Chapter from the book *Type 1 Diabetes - Pathogenesis, Genetics and Immunotherapy* 
Downloaded from: http://www.intechopen.com/books/type-1-diabetes-pathogenesis-genetics-and-immunotherapy
Innate Immunity in the Recognition of β-Cell Antigens in Type 1 Diabetes

Jixin Zhong\textsuperscript{1,2}, Jun-Fa Xu\textsuperscript{3}, Ping Yang\textsuperscript{1}, Yi Liang\textsuperscript{3} and Cong-Yi Wang\textsuperscript{1,2,3}

\textsuperscript{1}The Center for Biomedical Research, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, \textsuperscript{2}The Center for Biotechnology and Genomic Medicine, Medical College of Georgia, Augusta, GA, \textsuperscript{3}The Department of Clinical Immunology, Guangdong Medical College, Dongguan \textsuperscript{1,3}China \textsuperscript{2}USA

1. Introduction

Diabetes is a chronic disease caused by the inability to produce enough insulin by the pancreatic β cells or inappropriately use insulin by the peripheral tissues, and therefore, patients with diabetes are unable to control blood glucose to a normal level. Along with the industrialization and economic development, diabetes has gradually become a global health challenge as manifested by that it affects 5-10\% of the world population (Home, 2003). For example, in United States alone, approximately 21 million children and adults (around 7\% of the total population) have diabetes. Despite the significant advances in the development of therapeutic approaches for this devastating disease, the long-term outcome of diabetes, however, remains unsatisfied, as many complications could occur during the process of diabetes. Transient improper control of blood glucose level will result in the dangerous short-term complications such as diabetic ketoacidosis, nonketotic hyperosmolar coma, and hypoglycemia., while the life threading condition is the development of various long-term complications such as cardiovascular disease, nerve damage, chronic renal failure, retinal damage, and poor wound healing (Zhong et al., 2011). Given the fact that the administrated exogenous insulin cannot regulate glucose levels as accurately as the endogenous insulin released by the functioning pancreatic islets, diabetic patients are highly prone to the development of those complications. For example, patients with diabetes are 17 times more prone to kidney diseases (World Health Organization (WHO), 1994; Home, 2003), and diabetes also has become the most common cause of blindness in developed countries as manifested by that nearly half of the diabetic patients developed retinopathy (Amos et al., 1997).

There are two types of diabetes, type 1 and type 2. Type 1 diabetes (T1D), also called Insulin Dependent Diabetes Mellitus (IDDM) or juvenile diabetes, which is characterized by the selective destruction of the insulin-secreting pancreatic β cells by the autoreactive immune cells. Therefore, T1D is characterized by the absolute deficiency of insulin, and patients require injection of exogenous insulin for survival, which renders the blood glucose unable
to be regulated at a perfect level, leading to the persistent epigenetic changes which predispose to the change of gene expression and serve as risk factors for diabetic complications (El-Osta et al., 2008). Therefore, diverse complications are easily developed in patients with T1D. In contrast, **type 2 diabetes** is relevant to insulin resistance, usually caused by obesity. Insulin secretion in patients with type 2 diabetes could be normal, inadequate, or higher, but the peripheral tissues such as liver, muscle, and fat, have a low response to insulin.

It is believed that both genetic and environmental factors are implicated in the susceptibility of T1D. As one of the polygenic diseases, vulnerability to T1D involves more than 20 genetic intervals, among which loci within the HLA account for the most of genetic susceptibility (Bach et al., 2001; Onengut-Gumuscu & Concannon, 2002; Pociot & McDermott, 2002). In addition to genetic factors, a variety of environmental factors can affect T1D susceptibility. A majority of T1D are believed to be triggered by infections such as viral infection. Although less commonly, other environmental factors such as stress and certain chemical or drug exposure also appeared to be triggers for T1D.

Despite of extensive studies, the underlying mechanism of T1D is still not fully elucidated. Decades of clinical and experimental studies indicate that adaptive immune responses play a central role in the pathogenesis of T1D in both humans and NOD mice (Roep, 2003). As the major effector cells for β-cell destruction, T cells and T-cell-mediated adaptive immunity are considered to be the major factor for T1D. Autoreactive CD8+ T cells are confirmed to be critical for T1D pathogenesis in both patients and experimental animal models (Bottazzo et al., 1985; Conrad et al., 1994). Other than CD8+ T cells, self-antigen specific CD4+ T cells can also promote the production of autoantibodies against β cells by B lymphocytes. Therefore, most T1D related studies have been focused on adaptive immunity, while the role of innate immunity is overshadowed. Recently, accumulating evidence indicates that innate immunity also plays an essential role in the initiation and progression of T1D. For example, in addition to T cells, innate immune cells such as dendritic cells (DCs), macrophages, and natural killer (NK) cells are highly enriched in the insulitis lesion during diabetogenic process (Pietropaolo et al., 2007; Katz et al., 1995a; Rosmalen et al., 2000). Depletion of DCs and macrophages prevents the infiltration of lymphocytes into the pancreatic islets, and deletion of macrophages by silica almost completely prevents the development of diabetes and insulitis in diabetes-prone NOD mice and BB (BioBreeding) rats (Jun et al., 1999c; Lee et al., 1988d; Lee et al., 1988a; Oschilewski et al., 1985a). Furthermore, even temporary deletion of DCs and macrophages by clodronate-loaded liposomes for one week can tremendously postpone the onset of diabetes (Nikolic et al., 2005). Studies from our and other groups further provided strong evidence showing that diverse innate molecules such as high mobility group protein B1 (HMGB1) and heat shock proteins (HSPs) or innate receptors (e.g., Toll-like receptors and RAGE) are involved in T1D pathogenesis. Therefore, innate immunity has a key effect on the etiology of T1D.

