Macrophage Clearance Defects Prior to the Onset of Diabetes in NOD Mice

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ABSTRACT

An increased incidence of apoptotic β-cells in young nonobese diabetic (NOD) mice may result from decreased clearance of the dying cells from the pancreas. To investigate macrophage phagocytic ability *in vivo*, we examined 1) the time-course of peritoneal macrophage phagocytosis following thioglycollate (THG)-induced peritonitis and 2) the direct injection of apoptotic thymocytes. In those experiments, macrophages from NOD mice engulfed fewer apoptotic neutrophils and thymocytes as compared to control Balb/c macrophages. We also examined the production of cytokines by macrophages following apoptotic thymocyte stimulation *in vivo*. However, the level of transforming growth factor-β_1 (TGF-β_1) produced by peritoneal macrophages was not upregulated by apoptotic thymocytes in NOD mice.

These experiments demonstrate that macrophages from NOD mice have a defect in apoptotic cell clearance and an inability to upregulate TGF-β_1 following apoptotic cell stimulation. These results suggest that defective macrophage function may be involved in the initiation of autoimmunity in T1DM.

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ABBREVIATIONS

APC	Antigen presenting cell
BB	Biobreeding
BBdp	Diabetes-prone Biobreeding
BBdr	Diabetes-resistant Biobreeding
BSA	Bovine albumin
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FSD	Fractional standard deviation
NOD	Nonobese diabetic
NOR	Non-obese diabetes resistant
PEC	Peritoneal exudate cells
PI	Propidium iodine
PS	Phosphatidylserine
RT	Room Temperature
SLE	Systemic lupus erythematosus
T1D	Type 1 diabetes
TGF-β_1	Transforming growth factor-β_1
Th	T helper
TUNEL	Terminal dUTP nick end labelling

CHAPTER 1 BACKGROUND

1.1 TYPE 1 DIABETES

Type 1 diabetes (T1D) is an autoimmune disease characterized by an inflammatory reaction in and around pancreatic islets that is followed by the selective destruction of the insulin-secreting β-cells (1). The pathogenesis of T1D has two distinct phases: insulitis, in which a mixed population of monocytes (macrophages and dendritic cells (DCs)) and lymphocytes (T cells and B cells) infiltrate the islets; and diabetes, in which most β-cells have been eliminated by T cells, and residual β-cells do not produce sufficient insulin to regulate blood glucose levels. The preclinical period of T1D development is silent and extensive. Insulitis can continue for several years after it begins and then progress to overt diabetes (2). Sometimes, individuals can have insulitis for their lifetime and never develop diabetes (2).

Under some conditions, β-cell-derived antigens are exposed to antigen presenting cells (APCs) (such as DCs) in the islets. After recognizing and taking up β-cell-derived antigens, APCs migrate to the regional draining pancreatic lymph nodes and become mature. Here, DCs display the β-cell-derived antigens to naive β-cell-reactive T cells and activate them. The T cells then acquire the ability to migrate throughout the body. When autoreactive T cells enter the islets and encounter cognate β-cell antigen, they are stimulated, retained within the islets, and begin killing the β-cells (Figure 1) (3). To activate autoreactive T cells, self-

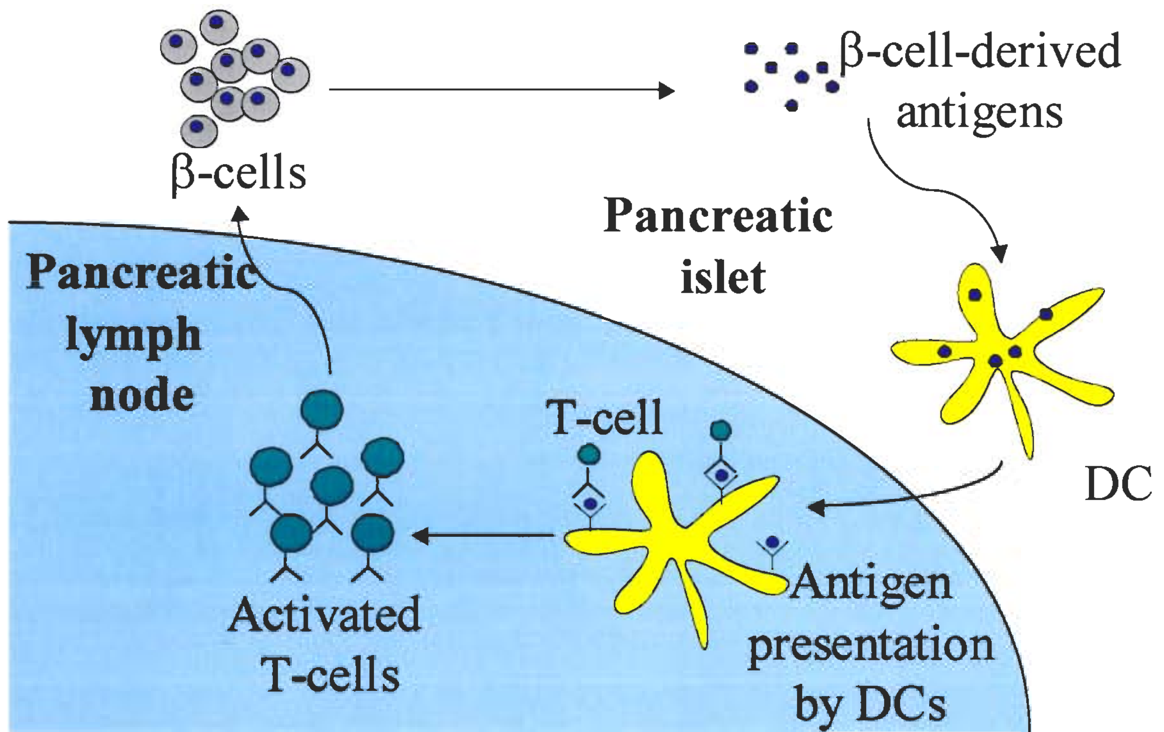


Figure 1: Proposed model of initiation of autoimmunity in T1D.
β-cell-derived antigens are taken up by DCs, in the islets, inducing maturation of the APCs and their migration to the immediately draining pancreatic lymph nodes. DCs display β-cell-derived antigens to naive β-cell-reactive T cells, activating them. The T cells acquire the ability to migrate through the pancreas, and kill β-cells.

antigens must be presented by APCs. Exposure of a large number of self-antigens to APCs may be the first step toward autoimmunity in T1D.

1.2 APOPTOSIS

1.2.1 MORPHOLOGICAL CHARACTERISTICS OF APOPTOSIS

Apoptosis eliminates unwanted, injured, or virus-infected cells. An important difference between programmed (apoptotic) versus accidental/toxic (necrotic) death is that programmed cell death results in the ordered fragmentation of the cell and the display of important signals. These signals lead to rapid phagocytosis of apoptotic cells by professional phagocytes (or neighbouring cells) without activation of the phagocyte and subsequent inflammation (4). Specific surface molecules on apoptotic cells facilitate recognition, binding and engulfment of apoptotic cells by macrophages. The importance of this process is highlighted by the redundancy of the phagocytic recognition, with macrophages utilizing several different receptors to engage and engulf dying cells.

Under normal conditions, the immune system is exposed to many millions of apoptotic cells each day. However, under physiological conditions free apoptotic bodies within tissues are rarely seen and mostly appear inside phagocytes that have avidly ingested the apoptotic cells as soon as they are generated. The lack of apoptotic bodies within tissues indicates the efficiency with which macrophages clear dying cells, and likely indicates the importance of the rapid removal of debris.

Distinct morphological changes characterize death by apoptosis (5). Initially, cells round up and detach from their neighbours, followed by condensation of both nucleus and cytoplasm, without disruption of intracellular membranes. The nucleus, however, fragments and characteristic membrane protuberances (blebs) appear. Apoptotic bodies form when blebs segment off, maintaining intact membranes. If apoptotic bodies are not rapidly engulfed by phagocytes, they may remain in the tissue and undergo secondary necrosis.

1.2.2 APOPTOSIS IN T1D

T1D mellitus occurs when there is an insufficient mass of functional β-cells to maintain normoglycemia. The mass of β-cells is determined by the balance between the rates of β-cell replication, neogenesis and cell death (6). In predisposed humans and animal models of T1D, the rate of β-cell destruction far exceeds the rate of new cell formation, such that ultimately few or no β-cells remain (7).

In models of both induced (7) and spontaneous (8) T1D, β-cells are eliminated by apoptosis. Importantly, in both models β-cell apoptosis precedes lymphocyte infiltration into the islets (insulitis), and continues after the appearance of immune cells in the islets. The incidence of apoptotic β-cells is higher in diabetes-prone animals than that of diabetes-resistant animals (9). These findings suggest that β-cell apoptosis may be important in both the initiation and perpetuation of β-cell directed autoimmunity.

Finegood and colleagues have previously observed a neonatal wave of β-cell apoptosis in normal rats (6). A similar neonatal wave of β-cell apoptosis also exists in the nonobese diabetic (NOD) mouse and biobreeding (BB) rat, peaking at ~12 days of age (9). However, the incidence of apoptotic β-cells was higher in diabetes-prone animals as compared to diabetes-resistant strains. This neonatal wave precedes the priming of T cells in autoimmune diabetes (10). The coincidence of the neonatal wave of β-cell apoptosis and the first signs of T cell infiltration into the islets suggests that neonatal β-cell apoptosis may be the initiating event for autoimmunity in T1D.

1.2.3 APOPTOSIS AND AUTO-ANTIGENS

Under normal circumstances the products of apoptotic cells may not lead to autoimmunity (11). However, recent evidence suggests that aberrant or excessive apoptosis may play a causative role in the development of autoimmune diseases, including T1D. Casciola-Rosen and colleagues (12) have shown that ultraviolet-induced apoptosis of keratinocytes leads to redistribution of several auto-antigens to apoptotic blebs of the dying cells. Injection of normal mice with syngeneic apoptotic thymocytes induces auto-antibody production (11). Those autoantibodies are common in systemic lupus erythematosus (SLE) (11). Excessive apoptotic cells in the tissue will become secondary necrotic if they are not quickly engulfed by macrophages. Apoptotic and secondarily necrotic cells may provide auto-antigens to APCs and initiate autoimmunity, as supported by the above studies. Most patients deficient in early components of the complement

system develop SLE, and C1q-deficient mice develop autoantibodies and glomerulonephritis characterized by apoptotic body deposition in the glomeruli (13). In this model, excessive apoptotic bodies are due to a defect in C1q mediated clearance by macrophages (14), suggesting the importance of clearance of apoptotic cells by macrophages in resolving inflammation.

1.3 PHAGOCYTOSIS OF APOPTOTIC CELLS BY MACROPHAGES

Macrophages have been reported to play a pivotal role in the pathogenesis of T1D. They are the first immune cells to be activated in the multiple low dose streptozotocin (STZ), NOD mouse, and BB rat models of T1D (15-17). Macrophage infiltration into the pancreatic islets precedes the appearance of T-lymphocytes (18).

To resolve inflammation, clearance of apoptotic cells by mononuclear phagocytes is critical (19). During apoptosis, changes to the outer surface of the plasma membrane, such as exposure of phosphatidylserine (PS), lead to rapid recognition and phagocytosis of the dying cell by APCs (19). Of the APCs, macrophages are considered to be the "professional phagocytes" due to the efficiency with which they engulf and subsequently degrade apoptotic cells (20).

Under physiological conditions, macrophage clearance of apoptotic debris occurs without initiating an immune response. However, deficient clearance of apoptotic cells may relate to initiation of immunity (9). Scott *et al* reported that mer^{kd} mice, which lack the intracellular kinase domain of c-mer (a receptor that involved in

recognition of apoptotic cells by macrophages), have diminished *in vitro* clearance of apoptotic lymphocytes (21) that is attributable to a macrophage defect. Cohen *et al* have reported that mer^{kd} mice exhibit delayed *in vivo* clearance of exogenously administered apoptotic cells, and spontaneously develop antinuclear antibodies (22). Macrophages may therefore provide a link between apoptosis and autoimmunity. The inability of macrophages to bind and phagocytose apoptotic debris may transform the important physiological process of cell deletion by apoptosis into a pathological event.

One of the consequences of apoptotic cell engulfment by macrophages is the prevention of tissue inflammation. An obvious mechanism of this anti-inflammatory effect may be the decreased availability of apoptotic and secondary necrotic bodies for engulfment by professional APCs such as DCs. The increased number of apoptotic cells due to a macrophage clearance defect may be engulfed by immature DCs, which become mature and present the auto-antigen to auto-reactive T cells (23).

In our laboratory, a mathematical model was developed to investigate the kinetics of β-cell death in diabetes-prone (BBdp) and diabetes-resistant BB (BBdr) rats (24). O'Brien et al calculated that the net rate of β-cell death is of similar magnitude in both BBdp and BBdr rats (Figure 2). However, Terminal dUTP nickend labelling (TUNEL) staining indicated that there was a higher incidence of apoptotic β-cells in the pancreas of neonatal BBdp rats (Figure 3). The number of apoptotic β-cells present in the pancreas at any one point in time is determined by

both the rates of apoptosis and the rates of clearance of dying cells. Therefore, an increase in the incidence of apoptotic β-cells suggests defective clearance of apoptotic cells by phagocytes may be present in diabetes-prone animals.

We have previously reported that macrophages from BBdp rats (24) and diabetes-prone NOD mice have impaired phagocytosis of apoptotic cells, compared to macrophages from diabetes-resistant BB (BBdr) rats (24), non-obese diabetes resistant (NOR) mice, insulin-dependent diabetes 5 (*Idd5*) congenic mice, Balb/c or C57B/6 mice (25) (Figure 4). The phagocytic defect was present in both peritoneal and bone marrow-derived macrophages *in vitro*. We hypothesize that this defect may lead to an increased number of apoptotic β-cells in the pancreatic islet and initiate autoimmunity in T1D (Figure 5).

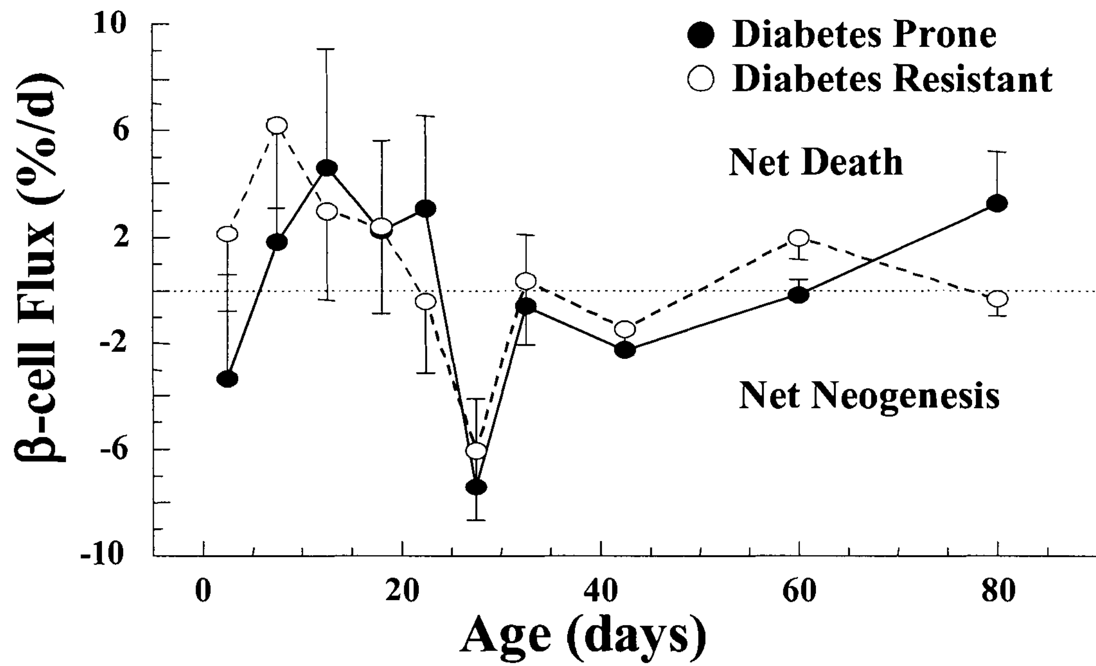


Figure 2: Age-dependent net β-cell neogenesis and net β-cell death in BBdp and BBdr rats as predicted by a mathematical model.
β-cell flux is the rate of cell death minus the rate of neogenesis. %/d: Percentage of total number of β-cells. The rate of β-cell death is of similar magnitude in both BBdp and BBdr rats. (Reprinted by permission from *Cell Death and Differentiation*, O'Brien et al, copyright 2002, Macmillan Publishers Ltd.)

