin (100-Pen/Strep) (GIBCO), and 5nl/ml β-mercaptoethanol (β-ME) (Bioshop Canada, Burlington, ON, Canada) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were subcultured once a week using 0.25% trypsin/EDTA solution (GIBCO). All cells used in the present study were between passage P19 and P36.

2.3.3 Induction and inhibition of apoptosis

Apoptosis was induced in MIN6 cells using staurosporine (STS) (Sigma). STS is a broad inhibitor of kinases and a known inducer of apoptosis. MIN6 cells were treated with 5μM STS for specifically indicated times in complete DMEM. To demonstrate inhibition of the apoptotic process, cells were treated with 100μM of Caspase Inhibitor I (Z-Val-Ala-Asp (OMe)-CH_2F (Z-VAD-fmk)) (Calbiochem, EMD Chemicals Inc., San Diego, CA) for one hour prior to the addition of STS.

2.3.4 Preparation of cell lysate

MIN6 cell lysates were obtained by homogenizing cells in CelLytic M mammalian cell lysis buffer (Sigma). One microgram per mL protease inhibitor cocktail (Sigma) was added to the lysis buffer right before use to prevent further protein degradation. Homogenized cells were subsequently centrifuged at 14,000 rpm for 15 minutes at 4 °C. The supernatant was collected and stored at -80°C until further use.

2.3.5 Collection of culture supernatant

Culture media from cells was centrifuged at 150 x *g* for 5 minutes to remove cellular debris. The supernatant was collected and stored at -80°C until use.

2.3.6 Protein assay

Protein concentrations of cell homogenates were determined by Bradford assay. A series of protein standards ranging from 0 to 1000 μg bovine serum albumin (BSA) were purchased from Bio-Rad (Mississauga, Ontario, Canada).

5μl of either a standard or a sample with an unknown protein concentration was tested in a 96-well microplate using 250μl of Quick Start™ Bradford Dye reagent (Bio-Rad). If necessary, the samples were diluted with nanopure H_2O (dH_2O). All standards and samples were prepared in duplicates. After a five-minute incubation at room temperature, the absorbance of the standards and samples was measured at 595nm by using a Rainbow microplate reader (SLT Labinstruments Ges.m.b.H., Salzburg, Austria). A standard curve was created by plotting the absorbance values on ordinate versus their concentration in mg/ml on abscissa. The protein concentration of the samples was determined using the standard curve. The final concentration of the samples was adjusted by multiplying by the dilution factor used, if any.

2.3.7 Western blot

Protein samples were diluted with NuPAGE® 4X LDS Sample Buffer (Invitrogen, Burlington, ON, Canada) at a ratio of 1:3; buffer to sample. The diluted samples were heated at 85°C for 10 minutes and fractionated on pre-cast sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Ready Gel® Tris-HCl gels, Bio-Rad). The gels were subjected to electrophoresis for 70 minutes at a constant voltage of 120V.

After electrophoresis, fractionated proteins were transferred from the gels to Immun-Blot™ polyvinylidene difluoride (PVDF) membranes (Bio-Rad) for 25 minutes at a constant voltage of 15V. The membranes were blocked with a blocking buffer (3-5% w/v non-fat dry milk (Bio-Rad) in Tris Buffer Saline with Tween 20 (Sigma) (TBST)) for 1 hour at room temperature while gently being

shaken. Membranes were then incubated with primary antibody overnight at 4°C with gentle agitation. The membranes were subjected to a series of washes with TBST followed by incubation with secondary antibody conjugated with horseradish peroxidase for 1 hour at room temperature with gentle shaking. All antibodies were diluted at an indicated ratio with the blocking buffer.

After another series of washes, the blots were incubated with Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare Bio-Science, Baie d'Urfé, Québec, Canada) for 5 minutes and the protein of interest was detected by exposing the blots onto Amersham Hyperfilm™ ECL™ (GE Healthcare Bio-Science) or Bioflex® MSI film (CLONEX corporation, Markham, Ontario, Canada). The films were developed using Kodak X-Omat 100A Processor (Kodak, Rochester, NY). Signals were captured by Image Analysis software, Image J (NIH, Bethesda, MD) and optical density was quantified by calculating mean grey value. Results of mean optical density values were expressed as mean ± SE.

2.3.8 Sample preparation for Western blot for HMGB1 detection

Supernatant samples from culture media from MIN6 cells treated with staurosporine were centrifuged through Amicon Ultra-4 centrifugal filter devices (Millipore, Millissauga, ON, Canada) with 10-kDa Nominal Molecular Weight Limit (NMWL) for 15 minutes at 4,000 *g* at room temperature. Supernatants were pipetted from the top reservoir of the filter device, which consisted of proteins equal to and bigger than 10-kDa. The collected supernatants were spun through a 100-kDa NMWL Ultrafree-0.5 centrifugal filter device (Millipore) for 30 min at

10,000 *g*. at 4°C. The portion that passed through the filter was collected and stored at -80°C until further use. Equal volumes of elute were subjected to Western blot without conducting a protein assay.

2.3.9 Cell viability assessment using trypan blue exclusion assay

2.3.9.1 Induction of cell death

MIN6 β-cells were seeded in a 24-well cell culture plate and cultured until cells reached approximately 85% confluency. Cells were washed three times with Dulbecco's-PBS (DPBS) (GIBCO) and FBS-free, low-glucose (5 mM) DMEM with 100-pen/strep containing varying concentrations (0, 20, 40, 60, 80 or 100 mM) of Streptozocin (STZ). Cells were then incubated for one hour at 37°C in a humidified atmosphere containing 5% CO_2. STZ-containing media were replaced with STZ-free, FBS-free, and high-glucose (25 mM) DMEM with 100-Pen/Strep and β-mercaptoethanol (β-ME). Cells were further incubated for various durations (1, 4, 8, 12, and 24 hours) at 37°C in a humidified atmosphere containing 5% CO_2 before samples were collected.

