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Steady-State Cell Apoptosis and Immune Tolerance - Induction of Tolerance Using Apoptotic Cells in Type 1 Diabetes and Other Immune-Mediated Disorders

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1. Introduction

Type 1 diabetes (T1D) is also named insulin-dependent diabetes mellitus. Because T1D is more frequently seen in children, it is also called juvenile diabetes. Currently the standard treatment for T1D is the daily use of exogenous insulin. Because of the poor compliance with insulin use, T1D patients often suffer from hyperglycemia or hypoglycemia (1; 2). In addition, T1D patients are at high risk for experiencing ketoacidosis due to a variety of different reasons (3-5). Many T1D patients will inevitably develop serious, chronic complications in organs such as heart and kidney (6-9). Therefore, T1D is a devastating disease for young individuals. Thus far, there has been no cure for T1D, and the search for its cure is a long-term goal in T1D research.

Regarding the pathogenesis of T1D, it is clear that T1D is an autoimmune disease that is mediated by pathogenic T cell responses to pancreatic islet β cells (10-12). The fundamental problem in T1D is the breakdown of immune tolerance to self antigens. More specifically, β cell antigen-specific T cells, which are usually well controlled in healthy individuals through various self tolerance mechanisms, attack insulin-producing β cells. Thus, to cure T1D, restoration of immune tolerance against β cell antigens is necessary. Numerous investigators have expended tremendous energy and efforts in finding ways to prevent or cure T1D; however, much work still remains.

During fetal development, the majority of self-reactive T cell clones are deleted in the thymus. This process is referred to as central tolerance. Some self-reactive T cell clones do escape T cell deletion mechanisms and are exported to the periphery, leading to a potential risk for development of autoimmune diseases. In healthy individuals, self-reactive T cell clones are not pathogenic, because of well-functioning peripheral regulatory mechanisms (13; 14). One of the major mechanisms by which self tolerance is maintained is through the steady-state processing of apoptotic cells during normal tissue turnover (14). Evidence has

shown that the impaired processing of apoptotic cells is associated with the pathogenesis of systemic lupus erythematosus (15; 16). In this chapter, we will focus on discussing the relationship between steady-state cell apoptosis and self-tolerance maintenance. In addition, we will discuss β cell apoptosis and T1D pathogenesis as well as the application of intravenous apoptotic cell delivery in restoring immune tolerance in T1D and other immune-mediated disorders.

2. Steady-state cell apoptosis and immune tolerance

2.1 Steady-state apoptosis

The effective clearance of apoptotic cells and subsequent regeneration of cells is crucial in organ development, tissue homeostasis, response to injury, and maintenance of the innate and adaptive immune system (17). In the spleen and liver, greater than 1×10^{11} circulating neutrophils are eliminated each day. The vast majority (95%) of thymocytes die by apoptosis due to negative selection. Another example exists in the retina where photoreceptor rods continuously renew their light-sensitive outer segments (17). The reality is that almost every cell in our bodies is replaced during a lifetime, and some types of cells turnover more than once. It was once thought that clearance of apoptotic cells was immunologically inert, but we now know that the uptake of apoptotic cells by phagocytes can induce immunosuppression or tolerance (discussed in greater detail below). Interestingly, some pathogens (*Plasmodium falciparum*) and tumors exploit the immune inhibitory pathways involved in apoptotic cell clearance to aid in their survival and perseverance in the body (17). Cell death and the ensuing removal of dying cells by phagocytes can have profound regulatory effects on the immune system.

2.2 Immature dendrite cells (DCs) and macrophages engulf apoptotic cells during tissue cell turnover

The fate of hundreds of millions of apoptotic tissue cells that die each day is to be engulfed as quickly as possible by antigen-presenting cells, especially immature dendritic cells and macrophages. DCs distributed in the peripheral tissues are able to sense “find-me” signals elicited by the apoptotic cells (18-23). DCs migrate toward the dying cells then bind the apoptotic cells through recognition of “eat-me” signals on their surface (20; 22; 24-26). After phagocytosing apoptotic cells, DCs home to the T cell zone of local draining lymph nodes where they present self tissue antigens in a tolerogenic fashion to self antigen-reactive T cells that have escaped from the thymus. The various mechanisms will be discussed in the following sections.