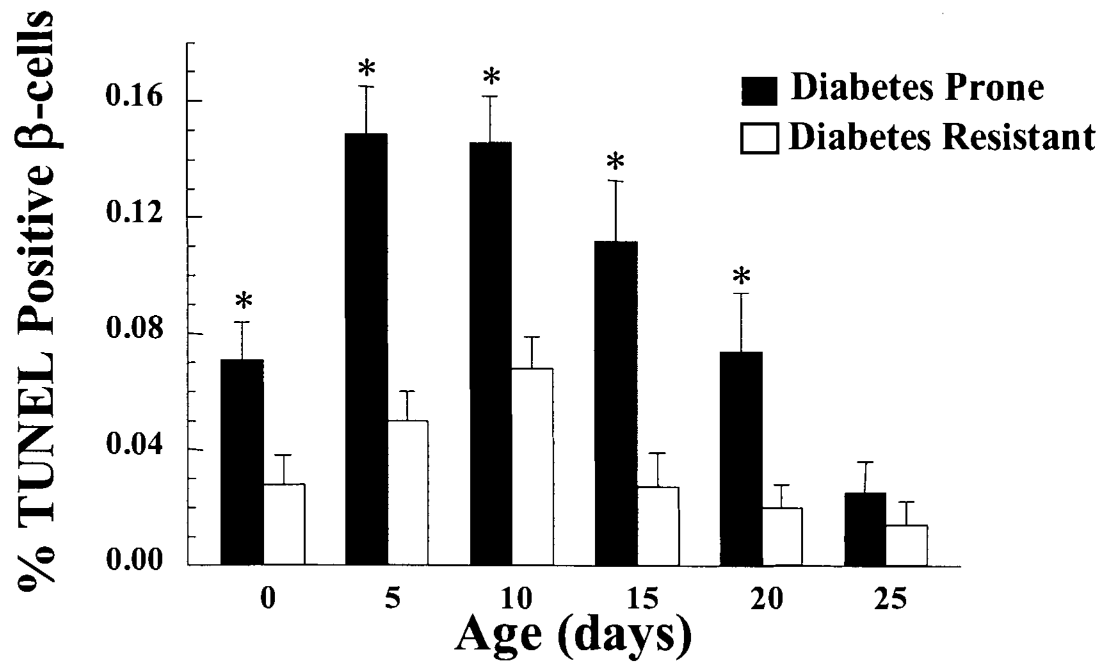


Figure 3: Age-dependent β-cell apoptosis (% TUNEL positive and insulin positive cells) in BBdp and BBdr neonates.
*P<0.05 between strains. (Reprinted by permission from *Cell Death and Differentiation*, O'Brien et al, copyright 2002, Macmillan Publishers Ltd.).

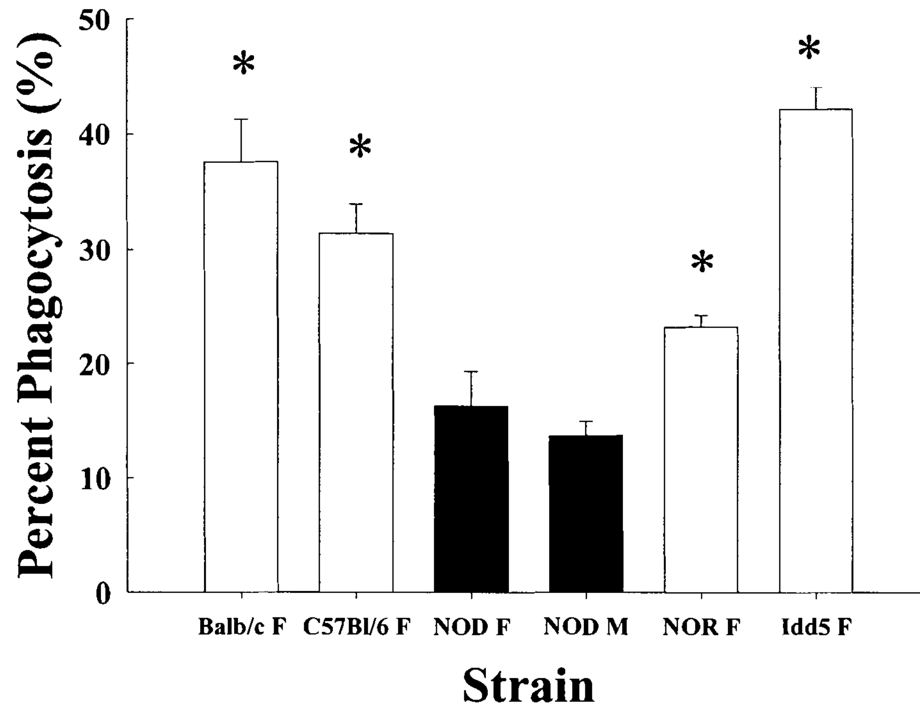


Figure 4: Impaired phagocytosis of apoptotic cells by macrophages from diabetes-prone NOD mice.
*P<0.05 vs. NOD females. (Based on data from O'Brien, Diabetes 51: 2002).

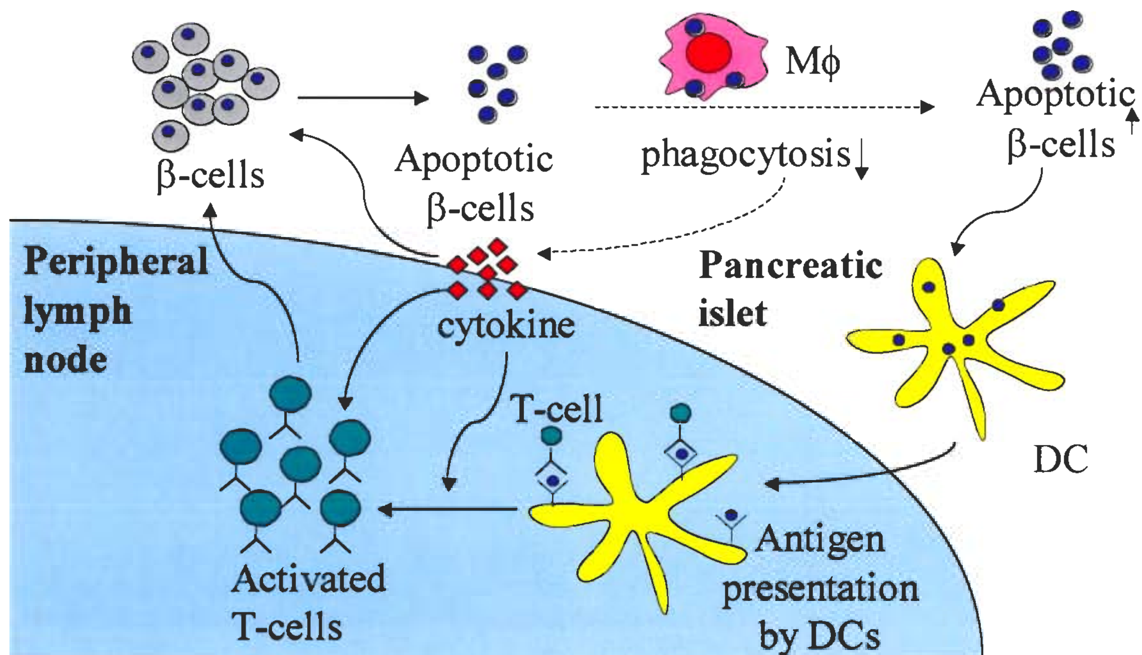


Figure 5: Proposed mechanism of autoimmune initiation in T1D.
β-cells undergo apoptotic death. Defective clearance of apoptotic β-cells by macrophages (Mϕ) may induce excessive apoptotic β-cells to accumulate in the islets, and impair cytokine secretion. The excessive apoptotic β-cells can be engulfed by DCs and initiate autoimmunity. At the same time, an impaired cytokine network may support the initiation of autoimmunity in T1D.

1.4 THE ROLE OF CYTOKINES IN T1D

1.4.1 CYTOKINES AND T1D

Cytokines are proteins secreted by many cells. Lymphocytes, monocytes and macrophages are the main immune cells responsible for cytokine secretion (26). Cytokines bind to membrane receptors on target cells and play an important role in modulating the immune response. Since monocytes and macrophages are present in most tissues, they serve to deliver cytokines locally in most organs.

There are many different ways to classify cytokines. One of them is related to the type of T helper (Th) cells that can secrete different cytokines. Th1 cytokines are known as pro-inflammatory cytokines, whereas Th2 and Th3 cytokines are anti-inflammatory. Th1 cytokines include interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-12. Th2 cytokines include IL-4, IL-10 and IL-13. Th3 cytokines include transforming growth factor-β (TGF-β). The role of the individual cytokines in the development of T1D is complex. In general, Th1 cytokines, such as IL-2 and IFN-γ, appear to play an immune-activating role, involving in the development of T1D; while Th2 or Th3 cytokines, such as IL-4 and TGF-β, play an immune-suppressing role, preventing T1D (27).

Some evidence suggests that an altered Th1/Th2 cytokine balance may be involved in both human and rodent (BB rat, NOD mouse) T1D (28-32). For example, there is defective production of IL-4 from T cells in recent onset T1D patients (33) and NOD mice (34; 35). Furthermore, increased plasma levels of

TNF-α, IL-2, and IFN-γ are observed in subjects with high risk of T1D, newly diagnosed T1D patients (28) and in diabetic children (IL-2 and IL-1β) (36). However, the mechanism by which the Th1/Th2 cytokine balance is disrupted is unknown. The factor(s) that disrupt(s) Th1/Th2 cytokine balance may play a crucial role in the initiation of the autoimmune response in T1D.

1.4.2 MACROPHAGES AND CYTOKINE PRODUCTION

Macrophages actively regulate the immune response via their production of cytokines and phagocytosis. The role of monocytes/ macrophages during inflammation includes antigen presentation, as well as the killing and engulfment of tumor cells, bacteria and other non-self cells or organisms. One of the most dynamic aspects of monocyte/macrophage function is the rapid synthesis of cytokine mRNA and protein following activation, and the release of the cytokines into the local environment (26).

Many signals trigger the production of cytokines by macrophages, including antigen presentation and the associated T cell contact, cytokine, immune complexes, and allo-antigens (including tumor antigens). In addition, the binding and/or uptake of particles or micro-organisms (including bacteria, viruses, fungi, parasites) by monocytes/macrophages can activate cytokine gene transcription (26). Apoptotic cells can also regulate the secretion of cytokines by macrophages (37; 38).

The macrophage response to phagocytosis of apoptotic cells is biased towards the release of anti-inflammatory mediators (38-40). *In vitro* phagocytosis of apoptotic cells suppresses the release of GM-CSF, IL-8, TNF-α, and thromboxane B2, but up-regulates TGF-β_1 and prostaglandin E2 (40). *In vivo* studies have also shown that apoptotic cells up-regulate TGF-β_1, and down-regulate TNF-α and MIP-2 (38). This reduction of inflammation results largely from the induction of TGF-β_1 secretion.

1.4.3 TGF-β_1

TGF-β is a 25-kDa nonglycosylated homodimeric protein consisting of two 12.5-kDa subunits linked by disulfide bonds (41). It is produced by most immune cells, including activated T cells, activated B cells, natural killer cells, macrophages, and DCs, and is stored within platelets in humans. It is a part of the super family containing TGF-α, bone morphogenic proteins, activins and inhibins. TGF-β comprises five members (TGF-β_1–β_5) and is highly conserved across mammalian species.

TGF-β plays an immunosuppressive role in the immune response. It is generally accepted that TGF-β is an important modulator of immune functions including cell growth and differentiation. TGF-β is classified as a Th3 cytokine, and can be produced by most mammalian cells (42; 43). TGF-β receptors are also found on most mammalian cells.

The most abundant types of TGF-β are TGF-β_1 and-β_2, which share 70% homology. TGF-β_1 is believed to be responsible for most of the immunosuppressive effects of TGF-β (44). Studies using mice having null mutations in the TGF-β_1 gene have clearly illustrated that this isoform is a critical regulator of immune cell differentiation and function (45; 46). Furthermore, loss of this gene is sufficient for the development of an autoimmune-like phenotype. Numerous studies have demonstrated that increased TGF-β_1 production correlates with the resolution of inflammatory responses, particularly in organ-specific autoimmune diseases (47). The protective capacity of TGF-β_1 is thought to occur through decreased generation of autoreactive T cells and enhanced generation of regulatory T cells (48).

TGF-β_1 also protects against the development of diabetes in NOD mice (49-52), and delays autoimmune destruction of β-cells after islet transplantation (53). TGF-β_1 can prevent autoimmune diabetes through several pathways, including directly inducing apoptosis of effector T cells (54), converting APCs to a tolerogenic phenotype (55), and directly regulating expansion of $CD4^+CD25^+$ regulatory T cells (48). Some evidence also suggests that TGF-β_1 can influence the immune response by polarizing T cell responses to the Th2 phenotype (50). TGF-β_1 promotes the bias of Th2 and Th3-type immune profile by two different mechanisms. First, TGF-β_1 impairs the ability of macrophages to produce IL-12 and express CD40 (56; 57). This ultimately reduces IL-12 responsiveness in T cells (58) resulting in decreased IFN-γ production and suppression of Th1-

mediated responses (58; 59). Second, TGF-β_1 down-regulates IL12 receptor protein expression in Th1 T cells resulting in the direct suppression of Th1 cytokine secretion (60).

1.4.4 APOPTOTIC CELLS REGULATE CYTOKINE SECRETION BY MACROPHAGES

The uptake of apoptotic cells by macrophages modulates macrophage function. The clearance of apoptotic cells by macrophages may also inhibit the release of pro-inflammatory cytokines (37; 40).

We have observed defective clearance of apoptotic cells by macrophages from NOD mice *in vitro*. This defect may lead to the abnormal expression of multiple cytokines. The disturbed profile of cytokines may prevent the induction of tolerance to endogenous β-cell antigens. For this reason, we hypothesize that the defective phagocytosis of apoptotic cells and imbalanced cytokine secretion by macrophages following uptake of apoptotic cells may be the first step towards autoimmunity in T1D (Figure 5).