### 2. Innate immunity and adaptive immunity

Given the fact that T1D is an autoimmune disorder, it would be logic to first introduce the immune system and its relevant defensive mechanisms, the innate and adaptive immune response. The **immune system** is defined as the collection of organs, tissues, cells, and molecules that protects host from various environmental threats such as tumor cells,
pathogens, toxins, and other foreign molecules. It includes thymus, spleen, lymph nodes, bone marrow, tonsils, and various cells and molecules such as white blood cells, antibodies, and cytokines. The **immune response** is the defensive mechanism of immune system to protect body against those invasions, which is built on two separate foundation pillars: the innate and adaptive immune response. The **adaptive immune response** is an antigen-specific process by which the immune system discriminates non-self antigens. As suggested by its name, adaptive immune response is highly adaptable, antigen-specific, but its response is relatively slow. It recognizes an unlimited number of antigens by antigen receptors or immunoglobulins which went through somatic hypermutation to acquire their high diversity. In contrast, the **innate immune response** is the primary defensive mechanism against environmental threats in a non-specific manner, which is evolutionally older than its counterpart, the adaptive immune system. The innate immune system can be found in all classes of plants and animals and is the dominant immune system in insects, plants, fungi, and multicellular organisms. It distinguishes invading molecules from host component by recognizing conserved constituents of foreign molecules. The differences and similarities between innate and adaptive immune response are described in Table 1.

<table>
<thead>
<tr>
<th>Properties</th>
<th>innate immunity</th>
<th>adaptive immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>non-specific</td>
<td>antigen specific</td>
</tr>
<tr>
<td>Action time</td>
<td>quick</td>
<td>slow, 2-6 days later than innate immune response</td>
</tr>
<tr>
<td>Persistence</td>
<td>short</td>
<td>long</td>
</tr>
<tr>
<td>Memory</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Antigens</td>
<td>conserved microbe-specific molecules, such as LPS, glycans, microbial DNA</td>
<td>divers proteins, peptides, and carbohydrates</td>
</tr>
<tr>
<td>Receptors</td>
<td>germ-line encoded</td>
<td>encoded in gene segments, its diversity relies on rearrangement</td>
</tr>
</tbody>
</table>

Table 1. Comparison of innate and adaptive immunity

The major function for the immune system is to defend against environmental threats and protect the body against disease by distinguishing and eliminating foreign or dangerous substance including pathogens, tumor cells and even transplanted organs. When encountering antigens derived from the host, the immune system in a normal individual is able to recognize them as self and decide not to respond. Dysfunction of the immune system may cause various immune disorders such as immunodeficiency and autoimmune diseases. Immunodeficiency is manifested by unable to respond to foreign or harmful antigens which results in both opportunistic and normal infections. In contrast, an autoimmune disease is caused by interpreting self-antigens as foreign or harmful antigens. In this case, the immune system in the patients cannot tell the difference between body’s own tissue and foreign antigens, resulting in an immune response that destroys their own tissues and cells. There are over 80 different types of autoimmune disorders including type 1 diabetes, systemic lupus erythematosus, multiple sclerosis, Grave’s disease, rheumatoid arthritis and so on. Autoimmune responses can be initiated by the following conditions: (1) the release of an
antigen that is usually expressed in a specific area and is not exposed to the immune system. For example, the fluid in the eyeball contains some antigens that are hidden from the immune system. Once the fluid is released into the bloodstream by injury, the immune system will recognize them as foreign antigens and react against them; (2) An antigen is altered. For example, antigens within the body can be altered by infections, drugs, and radiations. The altered antigens are then recognized by the immune system to initiate an autoimmune response; (3) Exposure of a foreign antigen with a similar conformation to the body’s natural antigen may trigger an autoimmune response against the body’s antigen as well as the foreign antigen; (4) Malfunction of the immune cells. For example, cancerous B lymphocytes may produce abnormal antibodies that attack body’s own antigens (Breecher & Dworken, 1986).