2.3 Dendritic cells acquire a tolerogenic phenotype following phagocytosis of apoptotic cells

DCs interact with apoptotic cells through specific receptors that can recognize molecules uniquely expressed on the apoptotic cell surface. Early studies demonstrated that DCs that had engulfed apoptotic cells were able to maintain an immature state as evidenced by low expression levels of major histocompatibility complex (MHC) molecules and co-stimulatory molecules such as CD80, CD86 (27; 28). More importantly, these DCs resisted maturation induction by lipopolysaccharide or CD40L. When these immature DCs presented antigens to the antigen-specific T cells, the latter became unresponsive to subsequent stimulation by

the same antigens (29; 30). The mechanisms underlying this process were unclear, but the recent discovery of TAM (Tyro-3-Axl-Mer) receptors on DCs or macrophages that specifically recognize ligands on apoptotic cells may help unravel the mystery (31). TAM receptors directly or indirectly interact with apoptotic cell surface molecules and work together with cytokines to transduce suppressive signals in DCs or macrophages (31). TAM receptors play important roles in regulating innate immunity, helping to avoid unwanted autoimmunity. It is worth mentioning here that DCs interacting with apoptotic cells may become immunogenic under certain circumstances (32; 33). An elegant study demonstrated that DCs phagocytosing bacteria-infected apoptotic cells became fully mature, showing strong T cell stimulatory activity, and induced inflammatory Th17 cells; whereas, DCs that had engulfed non-infected apoptotic cells acquired tolerogenic properties, inducing Foxp3+ regulatory T cells (33). This study suggests that following phagocytosis of dead cells during infections or inflammatory conditions, DCs or other antigen-presenting cells may actually trigger activation of self-reactive T cells, which may lead to autoimmunity or autoimmune disorders. More details regarding immunogenic and tolerogenic cell death were discussed recently (review by Green, et al) (32).

2.4 TGF- β is an important cytokine participating in immune tolerance induced by apoptotic cells

Several cytokines produced during the clearance of apoptotic cells are associated with the induction of tolerance. In particular, TGF- β is important in this process. TGF- β is an immunosuppressive cytokine that can be produced by apoptotic cells but more importantly, is secreted by DCs or macrophages that have phagocytosed apoptotic cells (34-37). Recently, TGF- β has been identified as an essential cytokine for the differentiation of adaptive Foxp3+ regulatory T cells (Tregs) (38; 39). Tregs play an important role in maintaining peripheral tolerance (40). Thus, increased levels of TGF- β produced during the processing of apoptotic cells likely contributes to apoptotic cell-induced tolerance through generation of Tregs. A recent report supplied evidence to support this idea by demonstrating that TGF- β is essential for anti-CD3 antibody treatment-induced autoantigen-specific tolerance in experimental autoimmune encephalomyelitis (EAE) (41). In this report, the authors clearly demonstrated that macrophages ingesting apoptotic T cells produced high levels of TGF- β , which subsequently induced Treg production, and consequently, EAE was prevented. However, under conditions where DCs and macrophages were depleted, the effect of anti-CD3 therapy was drastically compromised in preventing EAE because of the reduced levels of systemic TGF- β and Tregs (41). Hence, the local or systemic levels of TGF- β may be an important factor in determining the immunological outcomes associated with engulfment of apoptotic cells.

2.5 Phosphatidylserine on apoptotic cells mediates immunosuppression

The recognition of apoptotic cells by phagocytes involves highly specific receptor-ligand interactions. In the synapse between a phagocyte and an apoptotic cell, multiple Apoptotic Cell-Associated Molecular Patterns (ACMP) presented on the apoptotic cell surface interact with Pattern-Recognition Receptors (PRR) that are expressed by the phagocyte (42). One of the earliest events in apoptosis is the "flip-flop" and external exposure of the anionic phospholipid phosphatidylserine (PS), which is normally restricted to the inner leaflet of the plasma membrane. PS is an important "eat-me" signal that stimulates apoptotic cell