1.5 NONOBESE DIABETIC (NOD) MICE

The NOD mouse strain is an inbred strain of mice and well studied model of T1D. The model was developed in Japan in 1974 (61). NOD mice spontaneously develop a form of diabetes closely resembling human T1D and are a widely used animal model for human T1D. NOD mice exhibit the earliest stage of insulitis at 4-5 weeks of age (62). The cumulative incidence of animals exhibiting insulitis

reaches almost 100% by 30 weeks of age (63). T1D is seen in females by three months of age. The cumulative incidence of diabetes in female NOD mice rises to approximately 80% by 6 months of age. The incidence of diabetes development is significantly reduced in males (61). However, the precise reason for differences between male and female are not completely understood. Without the administration of exogenous insulin, animals that develop diabetes will die within a short period.

1.6 SUMMARY

Many studies suggest that impaired recognition and /or removal of apoptotic cells from pre-inflammatory foci contribute to autoimmune disease. Excessive apoptotic and secondary necrotic cellular bodies that remain in the tissue may be recognized by APCs, causing auto-antigens to be presented to naive T cells. Macrophages are professional phagocytes and the first immune cells to infiltrate the pancreatic islet. Therefore, defective functioning of macrophages, such as aberrant phagocytosis and/or cytokine secretion, may be the first step to disturbing the delicate balance between self-tolerance and self-reactivity.

Macrophages from BBdp rats and NOD mice demonstrate impaired uptake of apoptotic cells *in vitro* (24; 25). To determine the biological significance of these *in vitro* findings, we investigated the phagocytosis of apoptotic cells by peritoneal macrophages from diabetes-prone (NOD) and diabetes-resistant (Balb/c) mice *in vivo*.

Phagocytosis of apoptotic cells inhibits the release of inflammatory cytokines and promotes anti-inflammatory cytokine (TGF-β_1) production by macrophages both *in vitro* (40) and *in vivo* (38). It is possible that, in concert with defective phagocytosis of apoptotic cells, impaired cytokine secretion by macrophages may also play a role in the initiation of autoimmunity in T1D. Therefore, secretion of cytokines by macrophages after uptake of apoptotic cells in the NOD mouse model was also investigated.

1.7 HYPOTHESES

Phagocytosis of apoptotic cells by macrophages from diabetes-prone mice is reduced compared to diabetes-resistant strains *in vivo*. After phagocytosis of apoptotic cells *in vivo*, macrophages from NOD mice have impaired secretion of anti-inflammatory cytokines, such as TGF-β_1.

CHAPTER 2 METHODS

2.1 ANIMALS

Female and male NOD mice, and female Balb/c mice (5 weeks of age) were purchased from Taconic (Germantown, NY). Female Balb/c mice which exhibit the same MHC class I molecule $H\text{-}2^d$ as NOD mice (62) were used as controls. All animals were purchased from the supplier and housed in Simon Fraser University Animal Care Facilities. Maintenance and experimental manipulation of the animals were performed in accordance with the guidelines and regulations of the Canadian Council on Animal Care and approved by the Animal Care Committee at Simon Fraser University. Mice were given free access to standard laboratory chow and water *ad libitum*.

2.2 INDUCTION OF APOPTOSIS

Thymi were harvested from female NOD mice and Balb/c mice and rinsed in sterile PBS (Life Technologies, ON, Canada). Thymocytes were obtained by gentle mincing of thymi in a sterile environment to yield a single cell suspension. Cell viability was determined using light microscopy and trypan blue (Sigma, MO, USA). Mouse thymocytes were irradiated with 254 nm ultraviolet light for 10 min in RPMI 1640 medium (Life Technologies, ON, Canada) (100 units /ml penicillin/ streptomycin) and then cultured in RPMI 1640 medium containing 0.4% bovine serum albumin (BSA) (Serologicals Proteins, IL, USA) for 3 hours

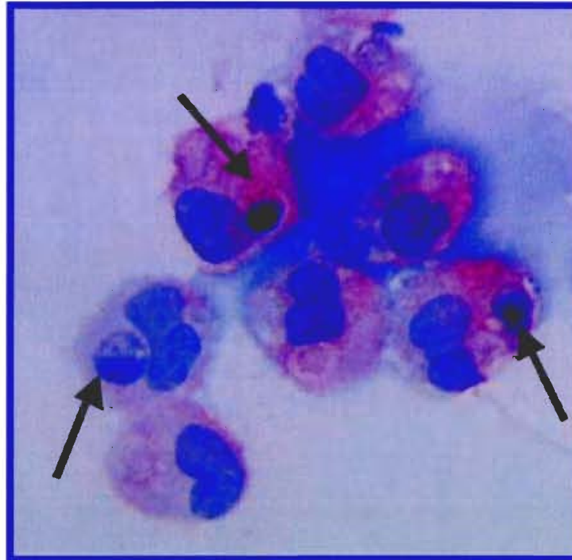
at 37° C, 5% CO_2. Irradiated thymocytes were washed in sterile PBS and resuspended in 0.5 ml sterile PBS before being injected into the peritoneal cavity of a mouse. Cell death was assessed after staining with Annexin V and Propidium iodine (PI) (Molecular Probes, OR, USA) (Appendix I) and observation under a fluorescent microscope. Early apoptotic thymocytes were counted and the percentage of early apoptotic thymocytes was calculated.

2.3 *IN VIVO* PHAGOCYTOSIS ASSAY

Previous studies by others have demonstrated that the phagocytosis of apoptotic cells by macrophages is a function of time (14). The peak of the phagocytosis index (the number of ingested apoptotic cells per 100 macrophages) is around 30 minutes after the injection of apoptotic cells. Therefore, we studied macrophage phagocytosis over time (0, 15, 30, 60, 120 min) after intraperitoneal (i.p.) injection of apoptotic thymocytes. F4/80 antigen is a specific marker for macrophages (64). Although F4/80 also has been found on the surface of eosinophils, eosinophils can easily be distinguished by the morphology of their specific nucleus.

Apoptotic thymocytes (40×10^6) in 0.5 ml PBS were injected into the peritoneal cavity of mice. At different time points (0, 15, 30, 60, 120 min) mice were sacrificed by CO_2 asphyxiation. Ice-cold RPMI 1640 medium (5 ml) was injected into peritoneal cavity of the mouse. The abdomen was gently massaged for 1 minute and then the injected fluid was removed with a needle and syringe. This process, known as lavage, allows collection of peritoneal exudate cells (PECs).

A



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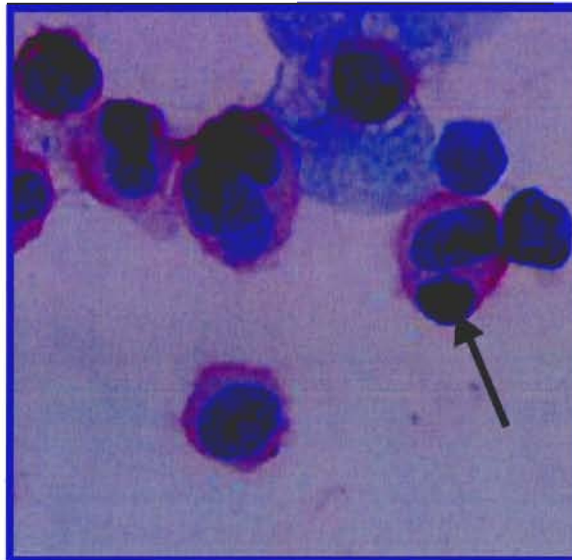


Figure 6: F4/80 immunostaining of macrophages from Balb/c (A) and NOD (B) mice.
Arrows point to macrophages that have engulfed apoptotic thymocytes.
Magnification, ×1000.

Cells were immediately stored on ice. Peritoneal cells were spun onto glass slides using a cytospin instrument and fixed in 2% paraformaldehyde (ACROS, NJ, USA) for 30 minutes at room temperature. Then cells were stained with a rat monoclonal antibody to murine F4/80 (CALTAG, CA, USA) and counter-stained with Harry's hematoxylin (Sigma, MO, USA) (Appendix II). Phagocytosis was evaluated by counting 1000 F4/80 positive cells (macrophages) per slide at 1000 × magnification under oil immersion. Macrophages were considered to have phagocytosed an apoptotic body when thymocytes were clearly visible within the perimeter of the macrophage (Figure 6). The results were expressed as the percent phagocytosis (the number of macrophages containing at least 1 apoptotic cell per 100 macrophages) and as phagocytic index.

2.4 THIOGLYCOLLATE-INDUCED PERITONITIS

The I.P. injection of thioglycollate induces peritonitis that leads to an acute influx of neutrophils, which subsequently undergo apoptosis. This occurs within hours and is followed by the recruitment of macrophages that phagocytose the apoptotic neutrophils (65). That provides us with a macrophage phagocytosis model using thioglycollate stimulation without requiring the addition of extra apoptotic cell bodies.

Mice were injected I.P. with 4% (w/v) sterile thioglycollate solution (Sigma, MO, USA). The amount of thioglycollate injected was based on the body weight of mice (0.83ml/g). At 0, 8, 24, 48, 72 and 96 hours mice were sacrificed by CO_2

asphyxiation. PECs were obtained by peritoneal lavage with 5 ml ice-cold PBS. Cells were immediately stored on ice. PECs were initially counted on a hemocyometer and then differential cell counts were conducted on cytospins stained with rat monoclonal antibody to mouse F4/80 and TUNEL staining (ApopTag kit, Intergen, Purchase, NY, USA) (Appendix III). One thousand PECs were counted to determine the relative percentages of neutrophils, macrophages and other cells. The number of neutrophils was calculated as the total number of PECs multiplied by the ratio of neutrophils. The number of macrophages was calculated as the total number of PECs multiplied by the ratio of macrophages. In order to determine the percentage of apoptotic neutrophils, one thousand neutrophils were examined in each sample. Neutrophils were considered apoptotic when they were TUNEL-positive. 1000 macrophages were counted, and the phagocytosis was expressed as percent phagocytosis and as phagocytic index. Only TUNEL-positive neutrophils that were clearly within the perimeter of the macrophage were counted.

2.5 MATHEMATIC MODEL FOR THIOGLYCOLLATE-INDUCED PERITONITIS

To better understand the result of thioglycollate-induced peritonitis, a mathematical model was developed by another member of our laboratory, Cheryl Dyck (Figure 7). The model is based on the experimental design and describes the recruitment of neutrophils to the peritoneal cavity and death by apoptosis after injection (i.p.) of thioglycollate. Macrophages are then recruited and remove

apoptotic neutrophils. The various parameters in this model are defined in Table 1. The number of apoptotic neutrophils at time t depends on K_{ap} and K_{me}. The number of macrophages that have engulfed 0 apoptotic neutrophil (M_0) or 1(M_1), 2 (M_2), 3 (M_3) apoptotic neutrophils depends on K_{me} and K_d.

2.6 *IN VIVO* CYTOKINE ASSAY

To induce peritoneal inflammation, mice were injected I.P. with 1 ml of 4% sterile thioglycolate once a day. After 3 days, 40×10^6 apoptotic thymocytes from female Balb/c mice in 0.5 ml sterile PBS were injected into the peritoneal cavity of thioglycolate-treated mice. Control mice of the same strain were injected intraperitoneally with 0.5 ml sterile PBS. After 1 hour, mice were sacrificed by CO_2 asphyxiation. PECs were recovered by peritoneal lavage with 3 ml of ice-cold PBS. Cell suspensions were immediately stored on ice and cytospun onto slides for immunostaining. After centrifugation of lavage fluid (16000g for 5 minutes), supernatants were stored at –70°C for cytokine ELISA.

2.7 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Interleukin (IL)-10, IL-12 heterorodimer p70, interferon (IFN)-γ, tumor necrosis factor (TNF)-α were measured by ELISA using Pharmingen kits. TGF-β_1 ELISA was performed using R&D Systems Inc. kit (Minneapolis, MN, USA). Since the ELISA assay measures only active TGF-β_1, total TGF-β_1 was measured after

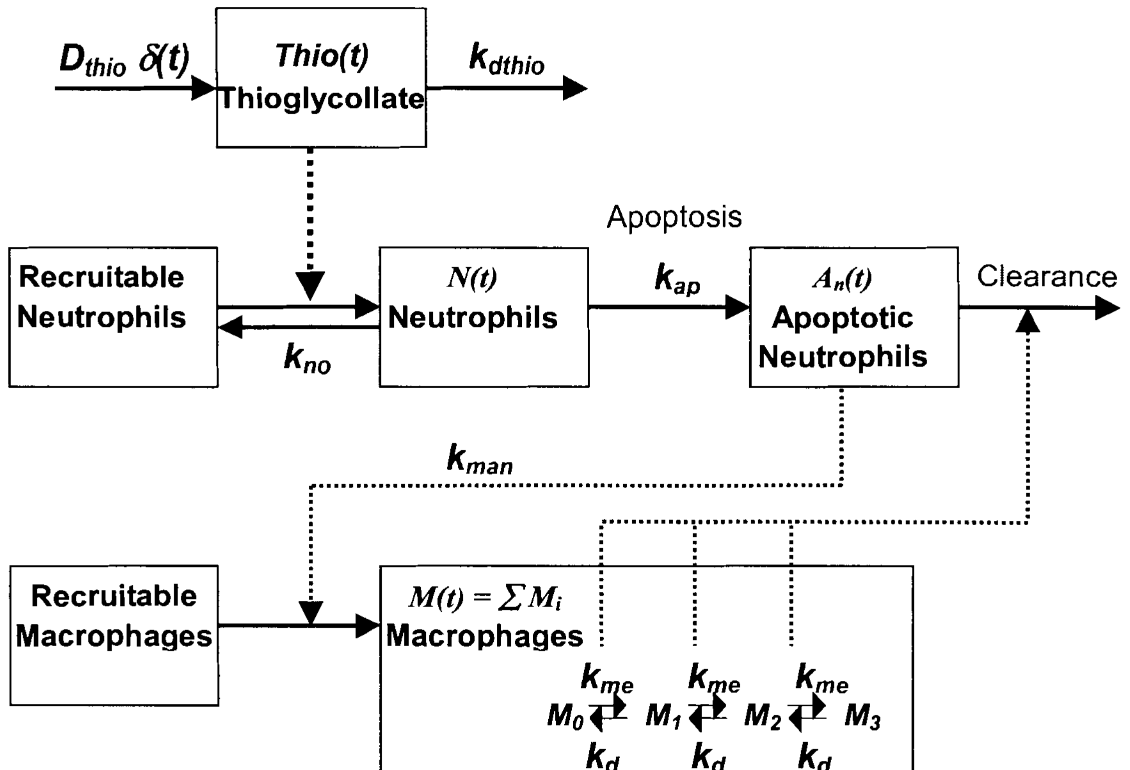


Figure 7: The relationship of parameters in the mathematical model of the thioglycollate-peritonitis experiment.

Table 1: The definition of parameters in the mathematical model of the thioglycollate-peritonitis experiment.