2.3.9.2 Trypan blue exclusion assay

At the end of each incubation period, the media was gently aspirated and cells were treated with 0.25% Trypsin/EDTA solution (GIBCO) for 1 to 2 minutes to be dissociated from the plate. Complete DMEM was then added to stop the action of the trypsin. An equal volume of cell suspension and 0.4% trypan blue solution (Sigma) were mixed, and viable cells were counted using a haemacytometer (VWR International). Viable cells were expressed as a

percentage of total viable cells treated with 0 mM STZ at any given time point to account for the possibility of normal growth of MIN6 cells during the period.

2.3.10 Flow cytometry

2.3.10.1 Induction of cell death

In order to label cells for analysis by flow cytometry, single cell suspensions were prepared by trypsinization before STZ treatment. MIN6 β-cells were trypsinized and resuspended into FBS-free DMEM with 100-pen/strep and β-ME. Cells were counted and a suspension of cells was seeded into a 5 ml polypropylene tube (BD Bioscience, Mississauga, ON Canada) at 1/4 X 10^6 cells/ml. Either 0, 10, 20, 40, or 60 mM of STZ was added, and the cells were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 hours.

2.3.10.2 Labelling of cell suspensions

Annexin V is a phospholipid-binding protein that has a high affinity for phosphatidylserine (PS), which is translocated from the inner to the outer leaflet of the plasma membrane in the early stage of apoptosis process. 7-Aminoactinomycin D (7-AAD) nucleic-acid dye is an intercalating agent used for the exclusion of nonviable cells in a flow cytometric assay. When cell membrane integrity is compromised, such cells are stained with 7-AAD. Hence, it can be used as a marker of cell viability. 7-AAD positive cells were considered necrotic.

Cells were centrifuged at 900 rpm for 5 minutes at 4°C, and STZ-containing media were decanted. Cells were resuspended with ice-cold, sterile

DPBS and transferred to a 5 ml polystyrene round-bottom tube (BD Bioscience). Cells were centrifuged again at 900rpm for 5 minutes at 4°C, and DBPS was discarded. Cells were then resuspended with 100 μl of Annexin V-binding buffer (1x) (BD Pharmigen, BD Bioscience). Annexin-V conjugated with Phycoerythrin (PE) (BD Pharmigen, BD Bioscience, 3μl) was added to each cell suspension, followed by the addition of 3 μl of 7- AAD (BD Pharmigen, BD Bioscience). Cells were incubated for 15 minutes in the dark. Three hundreds microlitre of Annexin V-binding buffer (1x) were added before further analysis.

2.3.10.3 Necrotic cell detection and analysis

Necrosis was identified by scanning the labelled cells by flow cytometry (FACSCalibur System, BD Biosciences, San Diego CA). MIN6 cell suspensions were labelled with PE-conjugated Annexin V and 7- AAD as described earlier. In each treatment group, 100,000 were analysed. These events were plotted on a grid comprised of four quadrants. Cells that were both Annexin V-positive and 7-AAD positive appeared in the top right quadrant of the flow cytometry dot-plot and were considered late necrotic. Cells that were only 7-AAD positive became visible in the top left quadrant of the flow cytometry dot-plot and were considered early necrotic. The sum of these two populations produced the total necrotic cell population. Since externalisation of PS occurs in the earlier stages of apoptosis, Annexin V-positive and 7-AAD negative cells were identified as early apoptotic and appeared in the bottom right quadrant of the dot-plot. Double negative cells, which were seen in the bottom left quadrant of the dot-plot, were considered viable. Analysis was conducted using CellQuest software (Becton Dickinson).

All flow cytometry experiments and analyses were performed in the laboratory of Dr. Jan P. Dutz at The British Columbia Child and Family Research Institute in Vancouver.

2.3.11 Sample preparation for Western blot for HMGB1 from STZ-treated MIN6 cells

MIN6 β-cells were seeded in a 24-well cell culture plate and incubated until cells reached approximately 85% confluency. Cells were washed three times with DPBS, and FBS-free, low-glucose (5 mM) DMEM with 100-pen/strep containing increasing concentrations (0, 20, 40, and 60 mM) of STZ. Cells were then incubated for one hour at 37°C in a humidified atmosphere containing 5% CO_2. STZ-containing media was replaced with STZ-free, FBS-free, and high-glucose (25 mM) DMEM with 100-pen/strep and β-ME. Cells were further incubated for 4 hours at 37°C in a humidified atmosphere containing 5% CO_2 before media samples were collected.

Supernatant samples from culture media from MIN6 cells treated with STZ were centrifuged through an Ultrafree-0.5 centrifugal filter device (Millipore) with 10-kDa NMWL for 30 minutes at 10,000 *g* at 4°C. Media samples that did not pass through the filter were collected and stored at -80°C until further use. Equal volumes of elute were analysed by Western blot without conducting a protein assay.