phagocytosis by DCs or macrophages. PS recognition receptors can either bind PS directly or bind indirectly through soluble bridging molecules. Receptors that directly interact with PS include members of the TIM (T cell immunoglobulin domain and mucin domain) family (43-49), BAI1, the seven transmembrane brain angiogenesis inhibitor 1(50), and the atypical EGF-motif containing membrane protein Stabilin-2 (20). MFG-E8 has been identified as a bridging molecule that can simultaneously bind PS on apoptotic cells with high affinity (51-54) and engage integrin $\alpha\beta3$ on phagocytes. Gas6 (growth arrest specific gene 6) and protein S bridge PS to TAM family of receptors on phagocytes (31). Additional membrane proteins important in binding apoptotic cells include CD36, CD14, CD68, and thrombospondins (55-58). It is believed that PS receptors not only help to physically tether the apoptotic cells to the phagocytes but also generate intracellular signals that stimulate endocytosis. Important for the induction of tolerance, binding of PS to its receptor MerTK on DCs or macrophages induces downstream signals which result in down-regulation of NF- κ B and inhibition of pro-inflammatory cytokine production (59-61). It has also been reported that PS through the bridging molecule Gas6 protein binds to AXL/Mer family members to suppress NK- κ B activation and inflammatory mediators such as IL-1 and iNOS (62). Recently, it was shown monocyte-derived DCs incubated with PS liposomes assumed immature DC characteristics and were unable to stimulate T cells (63; 64). Our data showed that water-soluble PS (C6) dramatically inhibited T cell proliferation in response to stimulatory anti-CD3 and anti-CD28 antibodies (Xia, unpublished data). We also discovered that PS could be released from apoptotic cells, as supernatants from apoptotic cells efficiently blocked anti-PS antibody binding to apoptotic cells (xia, unpublished data). Therefore, PS has proven to be an important factor in apoptotic cell-induced immunosuppression.

3. β cell apoptosis and type 1 diabetes pathogenesis

It has been widely accepted that diabetes, including T1D and T2D, is associated with excess death of pancreatic β cells. However, evidence that pancreatic β cells undergo a wave of cell death in the early age of normal individuals suggests that early β cell death may be beneficial for avoiding autoimmunity. In line with this, results from an animal study demonstrated that induction of β cell death at an early age in NOD mice protected the animals from developing T1D (65). Despite contradictions, β cell death may play distinct and different roles in autoimmune processes depending on the environment where β cell death occurs. The following will discuss β cell apoptosis and T1D pathogenesis in hopes of clarifying certain issues that are clouded in confusion.

3.1 Two waves of β cell death in T1D

Animal studies have shown that pancreatic β cells only undergo a single wave of β cell death at an early age in normal strains of animals, whereas, in T1D prone animals, there are two waves of β cell death. The first wave occurs at ages similar to normal strains, yet, the second wave of β cell death follows a few weeks later (66; 67). The second wave of β cell death is thought to be mediated by autoimmune attack (66; 68). However, it is still unclear whether the first wave of β cell death is associated with the second wave, and why the second wave of β cell death occurs only in T1D prone animals. A few lines of evidence show that inefficient processing of dead β cells at an early age might contribute to the

development of autoimmune responses in the pancreatic islets (66; 67). Unfortunately, the role of early β cell death in T1D pathogenesis remains poorly understood.

3.2 β cell death in self tolerance or autoimmunity

While both environmental and genetic risk factors contribute to the susceptibility of an individual to T1D, the exact mechanisms that initiate T1D autoimmunity remain elusive (69). Accumulating evidence supports the idea that the exposure of β cell antigens resulting from early β cell death may be an initiating factor. If this is really the initiating factor, why does autoimmunity only occur in T1D prone animals but not in normal strains? As we discussed earlier, apoptotic cells are immediately processed by phagocytes in vivo in order to maintain self tolerance. A key in this process is the quick and efficient clearance of apoptotic cells after early stages of cell apoptosis, which prevents reactions to self-antigens. Otherwise, the apoptotic cells may advance to late stage apoptosis or to a necrotic stage at which point the dead cells may cause inflammatory responses and potentially lead to autoimmune disorders, such as SLE, as mentioned previously. It has been reported that in T1D animal models, phagocytes are defective in efficiently clearing apoptotic β cells during the first wave of islet β cell death (66). Failure to scavenge dead β cells could potentially initiate β cell antigen-specific autoimmunity, which subsequently leads to recruitment of additional inflammatory cells, including macrophages, DCs, and T cells to the islets to worsen autoimmune damage (68). This hypothesis, however, does not help explain the T1D protection mediated by streptozotocin-induced β cell death in NOD mice at young ages (65). In the study reported by Hugues et al, the authors demonstrated that NOD mice were protected from developing T1D when they were treated at 4 weeks of age with streptozotocin to induce cell death of a limited number of β cells (65). The data in the study also showed that there were more cells infiltrating the islets at an early time after streptozotocin administration, but the islet β cells were eventually protected. These findings suggest that β cell death induced by streptozotocin before autoimmunity begins may modulate autoimmune T cells to become tolerized, thereby protecting from T1D. The mechanisms underlying this protection were not well elucidated, but it is possible that early induction of β cell death creates a microenvironment that is more tolerogenic and characterized by increased TGF- β production by apoptotic β cells or the DCs and macrophages that phagocytose them. A recent report demonstrated that expression of TGF- β on islet β cells actually protected NOD mice from T1D (70), suggesting that local levels of TGF- β may play an essential role in the protection of β cells from autoimmune attack.