Since T1D is caused by the autoimmune responses that progressively destroy the insulin producing β cells, the role of adaptive immune response has long been proposed in T1D pathogenesis. The idea that T1D is an autoimmune disease first came from the observation that it usually occurred in association with other classic autoimmune diseases such as Grave’s disease, hypothyroidism, Addison’s disease and pernicious anemia (Eisenbarth, 1984), and histological examination showed a large amount of T cells in the insulitis lesion (Gepts, 1965). Further studies confirmed that insulitis happens only in the islets containing β cells, indicating that the autoimmune reaction in T1D is driven by β-cell-derived antigens (Roep, 2003). Autoreactive T-cell is believed to be the major mediator of β-cell destruction in both primary T1D and recurrent β-cell loss after islet transplantation (Pinkse et al., 2005). Circulating autoreactive T cells against different β-cell-derived antigens were detected in newly-onset diabetic patients (Velthuis et al., 2010), suggesting its role in T1D development. Treatment of monoclonal CD3 antibody has been shown to be able to protect T1D patients from autoimmune mediated β-cell destruction and preserve insulin production (Herold et al., 2002; Herold et al., 2009; Killestein, 2002). In addition, circulating autoantibodies against β cells are produced by B lymphocytes, another important component of the adaptive immune system, have also been detected in T1D patient. Nevertheless, the production of autoantibodies seems to be a consequence of β-cell destruction (Baekkeskov et al., 1982; Rodacki et al., 2006). Due to the fact that T-cell is the major effector cell in mediating β-cell destruction, adaptive immune response in T1D has been extensively studied and its role in T1D pathogenesis has been well established. However, accumulating evidence suggests that innate immune response is also essential to the pathogenesis of T1D.

3. Immune recognition of antigens

Antigens can come from both environment and body’s own tissues. However, the immune system reacts only to foreign or harmful substances under physiologic conditions. This is because each individual has its own identification molecules expressed on the surface of all cells, and the immune system is able to identify them during the recognition process. Major histocompatibility complex (MHC) is the most important identification molecule. MHC molecule in humans is also called human leukocyte antigens (HLA), while MHC in mice is termed histocompatibility-2 (H-2) (Kumanovics et al., 2003). In an effort to be identified in a large population, almost every individual has a unique set of MHC molecules different from others. Therefore, MHC molecules have an extremely large population diversity (Borghans et al., 2004). The diversity of MHC molecules comes from: (1) the polygenic of MHC locus; (2) the high polymorphic MHC locus, each MHC locus has many, even hundreds of
different alleles; (3) the co-dominantly expression of MHC. Thus, the combination of MHC molecules in each individual is almost unique. After positive and negative selection, T cells are tolerant to cells with self MHC molecules and potent to attack cells possessing different MHC molecules (such as foreign cells and mutated cells).

Fig. 1. **Molecular Structure of MHC Class I**: MHC class I protein is composed of two chains: α chain and β2 microglobulin. The α chain consists of a transmembrane region and three extracellular domains: α1, α2, and α3. MHC class I molecule is expressed on the membrane of all nucleated cells.

MHC regions are divided into three classes: class I, class II, and class III (Newton et al., 2004). MHC class I encodes heterodimeric peptide-binding proteins (the classical MHC class I molecules) and antigen-processing molecules (the non-classical MHC class I molecules). MHC class I protein has an immunoglobulin-like structure containing an α chain and a β2 microglobulin. The α chain consists of a transmembrane region and three extracellular domains (α1, α2, and α3) (Figure 1). MHC class I molecule is expressed in all nucleated cells. It presents cytosolic peptide (including self peptides and viral peptides synthesized by own cells) that is anchored to a cleft formed by α1 and α2 to TCR on CD8+ cytotoxic T cells. MHC class II is responsible for encoding peptide-binding proteins (the classical MHC class II molecules) as well as molecules modulating antigen loading (the non-classical MHC class II molecules). The MHC class II molecule also has an immunoglobulin-like structure and consists of two chains, one α chain and one β chain (Figure 2). Each chain contains a transmembrane region and two extracellular domains (α1 and α2 in α chain, β1 and β2 in β chain). MHC class II protein is expressed on the membrane of APCs and is responsible for presenting extracellular antigens.

Fig. 2. **Molecular Structure of MHC Class II**: MHC class II molecule has an immunoglobulin-like structure. It consists of one α chain and one β chain. Each chain contains a transmembrane region and two extracellular domains (α1 and α2 in α chain, β1 and β2 in β chain). MHC class II protein is expressed on the membrane of APCs and is responsible for presenting extracellular antigens.
The MHC class II protein is expressed on the membrane of antigen presenting cells (APCs). It loads processed extracellular peptide (e.g., peptides originating from microbes ingested in vesicles) on a cleft formed by α1 and β1 domain, and presents it to TCR on CD4+ helper T cells. MHC class III is responsible for encoding several secreted proteins such as complement components (C2, C4, and B factor), cytokines (TNFα, LTA, and LTB), and heat shock proteins. The function of MHC class III is different from class I and II, but MHC class III is located between them, so they are usually described together. In humans, the most intensely studied MHC class I genes are HLA-A, HLA-B, and HLA-C, while the most studied MHC class II genes are HLA-DP, HLA-DQ, and HLA-DR (Kindt et al., 2006).