2.3.12 Statistical analysis

All statistical analyses were conducted using SigmaStat 3.1. A Student's t-test was performed for STS treatment vs. control. A two-way analysis of variance (ANOVA) was performed for STZ concentrations (0, 20, 40, 60, 80, and 100 mM) and time of incubation (1, 4, 8, 12, and 24 hours) as independent variables and percent viable cells as a dependent variable. A one-way ANOVA was carried out for the STZ concentrations (0, 10, 20, 40, and 60 mM) as an independent variable and percent of necrosis as a dependent variable. One-way ANOVA were used again for the STZ concentrations (0, 20, 40, and 60 mM) as an independent variable and HMGB1 expression as a dependent variable. Post hoc analysis was done using Tukey's test. Results are expressed as means ± SE. Differences were deemed statistically significant at $p<0.05$.

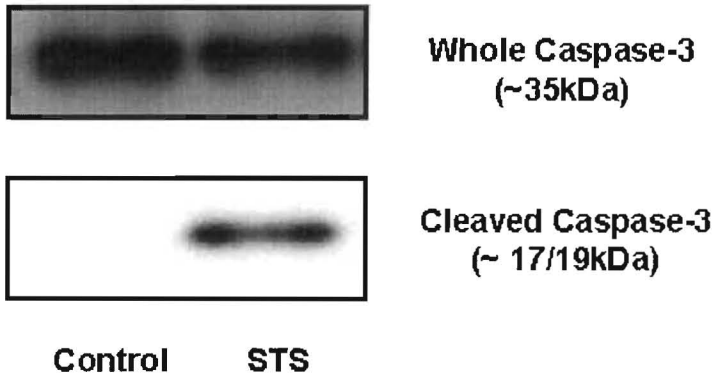
2.4 Results

To investigate the time-dependent release of HMGB1 from apoptotic β-cells, apoptosis was first induced in MIN6 β-cells by staurosporine (STS), a broad inhibitor of kinases. Induction of apoptosis was confirmed by analysing the expression of activated capase-3 at 4 hours after the induction. The lower panel of Figure 4A shows Western blotting of cell lysate from MIN6 cells treated with STS or from non-treated MIN6 cells using anti-cleaved caspase-3 antibodies. The protein band shown in the lower panel of Figure 4A corresponds to roughly 19kDa. The same membranes immunoblotted for cleaved caspase-3 were also labelled with antibodies against whole (uncleaved) caspase-3, and results are presented in the upper panel of Figure 4A. The protein band in the upper panel of Figure 4A is equivalent to a protein of approximately 35kDa. The presence of the cleaved caspase-3 band seen in lane 2 of the lower blot in Figure 4A confirms that cells were apoptotic compared to control.

Bands of cleaved caspase-3 were quantified for optical density (OD) and normalised by a corresponding OD of a band of whole caspase-3. The normalised OD was then plotted against treatment, which is displayed in the figure below.

Apoptotic MIN6 cells showed a greater than 16-fold increase in activation of caspase 3 compared to non-treated MIN6 cells. Student's *t*-test revealed that there was a significant difference in expression of activated caspase-3 between apoptotic and non-treated MIN6 cells (p=0.013).

A



B

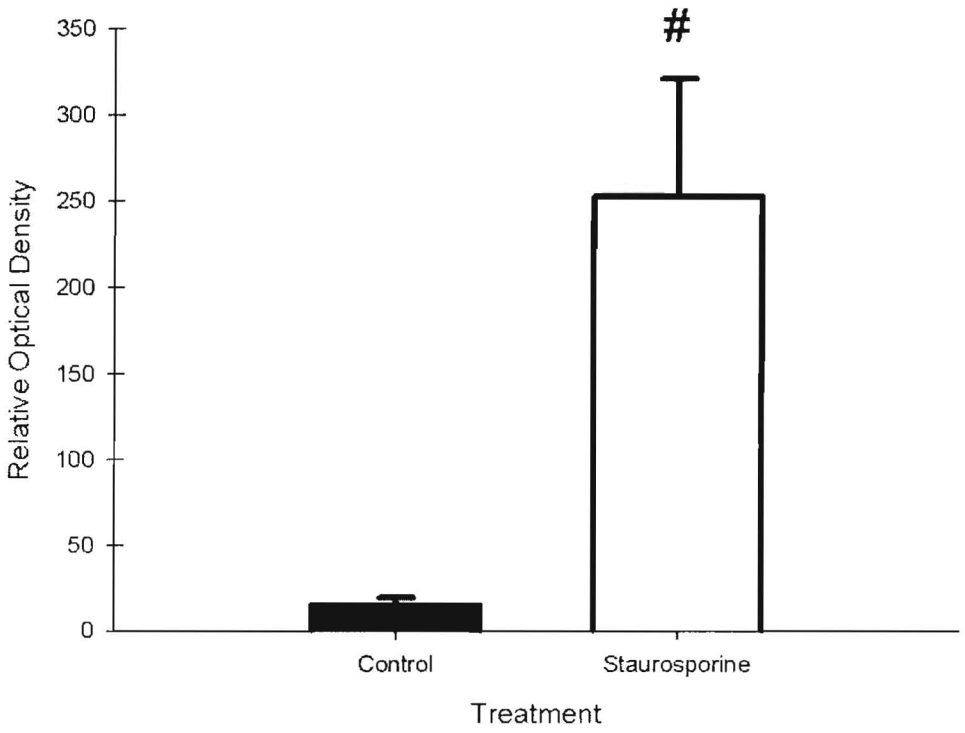


Figure 4. Protein expression of activation of caspase-3 in MIN6 cells treated by staurosporine (STS)

(A) Protein expression of activates caspase-3 in MIN6 cells by Western blot (B) Relative optical density of activated caspase-3 expression in MIN6 cells

MIN6 cells were treated with 10μM STS for 4 hours. Protein expression of activated caspase-3 was determined by Western blot. Results are shown as mean ± SE (n=4). #: P = 0.013 (Student's *t*-test)

In order to determine the time-dependent release of HMGB1 from apoptotic MIN6 cells, culture media was analysed. HMGB1 in culture media was detected by Western blot after 4 hours and 27 hours of the initial apoptosis induction. Western blotting of culture media from apoptotic MIN6 cells or non-treated control cells using an anti-HMGB1 antibody revealed a protein band corresponding to roughly 30kDa, as illustrated in Figure 5. The position of this band agrees with the approximate size of HMGB1 (~27kDa).