Regarding the relationship between β cell death and T1D pathogenesis, more attention has been focused on the investigation as to how innate and adaptive immunity induces β cell death leading to autoimmune diabetes. Based on histological analysis of the pancreas in T1D, DCs and macrophages appear first in the islets (66; 68). Thus, it is believed that DCs and macrophages are two important inflammatory cells in the initiation of autoimmunity. DCs and macrophages not only damage β cells directly through the secretion of pro-inflammatory cytokines such as TNF- α , IL-1, and IL-6, but more importantly, they present β cell antigens to antigen-specific CD4⁺ and CD8⁺ T cells, leading to adaptive autoimmune reactions against islet β cells. Preferentially induced Th1 cells in T1D can target β cells through direct killing or by secreting pro-inflammatory cytokines, such as IFN- γ (71). CD8⁺ cytotoxic T cells may kill β cells via the release of granzymes and perforin (72). Furthermore, soluble inflammatory mediators secreted by DCs, macrophages, or activated T

cells, such as IL-1 and IFN- γ , can induce Fas expression on β cells (71; 73; 74). Pancreatic histology of new onset T1D patients has shown that Fas+ β cells are surrounded by FasL+ activated T cells, (75-77) indicating that activated T cells may induce β cell death through FasL-Fas ligation during T cell and β cell interaction. There is still some debate about whether the up-regulation of Fas on β cells is associated with β cell death. Specific deletion of Fas from β cells using Cre-Loxp genetic manipulation leads to normal or slightly reduced incidence of T1D (78). Similar results were obtained with β cells over-expressing a dominant negative form of Fas (79). Another important factor potentially involved in β cell death is oxidative stress occurring in β cells during active inflammation in the local islets. It has been documented that β cells are highly sensitive to oxidative stress because of their inefficiency in making antioxidants (80-82). Streptozotocin and alloxan drug-induced diabetes is thought to be due to overproduction of reactive oxygen species (ROS) induced by the drugs. Supporting the role of ROS in β cell death, a study showed that genetic overexpression of the antioxidant thioredoxin in β cells significantly prevented NOD mice from developing T1D (83). Overexpression of antioxidants such as catalase or metallothionein reduces the susceptibility of β cells to cytokine-induced death in vitro (84-86). Recently, it was demonstrated that ROS participated in inflammatory processes by activating inflammatory cytokines via inflammasome-dependent or inflammasome-independent pathways (87; 88). Inflammatory cytokines such as IL-1 are secondary to oxidative stress and exaggerate β cell damage. Based on what has been described above, it is likely that T1D is a disease caused by multiple factors working together. Once autoimmunity starts, a vicious cycle appears to be initiated until all β cells are destroyed, leading to diabetes.

In summary, β cell death can play completely opposing roles in T1D pathogenesis. Steady-state β cell death that occurs in normal individuals, normal strains of mice or young T1D-prone animals provides protection from T1D. On the other hand, β cell death that occurs under inflammatory conditions seems to accelerate disease progression through exaggerating inflammation. Therefore, any measures capable of breaking this vicious cycle will potentially offer opportunities to restore homeostasis, facilitate self tolerance, and hopefully attenuate or cure T1D.