PARAMETER	DESCRIPTION	UNITS
N(t)	Number of peritoneal neutrophils	$\times 10^6$ cells / mL
M(t)	Number of peritoneal macrophages	$\times 10^6$ cells / mL
$A_n(t)$	Number of peritoneal apoptotic neutrophils	$\times 10^6$ cells / mL
M(0)	Number of macrophages that have engulfed 0 apoptotic neutrophil	$\times 10^6$ cells / mL
M(1)	Number of macrophages engulfed 1 apoptotic neutrophil	$\times 10^6$ cells / mL
M(2)	Number of macrophages engulfed 2 apoptotic neutrophils	$\times 10^6$ cells / mL
M(3)	Number of macrophages engulfed 3 apoptotic neutrophils	$\times 10^6$ cells / mL
Kme	Rate of phagocytosis of apoptotic neutrophils by macrophages	1/ ((10^6 cells / ml) / hour)
Kd	Rate of digestion of apoptotic neutrophils by macrophages	1/ ((10^6 cells / ml) / hour)
Kman	Rate of recruitment of macrophages	1/ hour
Kap	Rate of apoptotic death of neutrophils	1/ hour
Kno	Rate of neutrophil flux out of peritoneal	1/ hour
Kdthio	Thioglycollate disposal rate	1 / hour
Dthio	Thioglycollate volume	mL

preactivation with acidification to pH 2-3 for 10 minutes, and corrected to pH 6.5-7.5 prior to the ELISA assay (Appendix IV). Colour development was assessed using the microplate autoreader.

2.8 IMMUNOHISTOCHEMISTRY

Cytospin slides were fixed with filtered 2% (w/v) paraformaldehyde/30% (w/v) sucrose in PBS for 30 minutes at 37° C. Slides were incubated with 5% (w/v) bovine albumin / 10% (v/v) goat serum / 0.3% (v/v) Triton 100 in PBS for 1 hour at room temperature (RT). Then slides were stained with chicken anti-rh TGF-β_1 IgY (R&D Systems, Minneapolis, MN, USA) for 1 hour at RT, followed by donkey anti-chicken antibody-FITC conjugate (Chemicon, CA, USA) for 1 hour at RT. For double staining experiments, slides were further incubated with rat monoclonal antibody to mouse F4/80 red-Phycoerythrin conjugate (CALTAG, CA, USA) for 1hour at RT (Appendix V). Slides were analyzed by using fluorescence microscopy.

2.9 STATISTICAL ANALYSIS

The results from *in vivo* phagocytosis assay, *in vivo* cytokine assay and thioglycollate-peritonitis were analyzed using pre-planed *t* tests (two-tailed). A P-value < 0.05 was considered significant. Results are expressed as mean ± standard error.

CHAPTER 3 RESULTS

3.1 INDUCTION OF APOPTOTIC THYMOCYTES

To confirm that the rates of early apoptotic thymocytes in female NOD and Balb/c mice are not different, Annexin V and PI were used to e assess apoptotic death and exclude necrotic death. PI is used to assess plasma membrane integrity. Annexin V recognizes and binds phosphotidylserine on the surface of apoptotic cells. Annexin V^+ PI^- staining indicates early apoptotic death. Annexin V^+ PI^+ cells have undergone late apoptosis, e.g., secondary necrosis. In female Balb/c mice, the percentage of apoptotic thymocytes was very close to that of female NOD mice after irradiation with ultraviolet light (Figure 8).

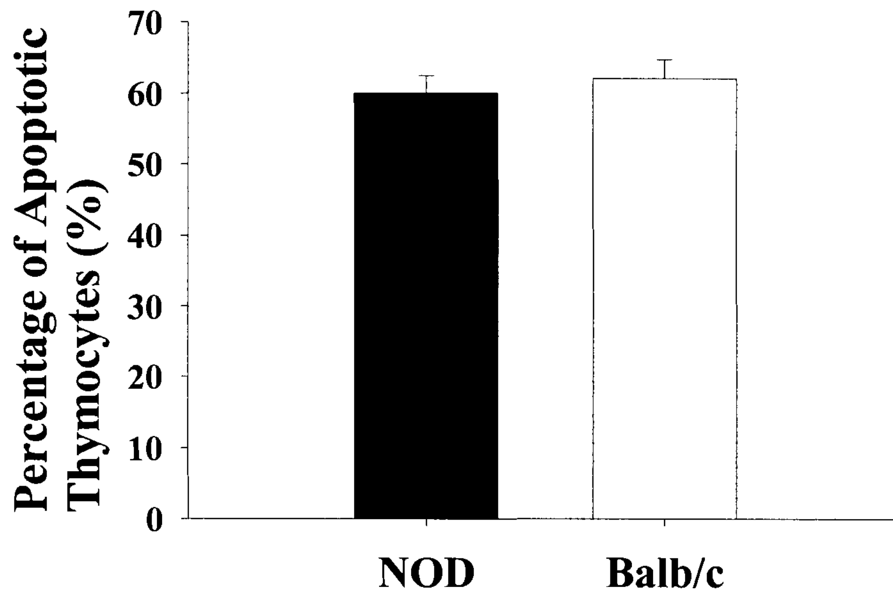
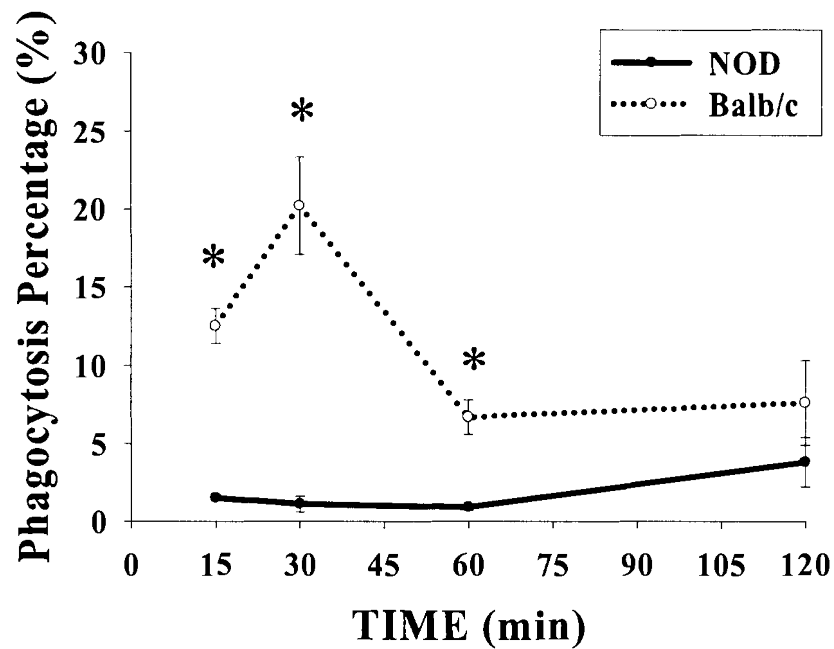


Figure 8: Induction of early apoptotic thymocytes from female NOD and Balb/c mice after irradiation with ultraviolet light.
Data are means±SE.

3.2 *IN VIVO* PHAGOCYTOSIS OF APOPTOTIC THYMOCYTES

Macrophages from female Balb/c mice exhibited a rapid response to apoptotic cells. Phagocytosis of apoptotic thymocytes by macrophages increased significantly at 15 minutes, reached the peak at 30 minutes (Figure 9) in these diabetes-resistant animals, and decreased at 1 hour. In contrast, clearance of apoptotic thymocytes by macrophages from female NOD mice did not show any increases over time. Compared with Balb/c mice, macrophages from female NOD mice exhibited defective clearance at 15, 30, 60 minutes after administration of apoptotic thymocytes ($P<0.01$ at all three time points vs Balb/c mice).

A



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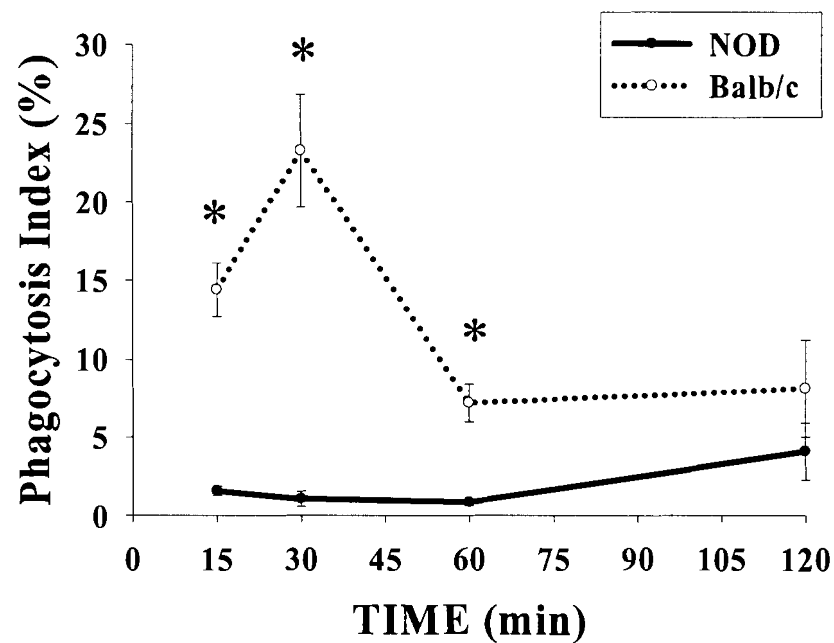

Figure 9: Time-course of phagocytosis of syngeneic apoptotic thymocytes *in vivo*. A: Percent phagocytosis of apoptotic thymocytes by macrophages. B: Phagocytosis index of apoptotic thymocytes by macrophages. Data are means ±SE. * P<0.01 vs Balb/c mice.

3.3 *IN VIVO* CROSS FEEDING

Sygeneic thymocytes were used in the time-course phagocytosis study. To determine the influence of different sources of apoptotic thymocytes on macrophages engulfment, we used apoptotic thymocytes from Balb/c mice to stimulate macrophages from both strains *in vivo*. Macrophages from female NOD mice showed defective engulfment of apoptotic Balb/c thymocytes compared with that of female Balb/c mice ($P<0.01$) at 15 minutes after administration of apoptotic thymocytes (Figure 10). The rates of engulfment of apoptotic Balb/c and NOD thymocytes by NOD macrophages were not different.

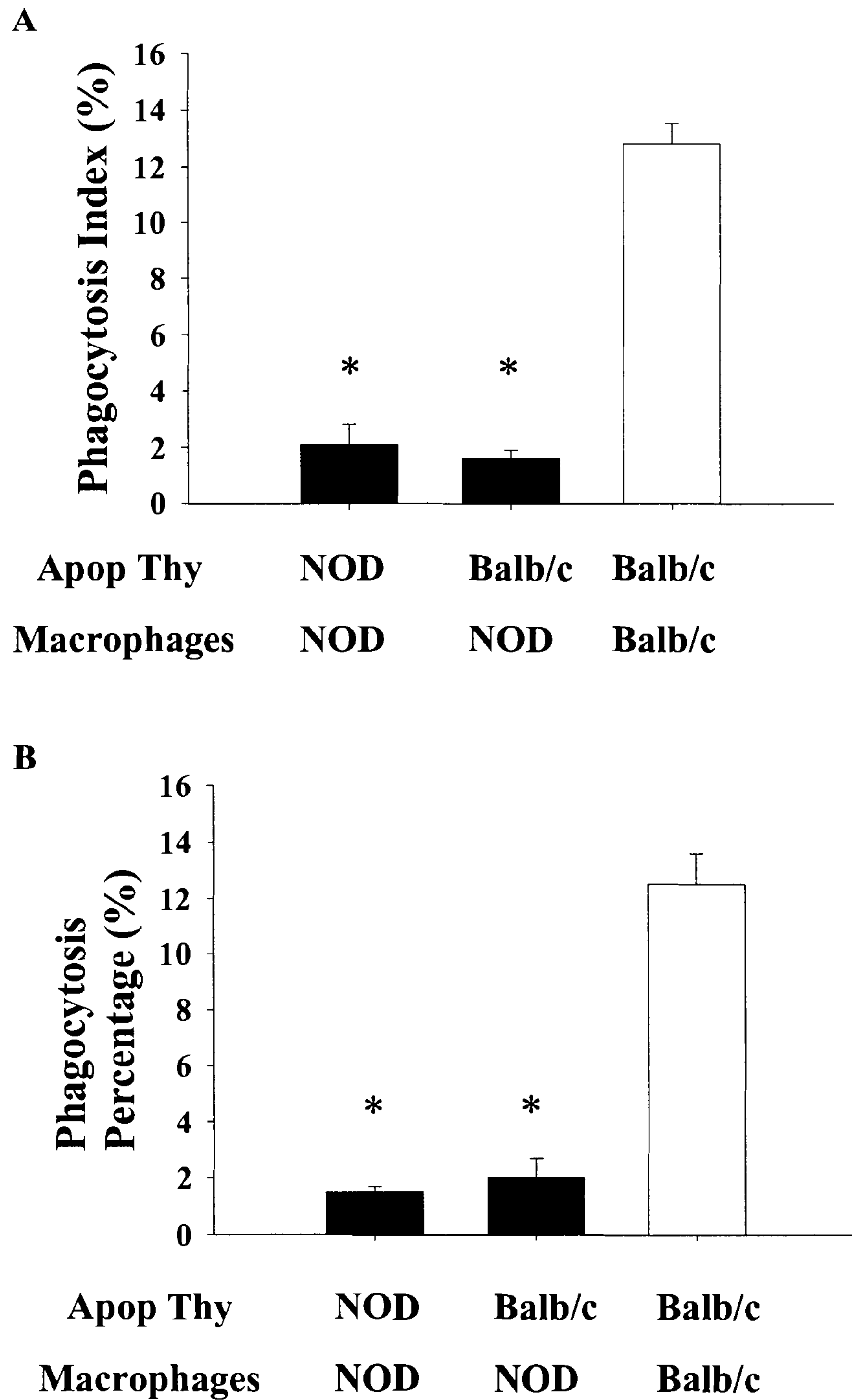

Figure 10: *In vivo* cross feeding with apoptotic thymocytes.
A: Percent phagocytosis of apoptotic thymocytes by macrophages. B: Phagocytosis index of apoptotic thymocytes by macrophages. Apop Thy: Apoptotic thymocytes. Data are means±SE. * $P < 0.01$ vs Balb/c mice.

3.4 THIOGLYCOLLATE-STIMULATED PERITONITIS

As demonstrated in Figure 11, the majority of resident peritoneal cells at 0 hour are macrophages in both female NOD and Balb/c mice. The number and morphology of resident macrophages was similar in both strains. Neutrophils were hardly found in resident PECs. The same number and population of PECs provided a similar initial condition between the two strains. The total number of PECs rapidly increased and reached a plateau after 24 hours. The stable number of PECs after 24 hours was accounted for by the balance of reduced number of neutrophils and increased number of macrophages (Figure 11 A). At 72 and 96 hours, the majority of PECs were positive for F4/80, i.e., macrophages. The number of macrophages was stable during the first 8 hours, and then began to increase until 96 hours in both strains. However, female NOD mice exhibited a slower increase in macrophages from 72 until 96 hours (Figure 11 B). After the initiation of peritonitis by I.P. injection of thioglycollate, the number of neutrophils was increased and this increase was maximal at 24 hours in female Balb/c mice and at 8 hours in female NOD mice (Figure 11 C). Although both strains showed an increase in neutrophils after administration of thioglycollate, a lower number of neutrophils was observed in female NOD mice from 24 to 48 hours. From the results, we suggest that female NOD mice not only exhibited a slower response, but also showed a smaller response upon thioglycollate stimulation, which was represented by the number of neutrophils and macrophages, compared to female Balb/c mice.