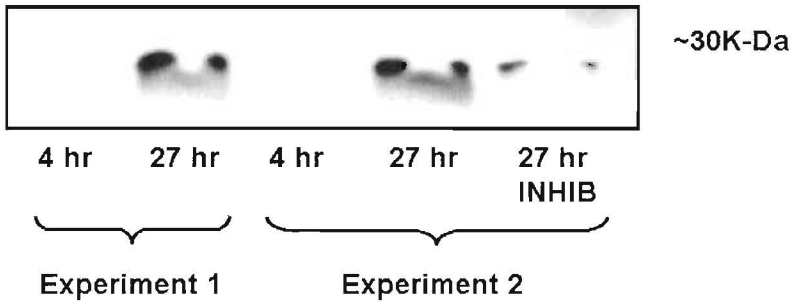


Figure 5. Protein expression of HMGB1 by Western blot

Representative Western blot is shown. Time indicates the duration that MIN6 cells were cultured with media contained with 10 μM staurosporine (STS). INHIB = caspase inhibitor added to media prior to STS treatment

Western blotting of the supernatant samples revealed that a negligible level of HMGB1 was detected in 4-hour samples while quite significant amounts were detected in 27-hour samples. Use of an inhibitor of caspase 3 to block the induction of apoptosis reduced HMGB1 expression in culture media at 27 hours after the initial treatment.

Now that the concept of time dependent release of HMGB1 from apoptotic β-cells was established, the next logical step was to explore the pattern of

HMGB1 extracellular release by necrotic β-cells exposed to increased dosages of STZ. Ideally, a positive correlation between necrosis and STZ dosage in MIN6 β-cells would be shown. However, the difficulty with this goal is that identifying and quantifying necrosis is not routinely performed, and there are no well-established methods reasonably available. To get around this problem, a cell viability assay was performed using trypan blue exclusion. Figure 6 illustrates the results from the trypan blue exclusion viability assay of MIN6 cells after treatment with incremental concentrations of STZ up to 100 mM.

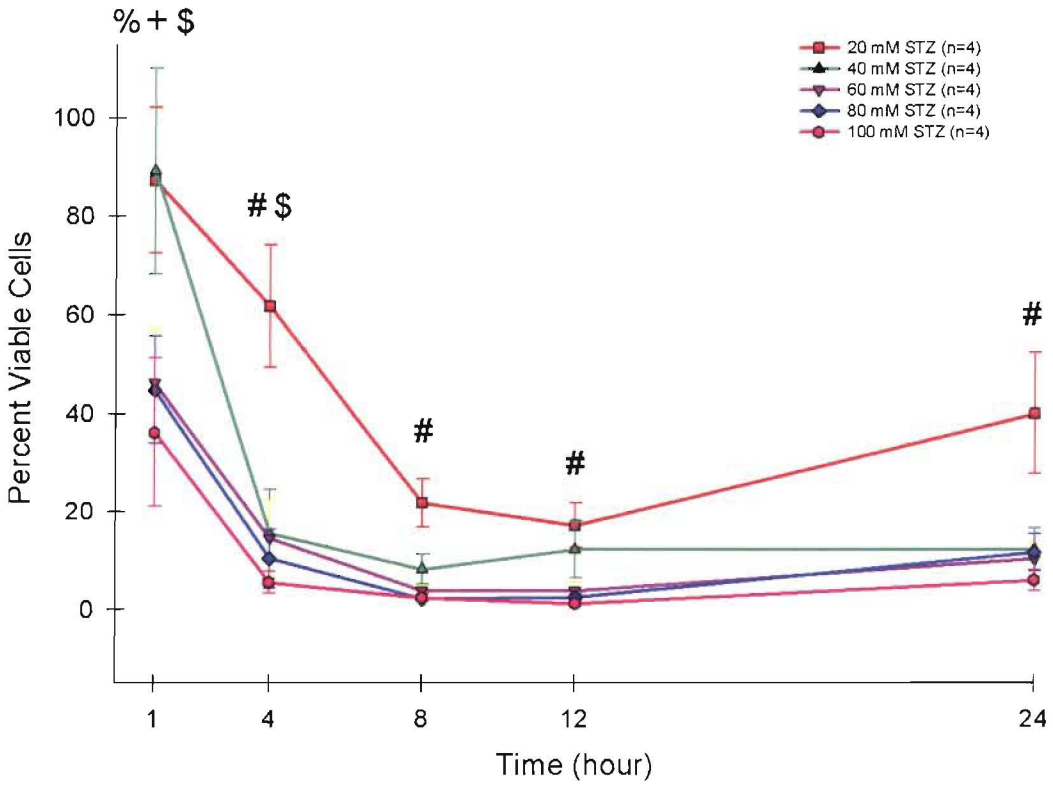


Figure 6. Percent viable MIN6 cells treated with various doses of streptozocin (STZ)

MIN6 cell viability is expressed as percentage of untreated control cells at each time-point. MIN6 cells were treated with indicated concentrations of STZ for one hour, and incubated in fresh STZ-free media for 1, 4, 8, 12 or 24 hours. Viable cells were determined by trypan blue exclusion assay. Results are shown as mean ± SE. #: $P<0.001$ against 0 mM STZ. $: $P<0.001$ against 20 mM STZ. %: $P<0.005$ against 40 mM STZ. +: $P< 0.001$ between 0 mM and 60, 80, 100 mM STZ.