4. Application of apoptotic cells in immune-mediated disorders

4.1 Extracorporeal photopheresis for the treatment of autoimmune disease

Intravenous delivery of apoptotic cells has been shown to have clinical efficacy in T-cell mediated autoimmune diseases. Extracorporeal photopheresis (ECP) is a novel immunotherapy that involves collecting peripheral blood mononuclear cells from patients and exposing them to a photoactivatable drug that induces programmed cell death or apoptosis. More specifically, the apheresis-based procedure involves connecting a patient through venous access to the THERAKOS™ UVAR™ XTS™ or CELLEX™ photopheresis medical device (shown below). Both photopheresis systems offer a point-of-care, patient connected, sterile, automated centrifugation platform that separates white blood cells from whole blood (89). Once white blood cells are concentrated into a collection bag, 8-methoxypsoralen (8-MOP), a naturally derived furocoumarin compound that readily intercalates into DNA (trade name=UVADEX®), is added and readily absorbs into the cells. 8-MOP-treated cells are then circulated across a photoactivation plate, which is exposed to 1.5-2 Joules/cm² of energy from an ultraviolet A (UVA) light source (90). Apoptotic processes are triggered in the treated cells, and they are returned to the patient. ECP is

currently approved by the U.S. Food and Drug Administration for the palliative treatment of skin manifestations associated with Cutaneous T cell Lymphoma (CTCL) that is refractory to other forms of therapy. ECP is available worldwide (>150 medical centers) (91) and has been utilized for over 20 years for the treatment of both allo-immune (graft vs host disease, GVHD, and graft rejection) and autoimmune conditions (Crohn's disease, T1D). This therapy is associated with a very rare incidence of serious adverse events and is generally well-tolerated by patients with no significant side effects (90; 92). In a few European countries, an alternative method has been utilized for performing photopheresis. Mononuclear cells are collected with a standard cell separator and UVA irradiation is performed in a laboratory setting using a stand-alone irradiator; however, no commercially available UVA irradiator is compliant with European standards marking for the sale and use of medical devices for performing ECP. While there are chain-of-custody issues and potential risks of infection associated with these 'open' photopheresis systems, there are reports of similar clinical efficacy between both techniques; however, no head-to-head comparison studies have been reported to date (93).

ECP is most commonly used to treat CTCL and GVHD. CTCL is a rare lymphoproliferative disorder characterized by the accumulation of malignant T lymphocytes in the skin. While clinicians have been successfully treating some CTCL patients with ECP monotherapy (94), others use ECP in combination with other therapies to improve outcomes (92). On average, response rates in CTCL range from 33% to 88% with ECP monotherapy; whereas, multimodality ECP response rates are comparable (94). Collectively across 19 studies, a combined overall response rate of 55.7% was reported across all stages of CTCL with 17.6% achieving a complete response (95). ECP is also highly beneficial for the treatment of GVHD. In a prospective phase II study involving steroid-refractory acute GVHD patients, 82% of patients with cutaneous involvement, 61% with liver involvement, and 61% with gut involvement achieved responses after ECP treatments. Interestingly, ECP not only facilitated tapering and eventual discontinuation of corticosteroids in responders but ECP therapy also improved overall survival (96; 97). A retrospective analysis of 71 patients with severe chronic GVHD demonstrated that patients who had received ECP showed an overall response rate of 61%, and complete responses were observed in 20% of patients (98). ECP therapy has also been reported to provide positive clinical outcomes for the treatment of rejection episodes associated with cardiac and lung transplantation (99). In conclusion, published data demonstrate that patients with conditions characterized by overactive inflammation and dysregulation of T cells can realize significant clinical benefit from ECP treatments. ECP seems to restore immune homeostasis without causing general immunosuppression in patients with inflammatory diseases. In fact, ECP-treated patients can respond normally to novel and recall antigenic challenges (100).

4.2 Apoptotic cell infusion and the use of ECP in type 1 diabetes

Intravenous infusion of apoptotic cells can significantly prevent type 1 diabetes in non-obese diabetic mice (101-103). More specifically, weekly delivery of ECP-treated spleen cells significantly delayed the onset of diabetes. The disease protective effects were enhanced when apoptotic cells were injected in combination with β cell antigens as demonstrated by a reduction in insulinitis and an increase in Foxp3⁺ Treg cells. Importantly, infusion of ECP-treated spleen cells did not induce global immunosuppression or exacerbate autoimmune responses in treated mice (101).