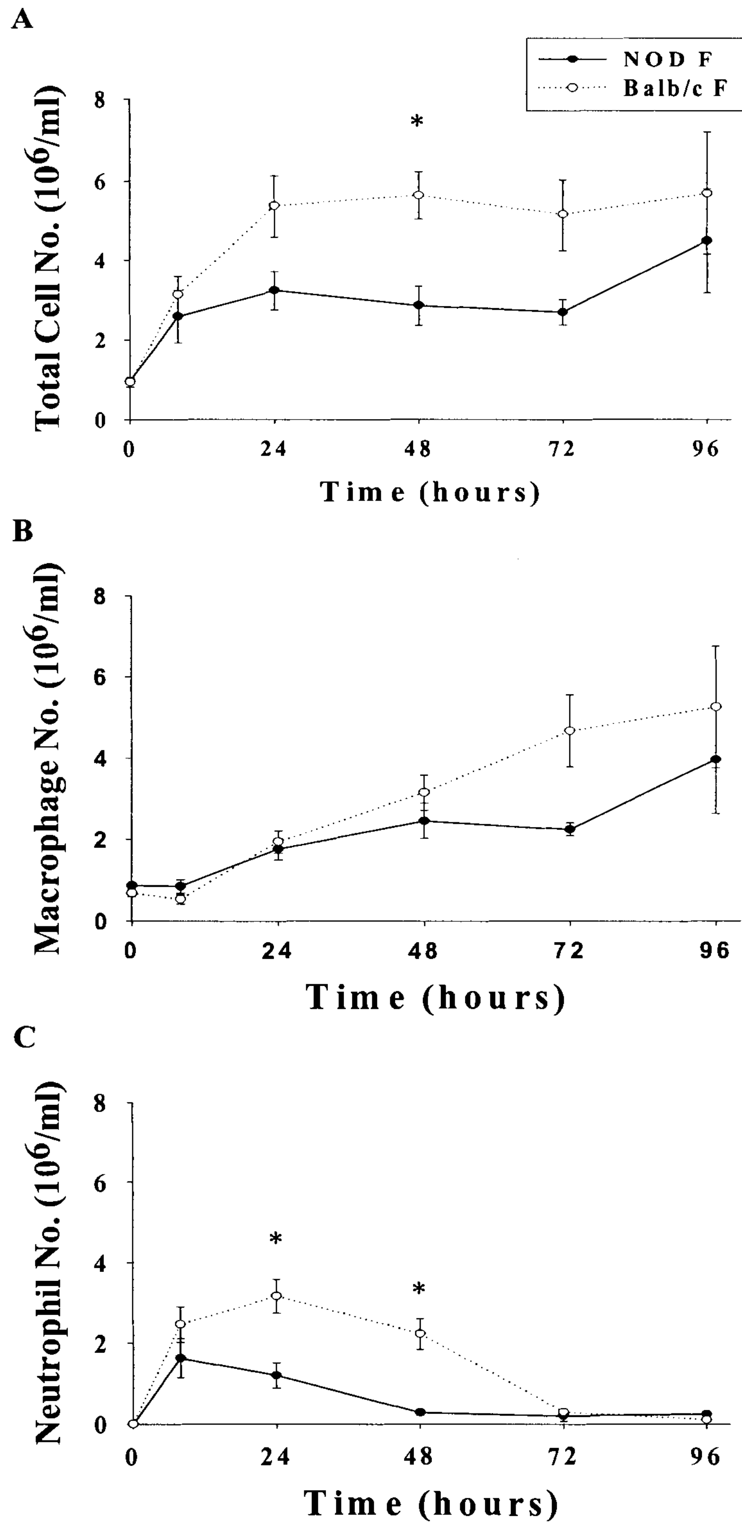

Figure 11: Peritoneal cell recruitment in female NOD and Balb/c mice upon thioglycollate stimulation.
The number of total cells (A), macrophages (B), neutrophils (C) is shown as cells per millilitre of lavage. Data are means±SE. * $P<0.05$ vs time matched NOD mice.

Compared with female Balb/c mice, female NOD mice showed lower response upon thioglycollate stimulation. The number of neutrophils in female NOD mice was less than that of female Balb/c mice early at 24 hours after the stimulation, until 48 hours. The recruitment of macrophages after the influx of neutrophils into the peritoneal cavity in female NOD mice was also lower than that of Balb/c mice from 72 hours to the end of the experiment, implicating the potential relationship between the recruitment of neutrophils and macrophages in this model.

The morphology of macrophages also changed over time (Figure 12). Vacuoles started to appear inside of them at 8 hours and continued to increase until 96 hours. At 96 hours, almost all the macrophages showed the same morphology in both strains. There was no difference in morphology between female NOD and Balb/c mice.

Neutrophils underwent apoptotic death after they were recruited into the peritoneal cavity and were then engulfed by macrophages. From our study, macrophages from female NOD mice had defective phagocytosis of apoptotic neutrophils compared with that of female Balb/c mice at 24 and 48 hours after initiation of peritonitis ($P<0.02$) (Figure 13). The number of apoptotic neutrophils is dependent on the rate of apoptotic death of neutrophils and the rate of phagocytosis of apoptotic neutrophils by macrophages. As a result of the clearance defect, the incidence of apoptotic neutrophils in female NOD mice was increased compared with that of female Balb/c mice ($P<0.01$) at 48 hours (Figure 14). The results are consistent with the observations of the phagocytosis of

apoptotic thymocytes by macrophages in vivo. After 72 hours, the number of neutrophils was close to that of the 0 hour time point in both strains. Macrophages completed cleanup of all the dead cells at that time, even in female NOD mice.

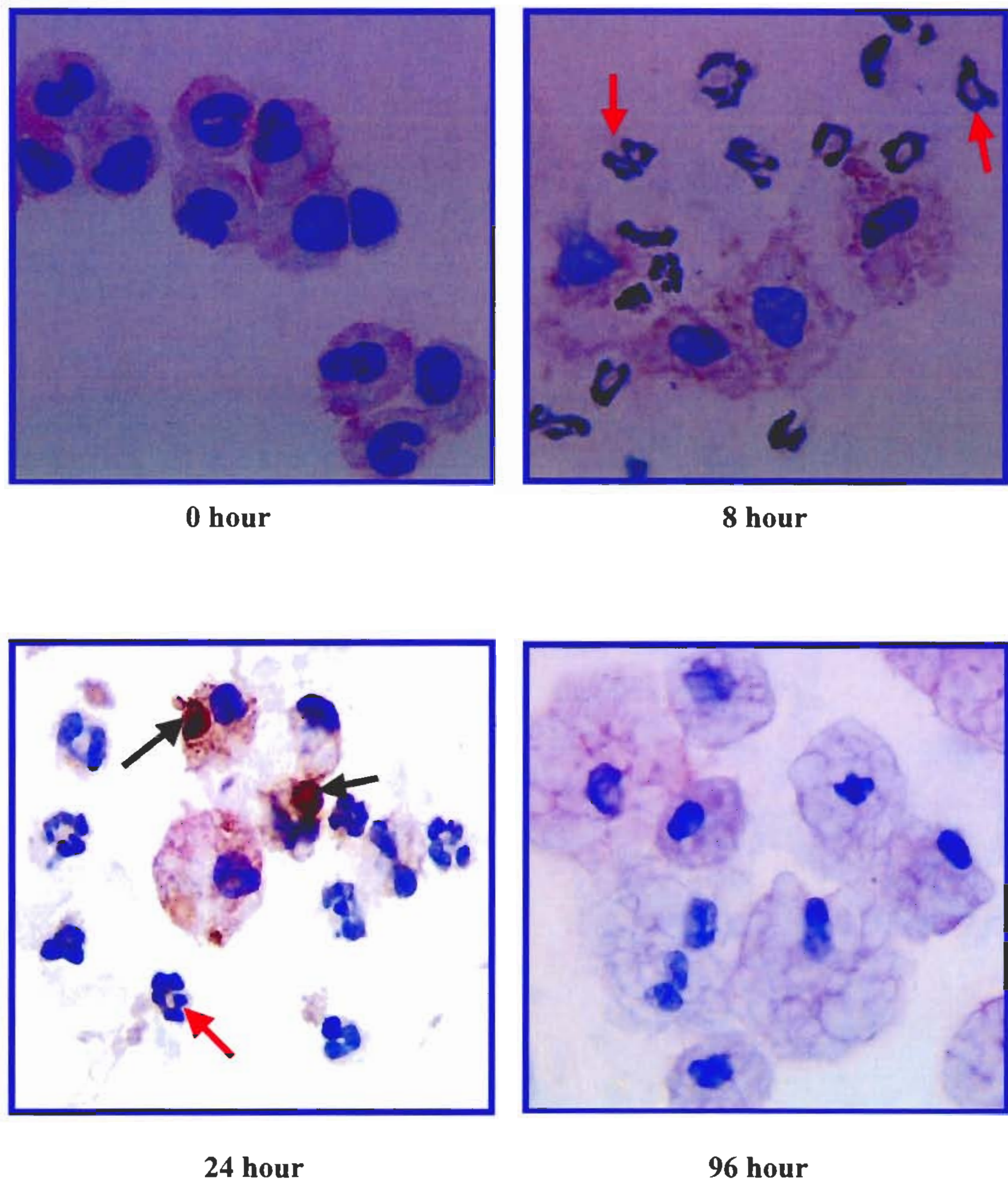

Figure 12: Morphologic changes of peritoneal cells over time after thioglycollate injection. Pink cells: macrophages. Black arrow: apoptotic neutrophils ingested by macrophages. Red arrow: neutrophils.

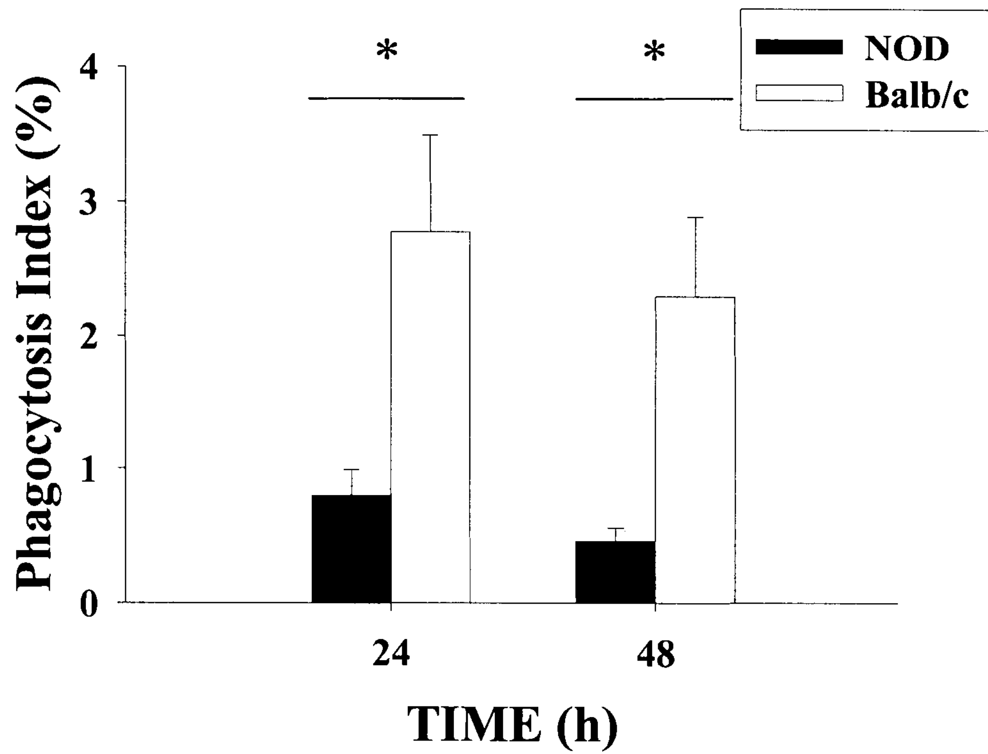

Figure 13: Clearance of apoptotic neutrophils by macrophages from NOD and Balb/c mice 24 and 48 h after thioglycollate injection.
Data are means±SE. * P< 0.02 vs Balb/c mice.

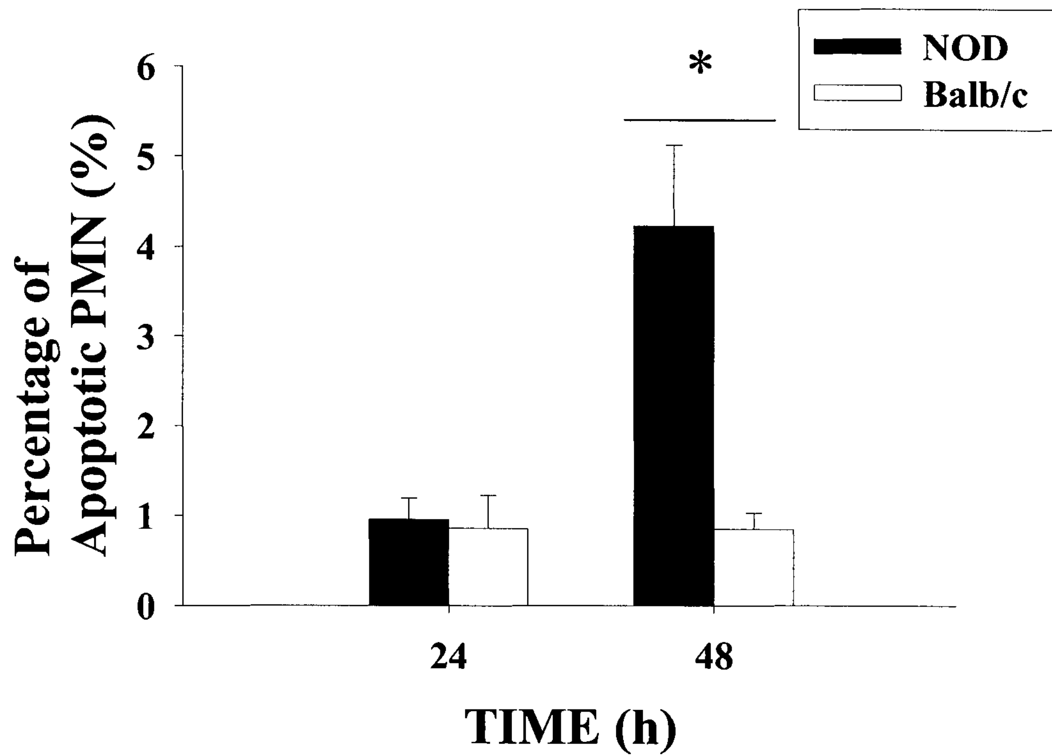

Figure 14: The percentage of uningested apoptotic neutrophils (PMN) from female NOD and Balb/c mice remained in the peritoneal cavity 24 and 48 h after thioglycollate injection.
Data are means±SE. * P< 0.01 vs Balb/c mice.

3.5 MATHEMATICAL MODEL FOR THIOGLYCOLLATE-STIMULATED PERITONITIS

To further confirm and better interpret the result, Cheryl Dyck in our lab established a mathematical model. The model can fit the results of the experiment very well (Figure 15). The fractional standard deviation (FSD) was used to measure the confidence (statistical precision) of parameters in this model. The FSDs of all the parameters were small, suggesting that these parameters are valuable in interpreting data from the experiment.

From the model, the rate of phagocytosis of apoptotic neutrophils (*Kme*) in NOD mice is 40% lower than that of Balb/c mice (Table 2). In this model, *Kme* was standardized by the number of neutrophils, apoptotic neutrophils and macrophages. Therefore, *Kme* represents the phagocytic ability of macrophages under the same ratio of macrophage to apoptotic neutrophil in NOD and Balb/c mice. Although the rate of digestion of apoptotic neutrophils (*Kd*) by macrophages in NOD mice is higher than that of Balb/c mice (Table 2), this did not accelerate the rate of phagocytosis by macrophages in NOD mice.