Less than half of cells were viable at concentrations equal to or higher than 60 mM after one hour of STZ-treatment ($p<0.001$ against 0 mM and 20 mM, $p<0.005$ against 40 mM). At 4 hours after the treatment, less than 20 percent of cells were viable at concentrations equal to or higher than 40 mM, whereas there were more than 60 percent viable cells at 20 mM STZ ($p<0.001$ against 0 mM, $p<0.01$ 20 mM against 40, 60, 80, and 100 mM). There were few viable cells at concentrations of 40 mM and greater at 8 hours or longer after the induction of cell death ($p<0.001$ against 0 mM). At 20 mM of STZ, viable cells were down to 22 percent and no major differences were observed thereafter except for a slight, statistically insignificant increase from at time 12 to time 24 hr.

To further characterise cell death, flow cytometry was conducted for MIN6 cells treated with increasing concentrations of STZ. To determine appropriate dosages, results from the previous experiment were evaluated. As is shown in Figure 6, there was no difference in percent viable cells above 60 mM. Thus, 80 mM and 100 mM STZ were no longer used in the study. STZ-treated MIN6 cells were labelled with Annexin V to quantify apoptosis and 7-Aminoactinomycin D (7-AAD) to assess necrosis before analysis by flow cytometry. Figure 7 is representative dot plot of MIN6 cells from flow cytometry analysis.

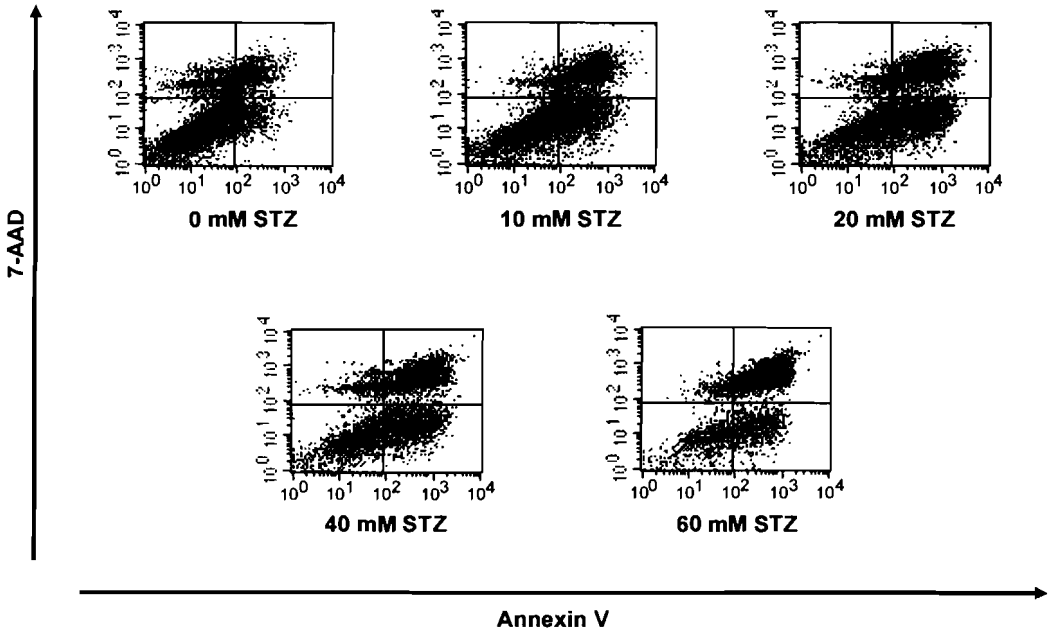


Figure 7. Representative dot plot of MIN6 cells treated with streptozocin (STZ)

Streptozocin-treated MIN6 cells were labelled with Annexin V for apoptosis and with 7-Aminoactinomycin D (7-AAD) for necrosis. Labelled cells were analysed by flow cytometry.

In this specific plot, nearly 85% of cells were 7-AAD positive at 60 mM, and approximately 55% of the population were negative for both Annexin V and 7-AAD at 0 mM. The dots in the upper right quadrant of the last plot indicate that the vast majority of cells treated with 60 mM STZ were necrotic. A population of Annexin V positive and 7-AAD negative cells is representative of early apoptosis and slightly decreased dose-dependently. As the STZ dosage increased from 0 – 60 mM, cells transitioned from the lower left to the upper right quadrant, suggesting the cells were increasingly necrotic at higher dosages. The dot plot visual data from Figure 7 is quantified and summarised from five independent experiments and shown in Table 1.