The recognition of adaptive immune system is based on the interaction of T cell receptor (TCR) and peptide-MHC (p-MHC) complex (Figure 3). TCR on T-cell membrane can only detect antigens presented on the surface of MHC molecules. The property of this recognition is called MHC restriction. TCR recognizes the residues on the peptide and residues from the MHC.

Fig. 3. Recognition of p-MHC by TCR: T cell cannot detect an antigen by itself. It can only recognize antigens presented by MHC on APC. APC uptakes foreign antigens and digests them into small peptides which are loaded onto the cleft of MHC molecules and then presented to TCR. Once they bind to p-MHC, TCR signal is transduced into T-cell via intracellular domain of associated CD3. T-cell is then activated and reacts to the invaders with corresponding antigen to clear them.
MHC molecules at the same time. TCR itself, however, lacks intracellular domain to transduce signal into T cells. On T-cell membrane, TCR forms a TCR/CD3 complex together with CD3. When binding to p-MHC, TCR signal is transduced into T cells via the intracellular domain of CD3 (Kuhns et al., 2006).

TCR consists of two chains, the α and β chain. Each chain is composed of two regions, the N-terminal variable (V) region and the C-terminal constant (C) region (Kuhns et al., 2006; Deng & Mariuzza, 2007). TCR is encoded by several segments, V, D, J, and C. The C region is encoded by C segment, while the V region is determined by V, D, and J segment. The V, D, and J segment have numerous copies. Despite of sharing the same genome, different T-cell clones have different TCR. The diversity of TCR originated from V region especially the complementary-determining regions (CDRs). Each TCR chain has three CDRs (CDR1, CDR2, and CDR3). CDR1 and CDR2 are encoded by V segment of the TCR gene, while CDR3 is generated from the V(D)J recombination (Vα and Jα segment recombination for α chain; and Vβ, D, and Jβ segment recombination for β chain) (Deng & Mariuzza, 2007; cha-Orbea et al., 1989). The diversity of TCR is believed to be generated by the following mechanisms: (1) the combination of α chain and β chain (each chain has two copies originated from father and mother, respectively); (2) V(D)J recombination (each TCR chain contains multiple gene segments, V, D, J segments, which need to be re-arranged by somatic gene re-arrangement during the development); (3) junctional diversity (additional bases will be inserted between segments during the V(D)J recombination, which results in the additional diversity of complementary-determining regions).

Normally, T cells do not provoke an immune response against self antigens, because autoreactive T cells are removed during the development of lymphocytes in the thymus (Deng & Mariuzza, 2007). Developing T cells are subjected to positive selection and negative selection in the thymus prior their presence in the periphery. Positive selection occurs in the cortex of thymus. The developing T cells that are unable to bind to MHC molecules on the thymic epithelial cells undergo programmed cell death (apoptosis), and as a result, only those cells with a high affinity to self MHC molecules on the thymic epithelial cells can survive. Those survived cells are next subjected to negative selection to get rid of autoreactive T cells. The medulary thymic epithelial cells and dendritic cells in the thymus display self antigens on MHC molecules. T cells with a high affinity to self-peptide-MHC complex, which are also called autoreactive T cells, undergo apoptosis and thereby to be removed from the T cell repertoire. During this process, T cells develop tolerance to those antigens present on the thymic DCs and medulary thymic epithelial cells. The ectopic expression of organ-specific antigens such as insulin in those cells is regulated by a transcription factor called AIRE (the Autoimmune Regulator) (Kindt et al., 2006). Therefore, loss of AIRE function impairs ectopic expression of organ-specific autoantigens such as insulin and thereby interferes T-cell negative selection, predisposing to the development of type 1 diabetes (Anderson et al., 2002; Pugliese, 2005).

Other than the involvement in T-cell recognition and T-cell negative selection, components of the innate immune system are also directly implicated in the recognition of pathogenic antigens, which is called pattern recognition. As the first line of host defense, the innate immune system is the first component to take an action on invading microbes. Innate immune recognition occurs in advance to adaptive immune recognition and determines the responsive consequence to the antigens. Host immune system including innate and adaptive immune system relies on the innate recognition to make the decision to respond or not to respond to a particular antigen.
4. Pattern recognition