The rate of apoptotic death of neutrophils (*Kap*) in NOD mice is lower than that of Balb/c mice. The number of apoptotic neutrophils we counted in our experiment is dependent on the rate of apoptotic death of neutrophils (*Kap*) and the rate of phagocytosis of apoptotic neutrophils by macrophages (*Kme*). Even though *Kap* in NOD mice is lower than that of Balb/c mice, the lower *Kme* in NOD mice finally results in the accumulation of apoptotic neutrophils.

The results of the mathematical model are consistent with the conclusions of the experiment. From both results, we demonstrated the deficient clearance of apoptotic cells by macrophages from NOD mice. The clearance defect of apoptotic cells by macrophages results in the accumulation of apoptotic neutrophils in the peritoneal cavity.

From our observations, NOD mice have a lower number of neutrophils, but an equal number of macrophages compared with that of Balb/c mice at 24 and 48 hours, suggesting that neutrophils from NOD mice might have an enhanced ability to recruit macrophages. Consistent with our observation, the rate of recruitment of macrophages by neutrophils (*Kman*) is higher in NOD mice compared with that of Balb/c mice.

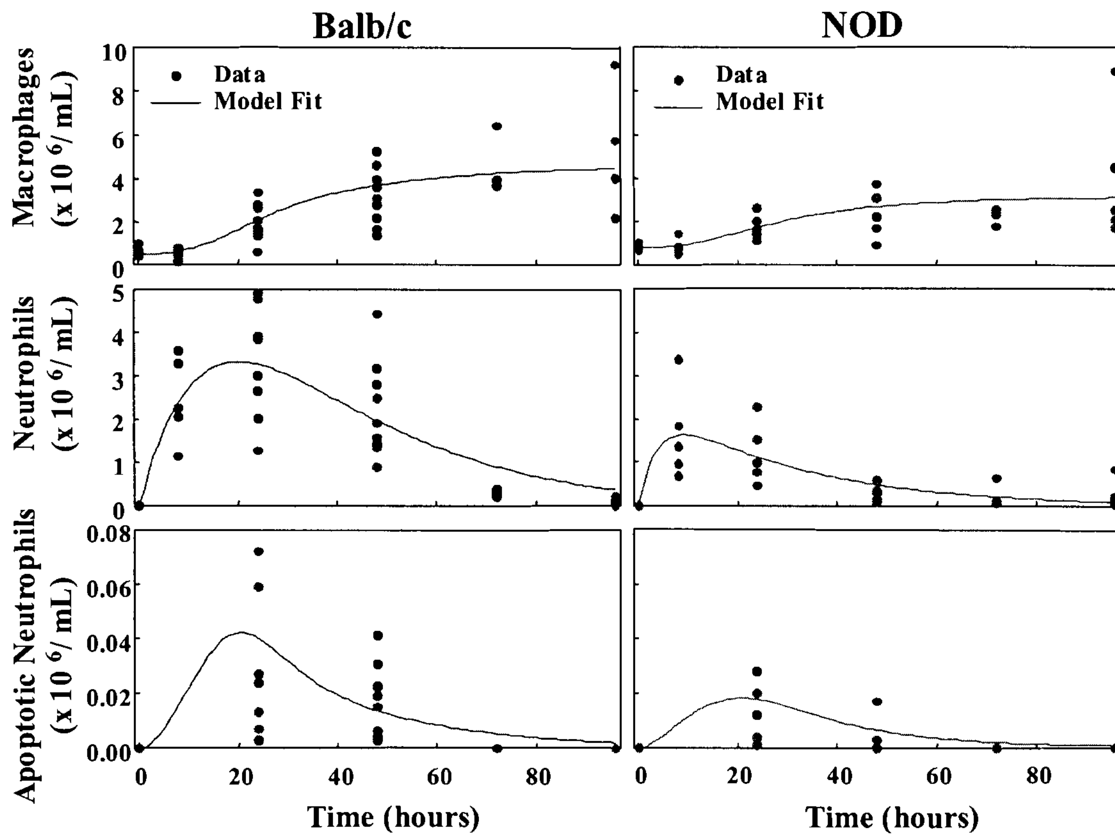

Figure 15: Model of the result of thioglycllate-peritonitis experiment.

Table 2: Parameters in the model of thioglycllate-peritonitis experiment.

Parameter	Balb/c		NOD	
	Value	**FSD**	**Value**	**FSD**
Kme	0.076	0.25	0.048	0.50
Kd	0.086	0.31	0.111	0.85
Kman	2.6	0.34	3.3	0.67
Kap	1.6E-03	0.26	1.1E-03	0.68
Kno	0.05	80.51	0.25	0.73
Kdthio	0.050	78.27	0.035	0.32
Dthio	0.45	0.17	0.57	0.54

3.6 *IN VIVO* CYTOKINE ASSAY

Under normal circumstances, the concentration of cytokines from peritoneal lavage fluid is undetectable. Thioglycollate causes the recruitment of a large number of macrophages to the peritoneal cavity (66) and provides the only means to obtain measurable levels of TGF-β_1 *in vivo*. Therefore, we used thioglycollate to recruit a large number of macrophages. It is possible that thioglycollate may activate macrophages and induce macrophages secreting more TGF-β_1. However, further investigation is required to support this hypothesis.

Before apoptotic thymocyte stimulation, the basal level of TGF-β_1 in thioglycollate-stimulated peritoneal fluid of female NOD mice was higher than that of female Balb/c and male NOD mice *in vivo* ($P<0.05$) (Figure 16). The peritoneal levels of TGF-β_1 from male NOD and female Balb/c mice ($P<0.05$) were increased significantly after apoptotic thymocyte stimulation. TGF-β_1 tended to be up-regulated by apoptotic thymocytes in female NOD mice, but there was no statistically significant difference compared to control female NOD mice. The levels of IL-10, IL-12 p70, IFN-γ and TNF-α were undetectable under all the conditions. Macrophages are the main cells in the peritoneal cavity to secrete cytokines (such as TGF-β_1) (38). Our data indicate that TGF-β_1 is a measurable cytokine secreted by peritoneal macrophages from both diabetes-prone and diabetes-resistant mice.

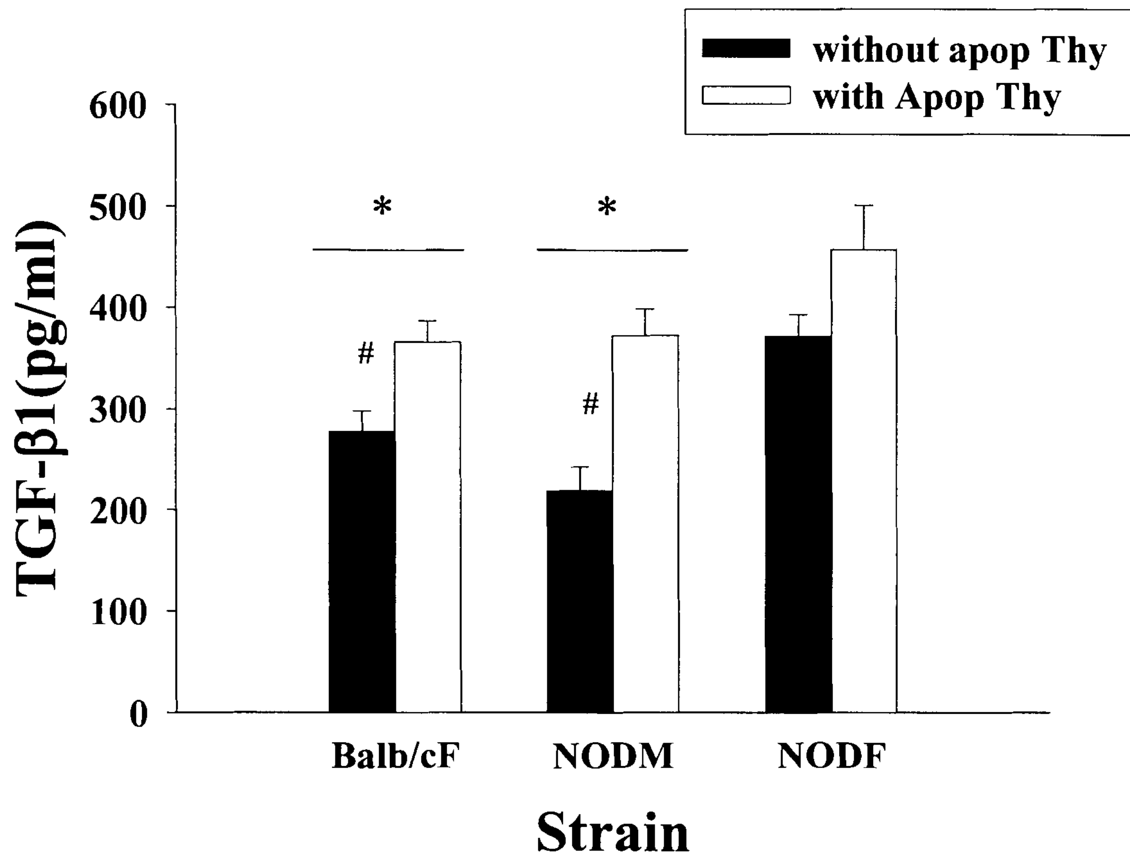

Figure 16: Peritoneal levels of TGF-β_1 following thioglycolate and apoptotic cell administration.
Apop Thy: apoptotic thymocytes. Data are means ± SE. * P<0.05 between treatment. # P<0.05 vs NOD females.

3.7 IMMUNOHISTOCHEMISTRY STAINING FOR TGF-β_1

Since other populations of cells (no more than 8% from our and other group observation (66)) also exist in the peritoneal cavity, we investigated whether macrophages are the main cells responsible for TGF-β_1 secretion. To do this, we stained the peritoneal exudates cells with anti-F4/80 antibody (a macrophages marker) and anti-TGF-β1 antibody. All the TGF-β_1 positive cells colocalized with F4/80 positive macrophages in female Balb/c mice and female NOD mice (Figure 17), indicating that macrophages are the major cell population in the peritoneal exudates producing TGF-β_1 in our experiment.

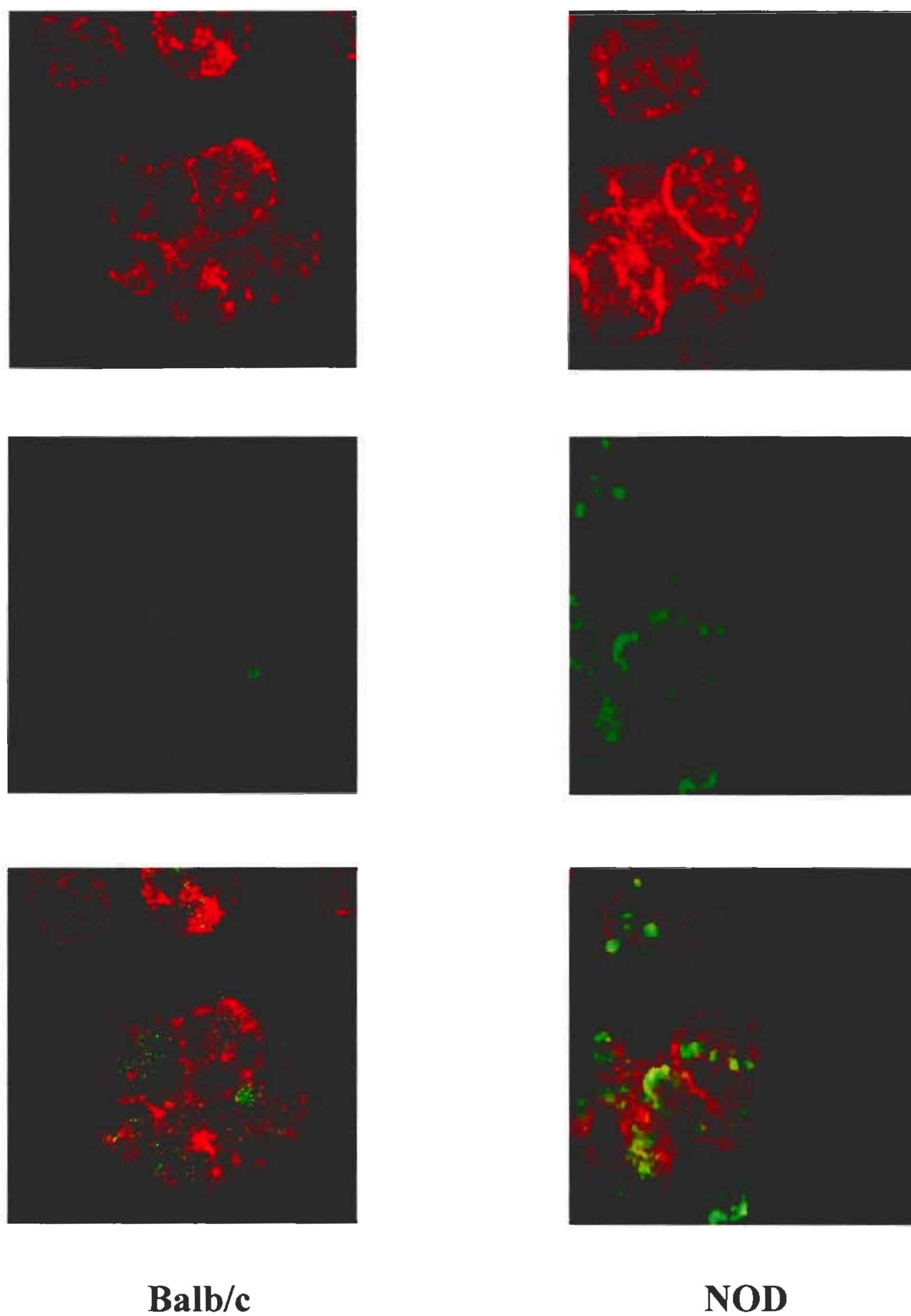

Figure 17: Immunostaining of peritoneal cells from female NOD and Balb/c mice after thioglycollate injection.
Green: Anti-TGF-β_1 staining. Red: Anti-F4/80 staining.

CHAPTER 4 DISCUSSION

4.1 *IN VIVO* PHAGOCYTOSIS OF APOPTOTIC THYMOCYTES

In our study, we found that macrophages from NOD mice exhibited impaired clearance of apoptotic thymocytes *in vivo*. At 15, 30, and 60 minutes following thymocyte injection, NOD macrophages were defective in the phagocytosis of syngeniec apoptotic thymocytes. There was no difference in phagocytosis between female NOD and Balb/c mice at 2 hours. This is consistent with other studies in SLE mice which demonstrate no difference in phagocytosis at the 2 hour time point (14). To exclude the influence of different sources of apoptotic thymocytes in macrophage engulfment, NOD macrophages were challenged by apoptotic Balb/c thymocytes or NOD thymocytes *in vivo*. The NOD macrophages showed defective uptake of apoptotic thymocytes from both strains, suggesting that the clearance defect in uptake was due to the NOD macrophages alone and not the apoptotic thymocytes. These data provide the first *in vivo* evidence for a defect in the clearance of apoptotic cells by macrophages from diabetes-prone mice. This is consistent with previous experiments done *in vitro* (25). Our results also support a previously mathematical model predicting an increased incidence of apoptotic β-cells arising from the defective clearance of apoptotic β-cells (24).