STZ (mM)	Viable (Double Negative)	Early Apoptosis (Annexin V Positive)	Early Necrosis (7-AAD Positive)	Late Necrosis (Double Postive)	ALL Necrosis (Both 7-AAD positive)
0	31.4 ± 5.2	15.4 ± 3.0	7.7 ± 1.5	28.8 ± 4.0	43.8 ± 2.6
10	29.6 ± 2.9	17.5 ± 1.2	3.8 ± 0.8	32.4 ± 1.6	43.4 ± 1.8
20	24.2 ± 1.5	13.9 ± 2.1	6.4 ± 0.7	35.5 ± 0.3	52.3 ± 0.8
40	13.7 ± 2.2	14.0 ± 2.5	4.9 ± 0.7	50.7 ± 3.7	66.8 ± 3.7
60	6.8 ± 0.5	12.0 ± 3.4	4.1 ± 0.7	60.4 ± 3.4	77.5 ± 3.3

(mean ± SE)

Table 1. Quantification of dot plots of labelled MIN6 cells treated with streptozocin (STZ)

In order to determine necrosis, the total amount of 7-AAD positive cells (both Annexin V negative and positive) were then plotted against concentration of STZ. As is shown in Figure 8 below, the number of 7-AAD positive MIN6 cells increased with greater concentrations of STZ.

At 60 mM, 77.5% of cells were 7-AAD positive, whereas only 43.8% were positive for 7-AAD at 0 mM. This confirms that the cells were increasingly necrotic at higher dosages. One-way ANOVA revealed a significant difference in 7-AAD positive cells between at 40 or 60 mM and at the rest of concentrations ($p<0.001$: 60 mM vs. 0, 10 and 20 mM; 40 mM vs. 0 and 10 mM. $p<0.02$: 40 mM vs. 20 mM).

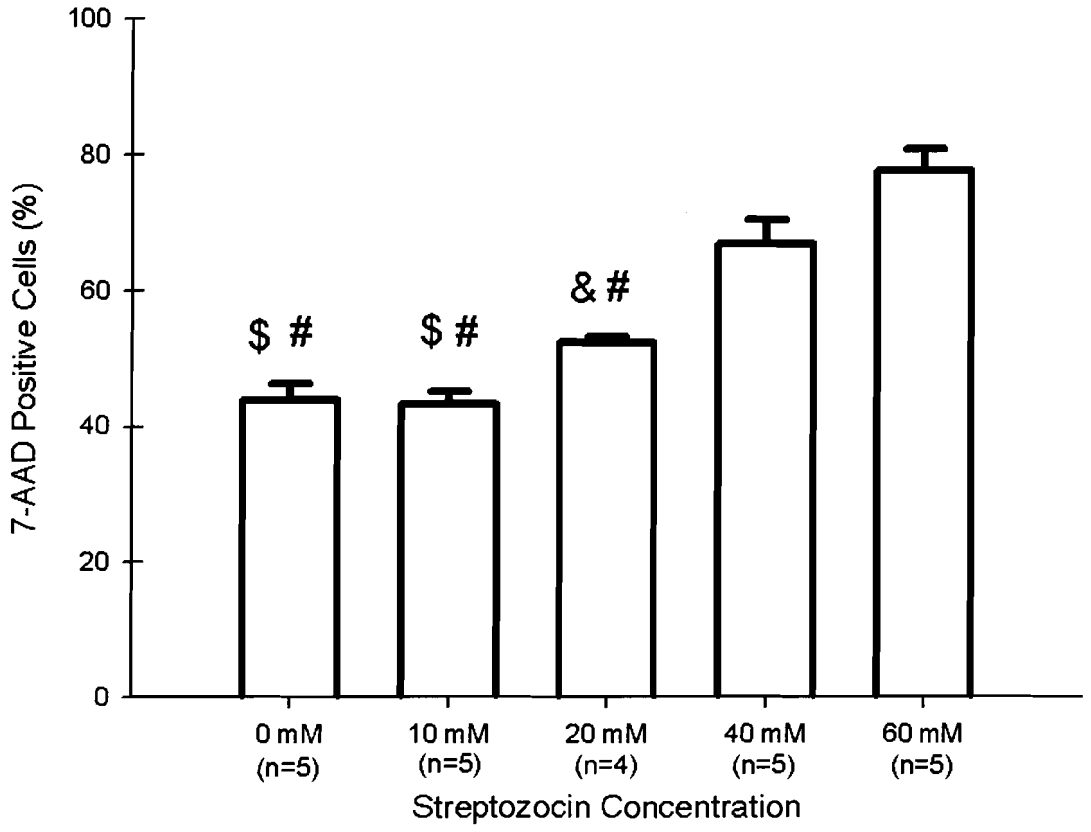
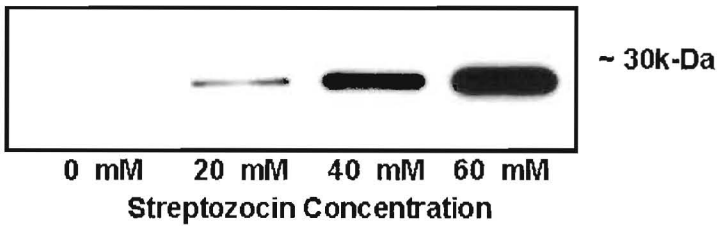


Figure 8. Percentage of necrotic MIN6 cells treated with increasing concentrations of streptozocin (STZ)

MIN6 cells were treated with increasing concentration of STZ to induce cell death for 2 hours. Necrosis was assayed by flow cytometry using 7-AAD labelling. Results are shown as mean ± SE. #: $P<0.001$ against 60 mM. $: $P<0.001$ against 40 mM. &: $P=0.018$ against 40 mM.

Now that the amounts of necrotic MIN6 cells at specific STZ dosages were established, the extracellular release of HMGB1 into culture media was analysed by Western blotting. STZ dosage of 10 mM was no longer used because 0 mM STZ and 10 mM STZ did not show any significant differences in the amount of necrotic MIN6 cells in the previous experiment.