Innate immune recognition is also known as pattern recognition. It refers to the detection of common molecular structural motifs or pattern unique to microorganisms or other innate danger signals by the binding of pattern recognition receptors (PRRs) to their ligands (Zhong et al., 2011). Unlike the adaptive immune system which has diverse antigen receptors to identify a large number of foreign antigens, the innate immune system recognizes conserved microbe-derived molecules using a limited number of germline-encoded receptors – PRRs. Due to their limited number, every pattern recognition receptor can identify a large amount of pathogen specific molecules which share a certain structural motif. According to the originality, the ligands of PRR are divided into 2 classes: pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). PAMPs are conserved components among microorganisms that can be discriminated from host molecules such as flagellin, lipoteichoic acid from Gram positive bacteria, LPS from Gram negative bacterial, and peptidoglycan and dsRNA from virus. They are unique to microorganisms and important for the survival and/or expansion of microorganisms. Following the recognition, an immune reaction against the PAMPs is initiated to eliminate the invaded pathogens. DAMPs are small intracellular molecules released by cells during injury and served as a danger signal to initiate tissue repair. Using PRRs, the innate immune system can sense DAMPs and sequentially initiate noninfectious inflammatory responses. DAMPs are usually small molecules belonging to nuclear or cytosolic proteins. For example, HMGB1, ATP, HSP, and DNA are examples of DAMPs that can be released into the extracellular matrix in the damaged tissues. They are then recognized by PRRs to induce noninfectious inflammations to clear cellular debris, limit tissue injury, and promote tissue repair.

In contrast to adaptive immune recognition that is accomplished by the members of a single family – the Ig super-family, innate immune recognition is mediated by several protein families including C-type lectins, leucine-rich proteins, scavenger receptors, pentraxins, lipid transferases, and integrins (Medzhitov & Janeway, Jr., 1997). According to their function, localization, ligand specificity, and evolutionary relationships, PRRs can be classified into two groups: signaling PRRs and endocytic PRRs. Upon binding to the microbial molecules, signaling PRRs transmit a signal into the host innate immune cells and induce the synthesis of regulatory molecules that are crucial to initiate inflammatory and immune response, such as cytokines and costimulatory molecules. They include membrane-bound Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors (NLRs). Endocytic PRRs are usually expressed on the cell surface of phagocytes and promote the attachment, engulfment and destruction of microbes by phagocytes, without inducing intracellular signals. They include mannose receptor (MMR), macrophage scavenger receptor (MSR), and opsonin receptors. Among those PRRs, TLRs are the best characterized PRRs. They play a major role in innate immune recognition and contribute to the initiation of inflammatory and immune responses.

TLRs belong to the evolutionarily conserved type I transmembrane proteins. There are a handful of members have been discovered so far, with 10 TLRs in humans (TLR1-10) and 12 TLRs in mice (TLR1-9, 11-13) (Beutler, 2004). The ligands for TLRs include various components of microbes that share the same motif. For example, TLR1/2/6, 4, 5, 9, and 11 can sense lipoprotein, LPS, flagellin, bacterial CpG DNA, and UPEC protein from bacteria; TLR3, 4, 7/8, 9 recognize dsDNA, RSV F protein, ssRNA, and viral CpG DNA from viruses;
whereas glycolipids, GIPLs, zymosan, and profilin-like protein derived from protozoa and fungi can be identified by TLR2/6 and TLR11 (West et al., 2006). Other than those PAMPs, certain DAMPs can also be recognized by TLRs (Johnson et al., 2003; Ohashi et al., 2000). For example, intracellular components as HMGB1 and HSP60 that are passively released from damaged cells can also bind to TLRs and induce TLR signaling (Ohashi et al., 2000; Erridge, 2010); oxidized beta2-GPI acts as another endogenous ligand of TLR to induce NF-κB activation and DC maturation (Buttari et al., 2005). TLRs consist of three domains: an N-terminal leucine-rich-repeat (LRR) domain, a single transmembrane domain, and a C-terminal intracellular TIR (Toll/IL-1 receptor) domain (Zhong et al., 2011; Takeda et al., 2003). The LRR domain is responsible for ligand binding, whereas the TIR domain is responsible for signaling. Upon the binding of PAMPs or DAMPs to LRR domain, TLRs initiate a cascade of signaling via TIR domain. By using a point mutation in TIR domain of TLR4 (P712H mutation), Poltorak and coworkers confirmed that TIR domain recruits downstream effectors and transduces intracellular signal for TLR (Poltorak et al., 1998). Binding of TLRs to their ligands induces diverse antimicrobial genes, proinflammatory cytokines, and chemokines. TLR signaling also can increase the expression of costimulatory molecules and promote antigen-presenting capability for APCs. Thus, innate recognition by TLR activates APCs to trigger inflammatory responses and initiates adaptive immune responses (Medzhitov & Janeway, Jr., 1997). TLR signaling is mediated via two types of pathways, the myeloid differentiation primary-response gene 88 (MyD88)-dependent and -independent pathway. Almost all TLRs except for TLR3 transmit intracellular signaling via MyD88-dependent pathway. Furthermore, MyD88-dependent pathway is the only pathway for TLR2, 5, 7/8, 9, and 11 (Zhong et al., 2011). Therefore, ligands for those TLRs such as peptidoglycans, flagellin, CpG DNA, ssRNA, and toxoplasma profilin-like protein cannot be sensed by MyD88 deficient cells (Takeda et al., 2003; Beutler et al., 2005; Adachi et al., 1998; Yarovinsky et al., 2005). Upon activation, MyD88 sequentially recruits IL-1 receptor-associated kinase-4 (IRAK-4) and IL-1 receptor-associated kinase-1 (IRAK-1) (Lin et al., 2010). Tumor necrosis factor receptor-associated factor-6 (TRAF-6) is subsequently recruited to MyD88/IRAK-4/IRAK-1 and then disassociate from the complex together with IRAK-1. TRAF-6 next sequentially activates c-Jun N-terminal kinase (JNK) and inhibitor of κB kinase (IKK), which in turn activates activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) to initiate the transcription of diverse pro-inflammatory cytokine and chemokine genes (Beutler et al., 2006) (Figure 4). However, MyD88 cannot explain all downstream effect initiated by some TLRs as manifested by that TLR3 and TLR4 signaling are not completely blocked by the deficiency of MyD88, indicating that there must be MyD88-independent pathways for TLR signaling (Covert et al., 2005; Kawai et al., 1999). Studies have now indicated that TLR3 and TLR4 can signal through IRF3 and finally activate NF-κB in MyD88 deficient cells. In MyD88-independent pathway, TLR signal is mediated by TRIF (TIR domain-containing adaptor inducing IFN-β) and TRAM (TRIF-related adaptor molecule), and finally results in the activation of NF-κB, AP-1, or IRFs (Akira et al., 2001; Hoebe et al., 2003; Yamamoto et al., 2003a; Yamamoto et al., 2002; Yamamoto et al., 2003b; Fitzgerald et al., 2003). Upon activation, NF-κB, AP-1, or IRFs subsequently induces a series of events such as promoting proinflammatory cytokine and chemokine production, recruiting leucocytes, activating APCs, and initiating an adaptive immune response (Medzhitov, 2001; Takeda et al., 2003; Kawai & Akira, 2006; Akira, 2003).
Fig. 4. **Signal Pathway of TLR**: TLRs transduce signal through MyD88-dependent and -independent pathway. Activation of MyD88 recruits IRAK-4, IRAK-1, and TRAF-6, and then sequentially activates JNK, IKK, AP-1, and NF-κB. MyD88-independent pathway is mediated by TRIF and TRAM, which finally leads to activation of NF-κB, AP-1, or IRFs.