Fig. 1. THERAKOS™ XTST™ Photopheresis instrument



Fig. 2. THERAKOS™ CELLEX™ Photopheresis Instrument

In the clinical setting, positive outcomes have been reported when ECP was used to treat patients with various autoimmune diseases. For instance, ECP has positively modified the disease course in systemic sclerosis, rheumatoid arthritis, atopic dermatitis, and systemic lupus erythematosus due to its ability to modulate immune processes (104). Because T1D is an immune-mediated disease with a defined diagnosis that follows a relatively homogeneous course, Ludvigsson et al (105) conducted a double-blind, randomized, placebo-controlled, prospective study to assess the efficacy of ECP in children newly diagnosed with Type 1 diabetes. A total of 49 patients were enrolled in the study across 3 different pediatric sites in Sweden. A total of 19 kids completed treatment in the active ECP treatment group; whereas, 21 patients completed treatment in the control group. Photopheresis was delivered using the first-generation THERAKOS™ UVAR photopheresis system in combination with an oral formulation of 8-MOP. ECP or sham pheresis was delivered on 2 consecutive days with the first treatment given within 5-6 days after the initial clinical diagnosis, and treatments were repeated after 2, 4, 8 and 12 weeks so that a total of 10 treatments were delivered over a 3 month period. The patients were followed for at least 3 years. Blood glucose levels, C peptide, HbA1c values, and other blood measures were monitored throughout the treatment period as well as during follow-up. Results demonstrate that the group actively treated with ECP had higher C peptide concentrations in the urine during the follow up period compared to the control group. Regarding HbA1c values, the proportion of children with HbA1c <6% was similar in the two groups during the follow up period. Interestingly, insulin doses/kg body weight required for stable blood glucose levels were always lower in the ECP-treated group, except at month 1 when photopheresis treatments were just beginning. The control group was more seriously ill than the ECP group with more weight loss, more ketonuria, higher HbA1c values and lower pH, which provides additional support for the efficacy of ECP in Type 1 diabetes. Collectively, the results from this study show that ECP had a long-term effect on the disease processes associated with diabetes in children, and there was a low frequency of adverse events associated with ECP procedures in this study. While it cannot be argued that the effectiveness of ECP was lower compared to other immune response modifiers, the safety profile of this modality does make it an attractive candidate for treating children with T1D. Since the study was conducted, there have been advances in the formulation of 8-MOP, advances in photopheresis device technology, and advances in the understanding of treatment algorithms and of the mechanism of action of ECP. As a result, additional studies are warranted to determine the optimal utilization of this treatment modality in order to reach its full potential in the T1D clinical setting.

4.3 ECP therapy in rheumatoid arthritis

A pilot study was conducted at Yale University by Malawista et al to investigate the effect of ECP on rheumatoid arthritis (RA) (106). Seven RA patients were treated on 2 consecutive days with ECP at 4 week intervals for 6 months. Joint scores and counts improved in 4 patients after 3 to 4 months of therapy. Responses were maintained for 2 to 3 months after completion of the protocol and no serious or toxic adverse events were reported (106). In a single-blinded, controlled, parallel group multicenter phase III clinical trial, the effect of ECP on joint count, joint score, bilateral grip strength, and physician assessment were compared to treatment with gold. In all categories, the 17 patients treated with ECP showed improvement over the 11

patients treated with gold. Univariate analysis of the results suggests that ECP treated patients responded in a comparable fashion to patients treated with gold (107; 108).

4.4 ECP therapy in crohn's disease

Crohn's disease (CD) and ulcerative colitis (UC) are collectively known as inflammatory bowel disease (IBD) and are chronic inflammatory conditions affecting the gastrointestinal tract in which seemingly innocuous luminal antigens stimulate mucosal CD4⁺ T cell mediated autoimmune responses in genetically susceptible individuals (109). A delicate balance of inflammation involving pro-inflammatory cytokines (TNF- α , IL-1, IL-6, IL-8, and IL-12) and anti-inflammatory cytokines (IL-4, IL-10, IL-11, and IL-13) occurs at the intestinal mucosa under normal conditions; however, once that balance is perturbed, chronic inflammation characterized by persistent T cell activation ensues. Current treatment guidelines recommend the use of aminosalicylates, glucocorticosteroids, and immunomodulators (azathioprine, 6-mercaptopurine, cyclosporine) to treat UC and CD; however, these medical approaches have limitations in efficacy and safety (110). Emerging therapeutic interventions targeting immunopathogenic mechanisms in CD include anti-TNF biologic therapies (eg. infliximab, etanercept, and adalimumab), inhibitors of proinflammatory cytokine receptors (anti-IL-6R), and anti-leukocyte adhesion therapies (natalizumab); however, long term safety still needs to be fully evaluated (110). Because of its safety and effectiveness in diseases mediated by pathogenic T cell populations, ECP has also been used to treat Crohn's disease.