4.2 THIOGLYCOLLATE-INDUCED PERITONITIS

4.2.1 DEFECTIVE UPTAKE OF APOPTOTIC NEUTROPHILS IN THIOGLYCOLLATE-PERITONITIS

Thyioglycollate-induced peritonitis is a common model to investigate the physiological mechanism of neutrophil and macrophage recruitment. Neutrophils die rapidly by apoptosis after thioglycollate-induced recruitment, and are then engulfed by macrophages. For this reason, this model has also been used to study the clearance of apoptotic cells in autoimmune disease (66). Consistent with the *in vivo* thymocyte feeding experiment, we found that macrophages from female NOD mice showed impaired uptake of apoptotic neutrophils compared with that of Balb/c mice. Our observation suggests that macrophages from female NOD mice have defective clearance of different apoptotic cells, and that the target of the defective clearance is not restricted to a specific cell type.

The inefficient clearance of apoptotic neutrophils by macrophages after thioglycollate stimulation could lead to the accumulation of apoptotic neutrophils in the peritoneal cavity in female NOD mice. Although the ratio of apoptotic neutrophils to total neutrophils was initially not different, a significant increase in the ratio of apoptotic neutrophils in NOD mice was observed at 48 hours. This observation is also consistent with previous mathematical models that predict an increased number of apoptotic β-cells due to the defective clearance (24). After 72 hours, the macrophages completely cleared all the neutrophils in both NOD and Balb/c mice. This is due to the termination of neutrophil recruitment resulting in a decline in apoptotic cells available for macrophage clearance. In this

experiment, neutrophil recruitment was only a transient even. Therefore, the delayed clearance might be compensated over time. Nevertheless, the delayed clearance may still give APCs the opportunity to capture remaining apoptotic bodies before their clearance by macrophages (67). In pathological conditions in which apoptosis is an ongoing or continuing process, the delayed clearance will ultimately fail to eliminate the continuous supply of apoptotic cells causing accumulation of apoptotic cells in the tissues and facilitating capture by pro-inflammatory APC.

The thioglycollate-induced peritonitis experiments were done *in vivo*, which may introduce some variation between the two different strains, such as the number of recruited neutrophils. These variations will affect comparisons between different strains and possibly lead to misinterpretation of the results. In order to obtain reliable comparisons, Cheryl Dyck developed a mathematical model for our experimental data. In this model, the effect of these variations can be taken into account.

From the above experiment, the number of neutrophils in NOD mice at 24 and 48 hours is significantly lower than that of Balb/c mice. Because macrophage phagocytosis is affected by the concentration of apoptotic cells, one could question whether the results really reveal differences in clearance between the two strains, or whether it simply reflects a change of engulfment rate due to different concentrations of apoptotic cells. From the mathematical model, the rate of clearance of apoptotic neutrophils (*Kme*) in NOD mice is lower than that of

Balb/c mice. Because the model has considered *Kme* as a function of the number of neutrophils, apoptotic neutrophils and macrophages, *Kme* reflects the phagocytic ability of macrophages from these two strains at the same ratio of macrophages to apoptotic neutrophils. The defective engulfment by macrophages in NOD mice predicted by *Kme* is consistent with our experimental results and suggests that the clearance defect does not result from the lower number of neutrophils in NOD mice.

The rate of apoptotic death of neutrophils (*Kap*) in NOD mice was lower than that of Balb/c mice. However, the lower *Kap* in NOD mice does not explain the higher ratio of uningested apoptotic neutrophils to all neutrophils in the peritoneal cavity at 48 hours after thioglycollate injection. The only explanation for the higher ratio of uningested apoptotic neutrophils to total neutrophils in NOD mice is a decrease in the phagocytic ability of macrophages (*Kme*) in this experiment. In conclusion, the reduced uptake of apoptotic neutrophils by NOD macrophages resulted in the accumulation of apoptotic neutrophils in the peritoneal cavity.

4.2.2 DIFFERENTIAL RESPONSE TO THIOGLYCOLLATE IN NOD AND BALB/C MICE

In NOD mice, the number of recruited neutrophils and macrophages after thioglycollate injection was less than that of Balb/c mice, demonstrating a weaker inflammatory response in this strain of diabetes prone mice. The same phenomenon was observed in SLE-prone mice (66). However, unlike the SLE-prone mice, we did not observe any morphological differences in macrophages

between NOD and Balb/c mice. One possible explanation for the discrepancy between studies is the dose of thioglycollate used. In the study using SLE-prone mice, each mouse was injected with 1 ml of thioglycollate (66). In this study, we adjusted the dose of thioglycollate based on the body weight of the mouse allowing for the accurate administration of thioglycollate and standardization of the dose. The mathematical model also assumed that the dose of thioglycollate (*Dthio*) injected into the peritoneal cavity is very close between the two strains and suggested that equal stimulation was provided in both strains. The adjusted dose of thioglycollate provides equal stimulation to macrophages in both strains, and eliminates morphological differences in macrophages due to inadequate stimulation by thioglycollate.

Thioglycollate stimulates resident peritoneal endothelial cells to produce chemokines such as L-selectin and intercellular adhesion molecule-1 (ICAM-1), which then causes neutrophil recruitment (68). Following recruitment into the peritoneal cavity, neutrophils will die by apoptosis causing the secretion of chemokines by those dead cells and the recruitment of macrophages (69). In our study, we observed that the NOD mice recruited fewer neutrophils in response to thioglycollate compared to Balb/c mice at 24 and 48 hours. However, the number of macrophages recruited into the peritoneal cavity in NOD mice was close to that of Balb/c mice, suggesting that the number of neutrophils influences macrophage recruitment but differs between these two strains. The mathematical model predicted that the rate of macrophage recruitment by neutrophils (*Kman*) in NOD mice is higher than that of Balb/c mice. Therefore, a lower number of neutrophils

in NOD mice can actually recruit the same number of macrophages as that of Balb/c mice.

4.3 TGF-β_1

Macrophages regulate the immune response via their production of cytokines and phagocytosis. Macrophages can secret both pro-inflammatory and anti-inflammatory cytokines. Under normal conditions, apoptotic cells suppress the secretion of pro-inflammatory cytokines by macrophages and up-regulate secretion of anti-inflammatory cytokines by macrophages (38; 40). The regulation of cytokine secretion is a major anti-inflammatory role of the apoptotic cell. To understand the secretion of cytokines by macrophages in T1D, we investigated both basal secretion of cytokines after thioglycollate stimulation and stimulated secretion of cytokines by NOD macrophages following the re-introduction of apoptotic cells *in vivo*.

In our study, TGF-β_1 was the only detectable cytokine. This is consistent with other studies demonstrating that TGF-β_1 is the predominant suppressive cytokine produced by macrophages (38). We observed that macrophages from female NOD mice produced a high basal level of TGF-β_1 compared with Balb/c mice prior to apoptotic thymocyte stimulation. This result is consistent with data from another group who performed *in vitro* experiments, using lupus-prone mice (70). Although TGF-β_1 levels increased in response to apoptotic cells in Balb/c mice, TGF-β_1 levels did not change in response to apoptotic cells in female NOD mice.

There are two possible explanations for the high basal levels of TGF-β_1 recorded in female NOD mice. First, it may arise from positive feedback due to insensitive TGF-β_1 receptors in the target immune cells (such as DCs, T cells, and even macrophages). However, there is no evidence to support defective sensitivity of TGF-β_1 receptors in T1D patients or animal models, so far. This issue requires further investigation. Second, the high basal level of TGF-β_1 may be a compensatory mechanism for additional defects that lead to autoimmunity (such as the disrupted profile of cytokines). If basal levels are already maximal, when excessive apoptotic cells appear, the level of TGF-β_1 will not increase further, and thus fail to suppress the initiation of autoimmunity. This is supported by studies in which the overexpression of TGF-β_1 in diabetes-prone mice prevented the development of T1D (49-52). However, it is not clear how much TGF-β_1 is required to maintain immune tolerance in T1D-prone mice.

These results are not consistent with the hypothesis that macrophages in T1D produce elevated basal levels of pro-inflammatory cytokines, such as IL1-β and TNF-α, and decreased basal levels of anti-inflammatory cytokines. However, this hypothesis has become controversial. For example, in one study, secretion of the pro-inflammatory cytokines IL1-β and TNF-α was not different between NOD, NOR and C57BL/6 mice (71). In contrast, another study found that macrophages from NOD mice have decreased basal mRNA levels of pro-inflammatory cytokines IL-1β, IL-12p70, TNF-α (72). It has been suggested that the elevated secretion of TGF-β_1 by macrophages may reduce production of pro-inflammatory

cytokines in normal mice (38). However, *in vitro* studies have demonstrated no difference in TGF-β_1 production by macrophages from NOD mice and made it difficult to link TGF-β_1 with decreased pro-inflammatory cytokine secretion by macrophages after thioglycollate treatment. *In vivo,* we demonstrated that NOD macrophages have elevated basal levels of TGF-β_1 secretion after thioglycollate treatment. Although *in vivo* and *in vitro* observations of TGF-β_1 secretion are different, the data suggest that macrophages in NOD mice could play a role in suppressing autoimmunity by elevated secretion of anti-inflammatory cytokines or by decreased secretion of pro-inflammatory cytokines. Nevertheless, as we point out above, the immunosuppression of macrophages from NOD mice may not be enough to prevent the initiation of autoimmunity.

The above groups also reported cytokine secretion by macrophages following apoptotic cell stimulation. However, these reports are also controversial. Instead of down-regulation of pro-inflammatory cytokines in C57BL/6 macrophages, NOD macrophages continued the production of IL1-β and TNF-α after challenge with apoptotic cells (71). From the first report and our observations, NOD macrophages exhibited the inability to regulate cytokine secretion following apoptotic cell stimulation. Nevertheless, apoptotic cells can also downregulate expression of IL1-β mRNA by NOD macrophages (72), suggesting that there could be some benefit from the apoptotic cells. However, if apoptotic cells are not removed quickly because of a macrophage clearance defect, they will become

secondary necrotic, and this may result in cytokine dysregulation by macrophages (71).

Necrotic cells are generally considered to play a pro-inflammatory role in the immune response and can also affect cytokine secretion. Although some studies suggested that necrotic cells cannot induce secretion of pro-inflammatory cytokines in normal mice (71; 73), NOD macrophages responded very differently. Unlike apoptotic cells, necrotic cells can elicit up-regulation of IL1-β and TNF-α mRNA by NOD macrophages (71). Therefore, the clearance defect of apoptotic cells may not only supply more of an antigen load for APCs, but may also lead indirectly (through stimulation of secondary necrosis) to the dysregulation of cytokine secretion by macrophages in NOD mice.

Macrophages resolve inflammation through two ways: phagocytosis and cytokine secretion. Phagocytosis of apoptotic cells by macrophages can regulate cytokine secretion. On the other hand, the clearance defect by macrophages could explain the inability of macrophages from female NOD mice to up-regulate TGF-β_1 secretion *in vivo*. Deficiency of transglutaminase 2 (TGase2), a protein involved in macrophage phagocytosis, prevented macrophage secretion of TGF-β_1 upon apoptotic cell stimulation (74). At the same time, macrophage phagocytosis of apoptotic cells was impaired in those mice (74). This report suggests a potential link between impaired phagocytosis and disrupted cytokine production, although not all the defects that resulted in impaired uptake of apoptotic cells can influence

cytokine secretion. It will be intriguing to study the relationship between cytokine secretion and phagocytic ability in diabetes-prone rodents.

4.4 APOPTOSIS, PHAGOCYTOSIS DEFECT AND AUTOIMMUNITY

Apoptotic cells have "eat me" tags (such as PS on the cell surface) (40) and can be effectively recognized and engulfed by their neighboring cells and professional phagocytes, especially macrophages. Phagocytosis of apoptotic cells by macrophages is very important in the remodeling of tissue during embryonic and fetal development (75). Clearance of dead cells is also an important housekeeping event for the homeostasis of normal tissue and organs. As the removal of apoptotic cells occurs before cell lysis, the release of immunogenic and potentially toxic intracellular contents in the surrounding tissue is avoided (39). Ip and colleagues demonstrated that apoptotic cells fail to stimulate DC maturation, and induce tolerance due to lower expression of MHC and co-stimulatory factors in those DCs (76). Uptake of apoptotic cells could also trigger the secretion of anti-inflammatory cytokines (38; 40). Therefore, apoptotic cells are generally thought to be immunosuppressive. However, many lines of evidence suggest that excessive numbers of apoptotic cells could induce autoimmunity. For example, external injection of apoptotic cells can induce a transient increase in auto-antibodies and immunoglobulin deposition in the glomeruli (11; 67). In this situation, the injection of exogenous apoptotic cells into mice mimics a defect in *in vivo* clearance, which finally results in the accumulation of apoptotic cells in the tissue.

Defective phagocytosis of apoptotic cells is one of the main pathological reasons leading to the accumulation of apoptotic cells in the tissues. Other autoimmune diseases, like SLE, also show insufficient uptake of apoptotic cells by macrophages *in vitro* and *in vivo,* and result in apoptotic body deposition in the tissues (66). If apoptotic cells are not engulfed by phagocytes, they progressively become later apoptotic or secondary necrotic (77). Secondary necrotic cells may elicit a pro-inflammatory response on phagocytes, and promote autoimmunity (78; 79). Furthermore, intranuclear components such as nucleosomes will be released and recognized by APCs, especially immature DCs. Following engulfment, immature DC will convert into a mature stage, begin expressing MHC molecules, prime naïve T cells (23), and finally initiate autoimmunity. Some *in vivo* observations from SLE-prone mice also support this theory (67). After phagocytosis of apoptotic cells, transplanted DCs from SLE-prone mice elicited auto-antibodies in both normal and SLE-prone mice. As a result, autoimmune disease accelerated in those SLE-prone mice (67).

The impaired clearance of apoptotic cells by macrophages from NOD mice was observed in this study. The defective phagocytosis might result in the accumulation of apoptotic β-cells in pancreatic islets of NOD mice, which has been previously observed *in situ* (9). Furthermore, the neonatal wave of β-cell apoptotic death in NOD mice precedes the lymphocyte infiltration (24). The neonatal wave of β-cell apoptotic death may be related to the acquisition of auto-antigens by APCs and later onset of autoimmunity in T1D. Recently, the role of dead β-cells in the priming of self-reactive T cells at an early age in NOD mice

was investigated. After transplanting dead NOD islets into 6 day old NOD mice, co-transferred BDC2.5 T cells, which have a β-cell specific T cell receptor, were activated and proliferated in the PLN (80). To test the role of physiological apoptotic β-cells in the initiation of autoimmunity, the caspase inhibitor ZVAD was administrated at 24-48 hours before transfer of the T cells to block β-cell apoptosis. Treatment of 21-42 day old NOD mice with a single dose of ZVAD impaired activation of naïve BDC2.5 T cells (80). This observation suggests that the increased apoptotic β-cell death at a young age is an essential event to activate T cells and trigger autoimmunity in T1D. The accumulated apoptotic and secondary necrotic β-cells in the neonatal NOD mice may be recognized and engulfed by APCs, especially DCs, presented to T cells in the pancreatic lymph node (10; 80), and finally initiate autoimmunity in T1D.