A.



B.

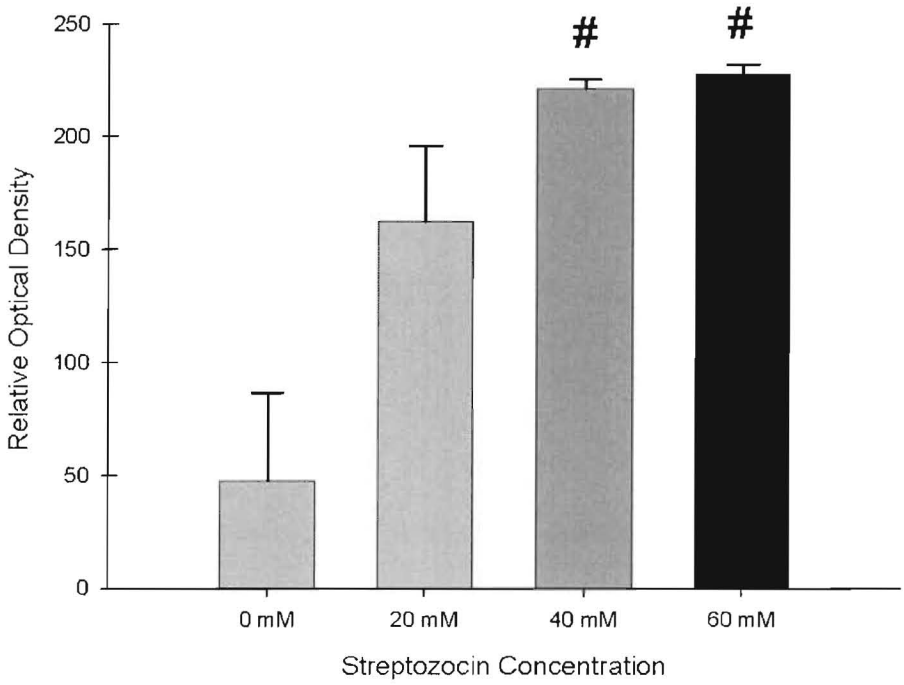


Figure 9. HMGB1 expression from culture media from MIN6 cells treated with increasing dosages of streptozocin (STZ)

(A) Protein expression of HMGB1 in culture media by Western blot. (B) Quantification of HMGB1 protein expression. MIN6 cells were treated with the indicated concentrations of STZ for one hour, and incubated for additional four hours. Supernatants were collected and assayed for HMGB1 expression by Western blot. Results are shown as mean ± SE (n=3). #: P≤0.007 against 0 mM treatment.

Figure 9A shows protein expression of HMGB1 in culture supernatant by Western blot, which revealed that HMGB1 release from MIN6 cells treated with STZ was increasing in a dose-dependent manner. In Figure 9B, optical density

(OD) of Western blots of HMGB1 protein expression was quantified and plotted against concentration of STZ. In concordance with the Figure 9A, 60 mM STZ-treated MIN6 cells released approximately 4.5 times greater amounts of HMGB1 into culture media than 0 mM STZ-treated MIN6 cells ($p<0.007$ 0 mM vs.40, or 60 mM). However, no significant difference was observed in OD between 0 mM and 20 mM, or between 20 mM and 40 or 60 mM.

2.5 Discussion

The extracellular release of HMGB1 from secondary necrotic MIN6 cells observed in the present study supports the notion presented by Bell *et al.* (37) that extracellular HMGB1 release may not be an exclusive feature of necrotic death, and suggests that HMGB1 release can occur as apoptosis proceeds. In Bell's work, HMGB1 release is blocked by a broad caspase inhibitor. Here, we observed otherwise. From cells treated with STS and an inhibitor, a reduced amount of HMGB1 release was detected compared to HMGB1 released from cells treated with STS alone. Use of the same inhibitor only partially blocked the release of HMGB1 from STS-treated MIN6 cells. This may suggest that, in this specific cell type, other apoptotic pathways (i.e., caspase-independent pathways) were activated by the same pharmacological agent, or that some of the HMGB1 release observed in this study may be due to cell death from normal cell turnover of this cell line over a 27-hour period. Interestingly, it has been shown that HMGB1 is sequestered in the nucleus during apoptosis, and hence HMGB1 release does not occur even from secondary necrotic cells (81). It has also been shown *in vitro* that apoptotic cells release DNA into the extracellular milieu (36). Since DNA can be released from secondary necrotic cells and is associated with HMGB1, presumably HMGB1 could be released even after apoptosis. That notion agrees with observation in this work.

The dosage of STZ is what dictated whether cells undergo apoptosis or necrosis. From the flow cytometry analysis, we observed a dose-dependent increase of necrotic β-cells treated with incremental doses of STZ. This result

confirms previous work done by Saini *et al.* (63). In their study, only 20 percent of cells die by necrosis at 30 mM STZ. In addition, their results show a nearly equal apoptotic and necrotic population at 30 mM STZ. Furthermore, from the flow cytometry analysis, it is revealed that the number of early apoptotic cells does not change greatly with increasing concentrations of STZ. This contradicts Saini's work as well, as they showed a significant increase (from about 5% to 20%) in the apoptotic cell population from 20 to 30 mM STZ. These discrepancies may be due to the methodology utilised to identify dead cells. Here, we used flow cytometry analysis for cells labelled with Annexin V and 7-AAD to characterize cell death. This method is generally well suited for identification of early apoptotic cells. However, all primary and secondary necrotic cells read positive for 7-AAD. Saini and colleagues analysed cell death by light microscopy, which allows more detailed morphological analysis than flow cytometry. They even distinguished primary from secondary necrosis and categorise secondary necrosis as apoptosis, whereas the present study categorises secondary necrosis as necrosis.