NLR is another type of innate receptors. NLR proteins are a group of NOD domain containing intracellular receptors which detect the presence of PAMPs or DAMPs in the cytosol. NLRs are composed of an N-terminal protein interaction domain, a central nucleotide-binding oligomerization domain (NOD) and C-terminal leucine-rich repeat (LRR) domain (Chen et al., 2009). The N-terminal domain of the NLRs is critical for downstream signaling, and NOD domain mediates self-oligomerization that occurs during activation, whereas LRR domain is responsible for detecting PAMPs or DAMPs. Based on their N-terminal structure, NLRs are categorized into 5 subfamilies: NLRA which contains an acidic transactivation domain; NLRB which is characterized by the presence of a baculovirus inhibitor of apoptosis protein repeat (BIR); NLRC which possesses a caspase recruitment domain (CARD), NLRP which is manifested by a Pyrin domain; and NLRX whose N-terminal domain is unknown (Kawai & Akira, 2009). NLRs are expressed in epithelial, mesothelial, and immune cells including both APCs and lymphocytes. NOD1 and NOD2, members of NLRC, are the two well-characterized NLR proteins. They recognize peptidoglycan structure of pathogens. Recognition by NLRs induces self-oligomerization of NLRs and activates NF-κB and MAPK (Park et al., 2007; Hasegawa et al., 2008; Hitotsumatsu et al., 2008), which subsequently results in the release of IL-1 family of cytokines including IL-1β, IL-18, and IL-33 (Meylan et al., 2006; Fritz et al., 2006; Ting et al., 2006; Kanneganti et al., 2007; Yu & Finlay, 2008). There is feasible evidence supporting that NLRs play a role in the pathogenesis of autoimmune diseases. For example, NOD1 genetic variants modulate the host response to environmental bacteria and thus are associated with the development of allergic diseases such as asthma (Weidinger et al., 2005; Hysi et al., 2005; Eder et al., 2006). In addition, the polymorphisms of NLRs are also demonstrated to be associated with Crohn disease (Hugot et al., 2001). However, the role of NLRs in T1D pathogenesis is yet to be clarified (Eizirik et al., 2009).
In addition to TLRs and NLRs, other well-studied PRRs include Macrophage mannose receptor (MMR), macrophage scavenger receptor (MSR), and receptor for advanced glycation endproducts (RAGE) (Janeway, Jr. & Medzhitov, 2002; Kumagai et al., 2008; Li et al., 1996; Medzhitov & Janeway, Jr., 2000; Medzhitov, 2001; Medzhitov, 2007; Pearson, 1996). MMR is an important phagocytic receptor expressed on macrophages (Janeway, Jr. & Medzhitov, 2002). It is a member of the C-type lectin family and functions as a PPR to mediate phagocytosis of a variety of gram-positive, gram-negative bacteria, and fungal pathogens (Fraser et al., 1998). The microbial pathogens are then delivered into lysosomal compartment to be destroyed by lysosomal enzymes.