Reinisch et al (111) reported results from a prospective, open label, single center pilot study conducted in 10 patients to assess the efficacy of ECP in the treatment of steroid-dependent Crohn's disease. Response was observed in 8 of 10 patients after a median time of 10 weeks (range 8-19 weeks), and remission was seen in 4 of these patients after a median time of 20 weeks (range 19-23 weeks). Steroid maintenance doses were significantly reduced from baseline, and improvements in clinical performance and quality of life were observed in responding patients. Mean C-reactive protein (CRP) declined and intestinal permeability in patients with ileal and ileocolonic Crohn's disease decreased after ECP.

Recently, a phase II study investigating ECP in 28 patients with moderately active CD who were refractory or intolerant to immunosuppressants and/or anti-TNF agents was completed (112). ECP was performed twice weekly for 4 weeks then twice weekly every other week until week 12. Primary efficacy assessment was a decrease in Crohn's Disease Activity Index (CDAI) of > 100 from baseline or CDAI < 150 at week 12. A clinical response was obtained in 14 of 28 (50%) patients, 13 of whom responded by week 6. Remission was observed in 7 of 28 patients who also had a CDAI reduction of over 200 points. Seven of 14 patients who were intolerant or refractory to anti-TNF agents responded to ECP. ECP was well-tolerated with minor serious adverse events reported. The investigators concluded that ECP is safe and beneficial for patients with moderately active Crohn's Disease that is refractory to immunosuppression and/or anti-TNF therapies.

4.5 ECP therapy in other autoimmune conditions

In pilot studies, patients with atopic dermatitis, multiple sclerosis, pemphigus vulgaris, and systemic sclerosis have achieved promising results when treated with ECP (92); however, further investigation through large, prospective clinical studies is required in order to fully understand the utility of this complex therapeutic approach in treating autoimmune conditions.

5. Immunological consequences of apoptotic cell clearance

5.1 Apoptotic cells modulate dendritic cell function

To limit unnecessary collateral damage in healthy tissue, apoptotic cells are rapidly and efficiently removed or cleared by both professional (dendritic cells and macrophages) and non-professional (cells localized near the dying cell, eg., primary lens epithelial cells, astrocytes, microglia) phagocytes (113). As mentioned previously, apoptotic cells can potentially be immunogenic and prime immune responses. In this section, we will only focus on the role of apoptotic cells in the induction of tolerance.

Apoptotic cell clearance plays a critical role in the maintenance of central and peripheral self-tolerance because 1) removal of cell corpses prevents the release of potentially immunogenic intracellular materials from dying cells and 2) phagocytes present self-antigens derived from apoptotic cells to T cells in the draining lymph nodes, which leads to deletion or anergy of self-reactive T cells. Dendritic cells, in particular, play an important role in these ongoing self-tolerance mechanisms (114). While there are several factors involved in the induction of immunological tolerance, the generation of regulatory T cells by DCs that have ingested apoptotic cells is a critical aspect of peripheral self-tolerance (115-117).