In the present study, we found extracellular HMGB1 released from both primary and secondary necrotic β-cells. The observed HMGB1 release could lead to the initiation of β-cell-directed immunity. There are some potential underlying mechanisms to propose. First of all, HMGB1 is considered one of the damage-associated molecular pattern (DAMPs) that can induce maturation of DCs (70, 89). Mature DCs may process readily available apoptotic β-cells,

present β-cell-derived antigen, and prime naïve autoreactive T cells to destroy β-cells.

Secondly, HMGB1 can activate macrophages, which once activated release inflammatory cytokines (78, 83). Activated macrophages and inflammatory cytokines released from activated macrophages are associated with direct killing of β-cells (90). In fact, one such cytokine, interleukin-1 (IL-1) has recently been shown to kill β-cells directly by necrosis (38). As is shown in the polyarthritis model (31), an increase in TNF-α from activated macrophages may result in autoimmunity in NOD mice (91).

Thirdly, an initial HMGB1 release could drag the islet environment into an endless feedback loop of an HMGB1 signalling cascade. Activated macrophages and mature DCs release HMGB1 (78, 80). β-cells destroyed by CD8+ T cells could release HMGB1 in the same way as tumour cells cultured with cytolytic CD8+ T cells release HMGB1 (82). It has been shown that *in vitro* exposure of large quantities of apoptotic cells to macrophages can also stimulate the release of HMGB1 (87). This phenomenon may also occur in the pancreatic islets especially where clearance of apoptotic cells is impaired. Moreover, IL-1-induced necrotic β-cells have been shown to release HMGB1 *in vitro* (38). Therefore, the positive-feedback loop of HMGB1 could keep going until most β-cells have been destroyed.

Lastly, another possible mechanism by which HMGB1 induces inflammation is to activate immune cells via the receptor for advanced glycation

end product (RAGE). Extracellular HMGB1 may interact with RAGE (85, 92). RAGE is expressed in immune cells from the spleen of diabetic NOD mice induced by adaptive-transfer (93). RAGE is also expressed in the islets from diabetic NOD mice with an inflammatory infiltrate. Interestingly, blocking RAGE by soluble RAGE prevents transfer of diabetes in an adaptive transfer model and delays islet graft rejection (93). *In situ* expression of TNFα and IL-1β was decreased in the RAGE-blocked mice compared to control mice. Increased expression of IL-10 and TGF-β by RT-PCR in the isolated islets from RAGE-blocked mice was also noted, suggesting blocking of RAGE shifts the islet environment from inflammatory to more or less a regulatory one. Binding of extracellular HMGB1 to RAGE in the islets may lead to the secretion of inflammatory cytokines such as IL-1 and TNF-α, which results in the activation of immune cells. *In situ* expression of HMGB1 and HMGB1/RAGE-dependent inflammatory response in NOD mice needs further investigation.

3: Summary and Future Direction

In this study, the degradation of apoptotic cells by macrophages was investigated in diabetes-prone NOD and diabetes-resistant BALB/c mice. The results showed that macrophages from NOD mice exhibit reduced degradation of apoptotic cells *in vitro* compared to BALB/c macrophages. Aberrant phagocytosis of apoptotic cells has been reported in young NOD mice. Taken together, clearance of apoptotic cells is impaired in NOD mice. This thesis also reports that HMGB1 was released from both primary and secondary necrotic β-cells *in vitro*. There is accumulating evidence that HMGB1 is a potent danger signal that initiates inflammation. The aberrant clearance could result in high incidence of tissue-remaining apoptotic β-cells in NOD mice, especially following the neonatal wave of β-cell apoptosis. Secondary necrosis from impaired clearance of apoptotic cells could release HMGB1 in the pancreatic tissue of NOD mice. β-cells killed directly by necrosis by tissue-infiltrating cells could also build up necrotic β-cells in the pancreatic islets. HMGB1 released from those necrotic β-cells could initiate or exacerbate inflammation.

To further delineate the role of HMGB1 in the pathogenesis of T1DM and potentially recurrent immunity to transplanted islets, which is currently the only cure available for T1DM patients, it would be interesting to investigate 1) whether HMGB1 from β-cells induces inflammation, and 2) whether HMGB1 is essential

for the development of T1DM. To answer these questions, we could use the NOD mouse model, primary immune cells and β-cell lines. Specifically, we could examine the degree of activation of innate immune cells, such as macrophages and dendritic cells, in exposure to culture media from necrotic β-cells. Moreover, we could observe the severity of insulitis, the disease development, or islet graft rejection in NOD mice after blocking HMGB1 by means such as use of anti-HMGB1 antibodies, or to generate an inducible gene knockout mouse model to test the role of HMGB1 in T1DM.

4: Conclusion

Macrophages from NOD mice display a reduced rate of degradation of internalised apoptotic thymocytes *in vitro* compared to control BALB/c macrophages. HMGB1 is released from both primary and secondary necrotic β-cells into the extra cellular milieu. Increased presence of necrotic β-cells, possibly due to impaired clearance of apoptotic cells, leads to increased expression of HMGB1 that may exacerbate inflammation in the pancreatic islets of diabetes-prone NOD mice.

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