MSR is a member of scavenger receptor type A family. It also serves as a phagocytic pattern recognition receptor on macrophages. Blockade or genetic deletion of MSR on macrophages impairs the recognition of apoptotic cells by macrophages (Platt et al., 1996). The ligands for MSR include LPS, dsRNA, and lipoteichoic acid (LTA) (Pearson, 1996). Due to the defective LPS scavenging, loss of MSR increases the susceptibility to infection of various pathogens including Listeria monocytogenes, herpes simplex virus, and malaria (Thomas et al., 2000; Suzuki et al., 1997).

RAGE belongs to the immunoglobulin super-family. It is a 35kD multiligand transmembrane receptor (Neeper et al., 1992; Xie et al., 2008). Its major ligands are advanced glycation end products (AGE) and HMGB1. RAGE is composed of five domains: one cytosolic domain, one transmembrane domain, one variable domain, and two constant domains. The cytosolic domain is responsible for the signal transduction, and the variable domain is responsible for the binding of its ligand (Xie et al., 2008). RAGE signal activates NF-κB which sequentially induces the transcription of many pro-inflammatory genes. RAGE can also enhance the adhesion of leukocyte to endothelial cells and thus promote the recruitment of inflammatory cells (Chavakis et al., 2003).

5. Innate immune cells in the autoimmune response against β cells

Recent findings suggest that innate immune cells play an essential role in the initiation of β-cell-specific autoimmune response. Innate immune cells such as DCs, macrophages, and NK cells are found in a large number in the autoimmune diabetic pancreas in addition to lymphocytes. Furthermore, DCs and macrophages are the major population of infiltrating immune cells during the initial phase of autoimmune insulitis (Pietropaolo et al., 2007; Rosmalen et al., 2000; Charre et al., 2002; Delovitch & Singh, 1997; Jansen et al., 1994; Katz et al., 1995b; Lee et al., 1988c; Voorbij et al., 1989). Their presence at the pancreas precedes the infiltration of T and B lymphocytes. In addition to the early stage of autoimmunity, accumulation of innate immune cells is also observed during the later β-cell destructive insulitis process (Jansen et al., 1994). The density of DCs is much higher in diabetes-prone NOD mice immediately after birth than that of control non-diabetic strains. Therefore, the entry of macrophages and DCs is considered as the initial sign of autoimmunity in T1D pathogenesis (Medzhitov, 2001; Pearson, 1996; Takeda et al., 2003).

A wave of physiological β-cell death was found in newborns of both mice and humans, cumulating at early infancy (Finegood et al., 1995; Trudeau et al., 2000; Kassem et al., 2000). This physiological β-cell apoptosis, which is peaking at 14-17 days after birth in NOD mice, is proposed to be an initial stimulus for triggering islet-specific autoimmune response (Turley et al., 2003). Scavenger cells are believed to be responsible for the clearance of these apoptotic β cells. It was found that scavenger cells in the pancreas of NOD mice are...
abnormally higher than that of control strains and they persist in the NOD pancreas (Charre et al., 2002). This accumulation of scavenger cells is believed to be a result from the defective clearance of apoptotic β cells (Haskins et al., 2003; Mathis et al., 2001; Akirav et al., 2008).

It is proposed that the defective clearance of apoptotic β cells results in apoptotic β-cell accumulation which then leads to the secondary necrosis (Erridge, 2010). During the secondary necrosis process, β-cell-derived antigens could be released and then taken up and processed by APCs in the islets. After uptake of β-cell-derived antigens, APCs become matured and migrate into the immediate draining lymph nodes (pancreatic lymph nodes, PLNs). In the PLNs, naïve β-cell-reactive T and B lymphocytes are primed and activated by APCs carrying the β-cell-derived antigens. Upon activation, T and B cells then migrate into the islets to attack the insulin producing β cells (Jansen et al., 1994). Depletion of macrophages by liposomal dichloromethylene diphosphonate, a substance known to be toxic to macrophages, in NOD mice results in the inability of T cells to differentiate into β-cell-cytotoxic T cells. During the initiation of β-cell-specific autoimmune response, APCs present β-cell-derived antigens to T cells and provide IL-12 to promote Th1 responses. T cells in a macrophages-depleted environment differentiate toward Th2 cells rather than Th1 cells. Of note, substantial administration of IL-12 restores the susceptibility to diabetes in macrophages-depleted NOD mice (Jun et al., 1999b). Consistently, many other groups also confirmed that depletion of macrophages dramatically prevents diabetes and insulitis in CY (cyclophosphamide)-treated NOD mice or BB rats (Lee et al., 1988e; Lee et al., 1988b; Oschilewski et al., 1985b).