Apoptotic cells exert immunosuppressive effects when they are engulfed by phagocytes by modulating cytokine secretion profiles, blocking maturation, and inducing Tregs (118). Voll et al (119) were the first to report that the presence of apoptotic cells during monocyte activation increases the ability of these cells to secrete anti-inflammatory cytokines (IL-10) and decreases their ability to release pro-inflammatory cytokines (TNF- α , IL-1, and IL-12). Our data demonstrate similar results can be obtained *in vitro* when human monocyte-derived dendritic cells are incubated with ECP-treated cells prior to or during activation (unpublished data). In a mouse model of allogeneic bone marrow engraftment, intravenous infusion of apoptotic cells exerted their immunoregulatory functions through TGF- β dependent Treg induction which required host macrophages (34; 41). In fact, TGF- β is mostly responsible for the immunomodulatory effects of apoptotic cells by creating a suppressive microenvironment (34; 120-122). Not only do apoptotic cells regulate TGF- β secretion but engulfment of apoptotic neutrophils has been shown to reduce the ability of DCs to secrete IL-23 (123), which is involved in the differentiation of Th17 lymphocytes and may reciprocally affect Treg cell expansion (124). In addition to modulating cytokine production, engulfment of apoptotic cells reduces the ability of DCs to stimulate effector immune responses by decreasing the levels of co-stimulatory surface molecule expression and by inhibiting maturation even after an inflammatory challenge. In humans, Lamioni et al showed that the apoptotic leukocytes are cleared by antigen-presenting cells *in vivo* leading to the differentiation of antigen-presenting cells towards a more tolerogenic phenotype (125). Collectively, evidence suggests that upon engagement and engulfment of apoptotic cells, there is an increase in anti-inflammatory cytokines, a reduction of pro-inflammatory cytokines, and a diminution in the stimulatory capacity by APCs, which results in suppression of inflammation and cell-mediated immunity, eliciting immune tolerance (89).

5.2 Apoptotic cell infusion leads to the generation of regulatory T cells

Regulatory T lymphocytes play a critical role in self-tolerance and homeostasis by actively suppressing immune responses (126-128). In the periphery, Tregs are present as either naturally occurring or induced, and the classic Treg phenotype is CD4+CD25+ cells expressing the molecular marker Foxp3, which is a member of the forkhead/winged-helix

family of transcriptional regulators (129). There is evidence in both animal models and from clinical studies that apoptotic cell infusion induces Tregs. Wang et al showed that the survival of cardiac allografts was prolonged as a result of donor apoptotic cell infusion which induced Tregs (130). In another mouse model of cardiac allograft rejection, George et al showed that compared to untreated mice, the frequency of splenic CD4+CD25+Foxp3+ cells increased 2-fold in ECP-treated animals and graft survival was prolonged (131). In a murine model of contact hypersensitivity, Maeda *et al* demonstrated that intravenous infusion of ECP-treated leukocytes induced Tregs (132). These Tregs were shown to mediate suppression through IL-10 and were characterized as expressing glucocorticoid-induced TNF family-related receptor (GITR) and the surface molecule neuropilin-1 (133). In a murine model that closely reproduces the treatment of GVHD with ECP, the infusion of ECP-treated cells significantly reduced clinical GVHD scores and pathology, diminished mortality, and increased the number of Foxp3+ Tregs in the spleen and thymus (134). Extending pre-clinical findings to humans, Lamioni *et al* observed that ECP treatments promote a significant increase in regulatory T cells in the blood of cardiac and lung transplant patients compared to untreated healthy individuals and patients who received traditional immunosuppressants (125). Treg function in both GVHD and CTCL patients was reported to be strengthened as a result of ECP treatments (135). The frequency of circulating CD4+CD25+GITR+CD62L+Foxp3+ Tregs with suppressive function increased in GVHD patients treated with ECP, suggesting an association between elevation in Tregs and response to ECP treatments (136). Lastly, a recent report from the pediatric ECP T1D trial suggested that ECP effectively inhibited autoimmune processes against islet cells by maintaining regulatory T cell activity (137). Collectively, these results suggest that infusion of apoptotic cells or ECP treatments can mediate an upregulation of Tregs via modulation of cytokines and dendritic cells, which may help explain why ECP has therapeutic effects in malignancy, alloimmune conditions, and autoimmunity; however, additional research and clinical studies are warranted in order to fully understand the full potential of apoptotic cells in the treatment of diseases.

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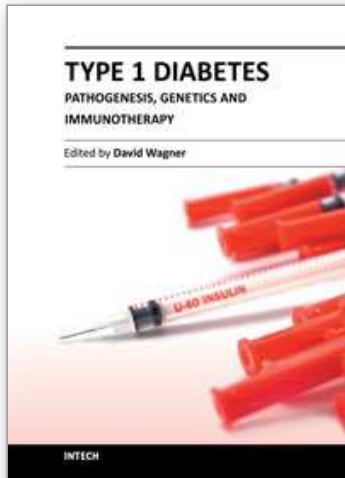
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This book is a compilation of reviews about the pathogenesis of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes. Areas including T cell development, innate immune responses, imaging of pancreata, potential viral initiators, etc. are considered.

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