4.5 MOLECULAR DEFECTS RELATED TO IMPAIRED CLEARANCE OF APOPTOTIC CELLS

The signalling pathway of phagocytosis by macrophages is not fully understood. In general, receptors of phagocytes tether apoptotic cells and activate downstream events in phagocytes, that may lead to cytoskeletal reorganization and engulfment (81). There are many surface receptors involved in the uptake of apoptotic cells, such as scavenger receptors (82), ABC-1 transporters (83), and CD14 (84). Deficiency of the Mer receptor (21) or PS receptor (85; 86) can also lead to impaired clearance of apoptotic cells. Mer knockout mice developed increased auto-antibodies compared to normal mice. The increase in auto-antibodies may be

associated with the defective clearance of apoptotic cells, suggesting a relationship between the impaired uptake of apoptotic cells and autoimmunity.

Many proteins, so called soluble bridging molecules, are also important in the recognition and clearance of apoptotic cells. These molecules include C-reactive protein, serum amyloid P component, mannose binding lectin (87), pulmonary surfactant protein A, surfactant protein D, and C1q (88). Mice lacking some of these proteins, such as C1q, also show defective engulfment of apoptotic cells (14). C1q deficient mice spontaneously developed auto-antibodies and glomerulonephritis. Patients with C1q deficiency develop severe SLE (89). Recently, two other molecules, milk fat globule epidermal growth factor 8 (MFG-E8) (90) and TGase2 (74), were described to play a role in the phagocytosis of apoptotic cells. Deficiency of MFG-E8 or TGase2 can result in impaired engulfment of apoptotic cells by macrophages. The MFG-E8$^{-/-}$ mice and the TGase2$^{-/-}$ mice both developed glomerulonephritis and auto-antibodies. Collectively, these investigations suggest that the deficient clearance of apoptotic cells may result in an autoimmune response, and initiate autoimmunity in diseases, like SLE and T1D. Identification of the proteins and/or receptors involved will be necessary to understand the signal transduction cascade leading to the clearance of apoptotic cells.

The serum proteins and macrophage receptors that are responsible for the defective clearance of apoptotic cells in NOD mice have not been fully addressed. Even though macrophages from T1D mice exhibit intrinsic defects in the uptake

of apoptotic cells (25), the lack of certain serum proteins may still play a role in macrophage clearance defects in the local microenvironment. Furthermore, abnormal expression of complement components (such as C1q), other proteins and even cytokines may contribute to the insufficient engulfment of apoptotic cells in islets, initiating and localizing autoimmunity in pancreatic islets. Further investigation of the molecular defects involved in these events should aid in the development of pharmaceutical agents for the treatment or prevention of T1D.

4.6 SUMMARY

In this study, the phagocytic ability of macrophages was investigated in NOD and Balb/c mice. Our results showed that macrophages from NOD mice have defective clearance of apoptotic cells *in vivo*. The presence of excessive numbers of apoptotic β-cells has been reported in young NOD mice and may initiate the autoimmunity in T1D. Taken together, the impaired engulfment of apoptotic cells may result in the accumulation of apoptotic β-cells. We also reported that the level of TGF-β_1 secreted by female NOD macrophages in response to thioglycolate treatment was higher than that in Balb/c mice. In response to apoptotic cell stimulation, the level of TGF-β_1 secreted by macrophages was increased in female Balb/c but not in female NOD mice. This high level of TGF-β_1 could be a compensatory response by macrophages to other defects that involve in the initiation of autoimmunity. However, this level of compensation may not be enough to prevent autoimmunity in T1D. The inefficient uptake of

apoptotic cells and insufficient anti-inflammatory cytokine (TGF-β_1) secretion by macrophages could be the first step in the initiation of autoimmunity in T1D.

4.7 CONCLUSIONS

Macrophages from NOD mice have defective clearance of apoptotic cells *in vivo*. This defect could result in the accumulation of apoptotic β-cells in pancreatic islets. The secretion of TGF-β_1 by macrophages from NOD mice is higher than that of control mice, but failed to be up-regulated by apoptotic cells. However, the high level of TGF-β_1 produced by NOD mice may not be sufficient in protecting those mice from autoimmunity. The defective clearance of apoptotic cells and insufficient secretion of TGF-β_1 may be involved in the initiation of autoimmunity.

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APPENDIX I ANNEXIN V AND PROPIDIUM IODINE (PI) STAINING PROTOCOL

Step1 - Annexin V Application

1. Take 1-2×10^6 thymocytes, wash in cold PBS 2X and centrifuge 1200RPM (200g) for 5 minutes.
2. Add 1ml Binding Buffer to 1×10^6 thymocytes. Take off 100 µl cell suspension in Binding Buffer for staining.
   Binding Buffer:
   Hepes - 2.383g
   NaCl - 8.182g
   CaCl – 0.278g
   2 L of dH_2O, then PH to 7.4
   Store at 2-8 °C
3. Add 7-8 µl Alexa Fluorescent 488 conjugated Annexin V, mix gently, and incubate 10 minutes at RT in dark.

Step2 - PI Application

1. Add 2 µl PI to cell suspension at 10 minutes after adding Annexin V. Incubate 5 minutes at RT in dark.
2. Add 350 µl Binding Buffer to cell suspension.
3. Place 350 µl of cell suspension into prepared cytospin clip.
4. Cytospin slides for 3minutes at 1200 RPM.
5. Coverslip slides using Glycerol/PBS (10:1).

APPENDIX II MACROPHAGE (F4/80) STAINING PROTOCOL

Step 1 - Cytoslide preparation

1. Place 350-400 μl of cell suspension into prepared cytospin clip.
2. Cytospin slides for 3minutes at 1200 RPM.
3. Fix slides with 2% paraformaldehyde 30 minutes at room temperature (RT) (place drop of fixative on slides).
4. PBS wash X3, then rinse slides at RT for 1hour (longer is better).

   Phosphate Buffer Saline (PBS):

   NaH_2PO_4 - 10.25g
   Na_2HPO_4 - 47.75g
   2L of dH_2O, then PH to 7.4
   NaCl - 350.65g
   Dilute 1:10

5. Take off slides, air dry.
6. Place cytoslides in PBS at RT for 5minutes minimum but longer is better (overnight if possible).
7. Wash slides 3X in PBS.

Step 2 - Blocking the Endogenous Peroxidase Activity

1. Incubate slides into 0.3% hydrogen peroxide (H_2O_2)/ Methanol at RT for 30 minutes.

Step 3 - Primary Antibody Application

1. Wash slides 3X in PBS.
2. Add 100 μl of 10% Lamb Serum (Life Technologies, ON, Canada) / PBS to each slide and incubate at RT for 30 minutes.
3. Tap off serum.
4. Add 50 μl of Rat monoclonal antibody to mouse F4/80 (1:25) (macrophage) to each slide and incubate at RT for 2 hours.

Step 4 - Secondary Antibody Application

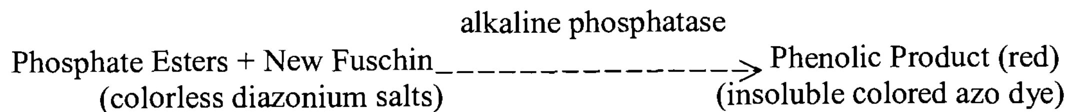
1. Wash slides 3X in PBS.
2. Add 100 μl of biotinylated anti-rat antibody (1:200) (Vector, Burlington, ONT, Canada) to each slide and incubate at RT for 1 hour.

Step 5 - ABC-Alkaline Phosphatase Application

1. Make up ABC- Avidin/Biotin Alkaline Phosphatase Complex (1:1000) (Vector, Burlington, ONT, Canada) 20 minutes before applying.
2. Wash slides 3X in PBS.
3. Add 100 μl of ABC-Alkaline Phosphatase Complex to each slide and incubate at RT for 1 hour.

Step 6 - Chromogen Visualization

1. Make up New Fuchsin Solution (Dako, Mississauga, ONT, Canada).
   3 drops Fuchsin chromogen
   3 drops Fuchsin activating agent
   Mix and incubate at RT 1 minute
   Add 1.76 ml Buffered substrate
2. Add 200 µl New Fuchsin Solution to each slide and incubate at RT for 10-20 minutes.
3. Wash in running tap water to stop reaction.
4. The chromogen is visualized by the following reaction:



Step 7 - Counterstaining

1. Place slides in Harris' hematoxylin for 30 seconds.
2. Wash slides in cold water.
3. Place slides in sodium borate for 5 seconds.
4. Wash slides in cold water.
5. Dehydrate slides in increasing concentrations of ethanol from 70% to 100%.
6. Place slides in xylene I for 5 minutes.
7. Place slides in xylene II for 5 minutes.
8. Coverslip slides using Permount mounting media (Fischer Scientific, NJ, USA).

APPENDIX III TUNEL AND MACROPHAGE (F4/80) STAINING PROTOCOL

Step 1 - Cytoslide prepration

1. To prepare cytoslides, follow Step 1 from macrophage staining protocol (Appendix II).

Step 2 - Antigen Retrieval

1. Place slides in 200ml 10mM Citrate Buffer (PH 6.0). Microwave for 5 minutes at power 40.
2. Add 80 ml of dH_2O to the container for rapid cooling. Transfer slides into PBS and wash slides 2X in PBS.

Step 3 - Blocking the Endogenous Peroxidase Activity

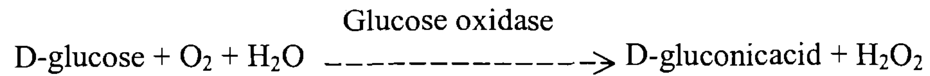
1. Incubate slides into 0.3% hydrogen peroxide (H_2O_2)/ PBS at RT for 30 minutes.

Step 4 - TUNEL staining

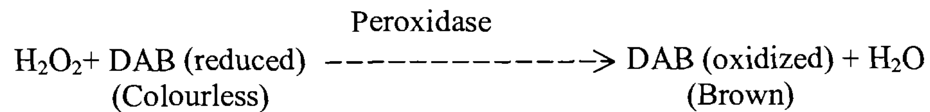
1. Wash slides 3X in PBS
2. Incubate slides with Equilibration Buffer at RT for 10 minutes.
3. Incubate with Terminal Deoxynucleotidyl Transferase (TdT) (1:4) at 37 °C for 45 minutes.
   TdT (1:4): 130ul TdT + 390ul Reaction Buffer
4. Transfer slides into Stop Wash Solution to stop reaction at RT for 10minutes.
   Stop Wash Solution: 1 ml Stop Wash Buffer + 34 ml dH_2O.
5. Wash slides 3X in PBS
6. Incubate with Anti –Digoxigenin Peroxidase (DIG) antibody at RT for 45min.
7. Wash slides 3X in PBS

Step 5 - Chromogen Visualization

1. Make up 3,3'diaminobenzidine tetrahydrochloride (DAB) solution.
   Tris (0.1M, pH 7.6) -100 ml
   D-Glucose -200 mg
   Ammonium Chloride -40 mg
   Glucose Oxidase -0.3 mg
   DAB -12.5 mg (Sigma, ONT, Canada) (add just before using)
2. Filter entire solution prior to using.
3. Develop in DAB solution for 4-5 minutes. Check under the microscope for brown staining in the nucleus.
4. The chromogen is visualized by the by the following reactions:



$$D\text{-glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} D\text{-gluconicacid} + H_2O_2$$



$$H_2O_2 + \text{DAB (reduced)} \xrightarrow{\text{Peroxidase}} \text{DAB (oxidized)} + H_2O$$

(Colourless) (Brown)

5. Wash in PBS and leave slides in PBS overnight.

Step 6 - Macrophage Staining

1. To perform macrophage staining, follow the macrophage staining protocol (Appendix II). Start from step 3 – step 6.

Step 7 - Counterstaining

1. Place slides in Harris' hematoxylin for 30 seconds.
2. Wash slides in cold water.
3. Place slides in sodium borate for 5 seconds.
4. Wash slides in cold water.
5. Dehydrate slides in increasing concentrations of ethanol from 70% to 100%.
6. Place slides in xylene I for 5 minutes.
7. Place slides in xylene II for 5 minutes.
8. Coverslip slides using Permount mounting media.

APPENDIX IV TGF-β_1 ELISA PROTOCOL

Step1 - Plate Preparation

1. Add 100 µl diluted capture antibody (anti-TGF-β_1 antibody) (1:180) in filtered PBS to each well of a 96-well microplate and incubate overnight at RT.
2. Aspirate each well and wash 3X with Wash Buffer.
   Wash Buffer: 0.05% Tween 20 in filtered PBS, adjust pH to 7.2-7.4
3. Add 300 µl Block Buffer to each well to block plate and incubate 2 hours at RT.
   Block Buffer: 5% Tween 20, 5% Sucrose, 0.05% NaN_3 in filtered PBS
4. Aspirate each well and wash 3X with Wash Buffer.

Step2 - Sample Activation

1. Add 0.1 ml 1 N HCl to 0.5 ml sample to activate latent TGF-β_1 to the immunoreactive form, mix well and incubate 10 minutes at RT.
2. Add 0.1 ml 1.2 N NaOH/0.5M HEPES each sample to neutralize acid.

Step3 - Assay Procedure

1. Add 100 µl sample or standard in Reagent Diluent per well and incubate 2 hours at RT.
   Reagent Diluent:
   BSA – 20 mg
   Tween 20 - 500 µl
   PBS - 100 ml
   pH 7.2-7.4, 0.2 µm filered.
2. Aspirate each well and wash 3X with Wash Buffer.
3. Add 100 µl detection antibody in Reagent Diluent (1:180) per well and incubate 2.5 hours at RT.
4. Aspirate each well and wash 3X with Wash Buffer.
5. Add 100 µl Streptavidin-HRP in Reagent Diluent (1:200) per well and incubate 20 minutes at RT in dark.
6. Aspirate each well and wash 3X with Wash Buffer.
7. Add 100 µl Substrate Solution (R&D systems, Minneapolis, MN, USA) to each well and incubate 20 minutes at RT in dark.
   Substrate Solution:
   Color Reagent A (H_2O_2) - 5 ml
   Color Reagent B (Tetramethylbenzidine) - 5 ml
8. Add100 µl Stop Solution (2N H_2SO_4) to each well.
9. Immediately measure the absorbance at 450 nm by microplate reader.

APPENDIX V TGF-β_1 AND F4/80 FLORESCENT STAINING PROTOCOL

Step 1 - Cytoslide prepration

1. Place 350-400 ml of cell suspension into prepared cytospin clip.
2. Cytospin slides for 3 minutes at 1200 RPM.
3. Fix slides with filtered 2% paraformaldehyde/30%sucrose in sterile PBS 30 minutes at 37 °C.
4. Wash slides 3X in sterile PBS.

Step 2 – Primary TGF-β_1 Antibody Application

1. Add 5% bovine albumin (BSA)/ 10% goat serum / 0.3% Triton 100/ PBS (PBST) to each slide and incubate at RT for 1 hour.
2. Tap off solution.
3. Add 50 μl of chicken anti-TGF-β_1 antibody (1:50) to each slide and incubate at RT for 1 hour.

Step 3 - Secondary TGF-β_1 Antibody Application

1. Wash slides 3X in sterile PBS.
2. Add 100 μl of Donkey anti-chicken antibody-FITC conjugate (1:500) to each slide and incubate at RT for 1 hour in dark.

Step 4 - F4/80 Antibody Application

1. Wash slides 3X in sterile PBS.
2. Add 100 μl of rat monoclonal antibody to mouse F4/80 red-Phycoerythrin conjugate (1:25) (macrophage)to each slide and incubate at RT for 1 hour in dark.
3. Wash slides 3X in sterile PBS.
4. Coverslip slides using Fluorescent Mounting Media and seal Coverslip slides with nail polish.