In addition to activating lymphocytes, macrophages are also directly implicated in the final stage of autoimmune-mediated β-cell destruction. Adoptive transfer of monocyte-depleted diabetogenic T cells failed to induce diabetes. Moreover, activated macrophages can directly kill β cells in vitro (Calderon et al., 2006; Jun et al., 1999a). By using a transgenic model, Martin et al. provided direct evidence suggesting that macrophages are able to directly destroy β cells without T and B lymphocytes (Martin et al., 2008). Chemokines are a group of proteins produced in response to cell/tissue damage or inflammatory stimuli to attract immune cells. They are subdivided into 4 subgroups: C, CC, CXC, and CX3C family. Transgenic expression of CCL2 (also known as MCP-1), a chemokine belongs to the CC family, under the control of insulin promoter recruits circulating monocytes into the pancreas. When the RIP-CCL2 transgene was bred into the Rag-1–/– mice in which they are deficient for mature T and B lymphocytes, these mice developed insulitis and diabetes spontaneously, and showed a similar time course with immunocomponent RIP-CCL2 transgenic Rag-1+/+ mice (Martin et al., 2008). This work demonstrates that macrophages are able to directly destroy β cells and play a more important role than what we thought. In addition, pro-inflammatory cytokines (e.g., IL-1β, TNFα, IL-6, and IL-12), as well as nitrogen and oxygen free radicals can be secreted into the pancreatic islets by activated APCs to directly destroy β cells (Beyan et al., 2003). Pro-inflammatory cytokines including TNFα and IL-1β produced by APCs and IFNγ produced by T cells can induce β cells to produce oxygen free radicals, nitric oxide, and peroxynitrite, which are highly cytotoxic to β cells themselves (Rabinovitch & Suarez-Pinzon, 1998). IL-12 secreted by macrophages in the pancreas is critical for T cells to differentiate into β-cell-cytotoxic T cells. Thus, macrophages can also promote T1D development by secreting IL-12 to enhance the differentiation of β-cell-targeting cytotoxic T cells. Deletion of macrophages decreases Th1 and Tc1 responses and thus prevents diabetes in NOD mice. In line with this observation, T cells can regain β-
cell-cytotoxic potential with the return of macrophages or macrophage-derived IL-12 (Lee et al., 1988e; Jun et al., 1999b). Free radicals released by immune cells or β cells (induced by pro-inflammatory cytokines) can cause lipid peroxidation of membranes, DNA fragmentation, protein cross-linking, and thus, directly destroy β cells (Gewirtz, 1999; Dean et al., 1986).

NK cells are another type of innate immune cells present in the lesion of insulitis. In a virus-induced diabetes model, Foldstrom and coworkers found that NK cells are critical for virus-induced autoimmune destruction of β cells (Flodstrom et al., 2002). Coxsackievirus B4 (CVB4) infection can induce autoimmune diabetes on SOSC-1-transgenic NOD mice. Depletion of NK cells can prevent β-cell loss on CVB4-infected SOSC-1-transgenic NOD mice and thereby blocks the development of diabetes (Flodstrom et al., 2002). Due to the ability to directly kill target cells and interact with APCs and T cells, the potential involvement of NK cells in T1D has been suggested in early 1980s. Splenic NK cells were shown to be able to destroy islet cells in both diabetes-prone BB rats and NOD mice (Flodstrom et al., 2002; MacKay et al., 1986; Koeveray, 1988; Nakamura et al., 1990). Deletion of NK cells prevent T1D development in mice induced with virus, streptozotocin and cyclophosphamide (Flodstrom et al., 2002; Maruyama et al., 1991a; Maruyama et al., 1991b). Nevertheless, the role of NK cells in T1D seems only to modulate the intensity or aggressiveness of autoimmune destruction, as NK deletion failed to prevent spontaneous T1D in NOD mice or BB rats (Edouard et al., 1993; Ellerman et al., 1993; Sobel et al., 1995).

6. Innate molecules in the recognition of β-cell antigen

Given the involvement of innate immunity in T1D pathogenesis, innate recognition by PRRs, the first event of innate immune response, is suggested to be involved in triggering autoimmune reaction against β cells. In patients with T1D, TLR-2 and TLR-4 and their downstream molecules including MyD88, TRIF, and NFκB in monocytes are significantly upregulated, demonstrating that TLRs and their downstream signaling contribute to the development of T1D (Devaraj et al., 2008).

As mentioned earlier, there is a wave of physiological β-cell death after birth. Defective clearance of apoptotic β cells during β mass turnover has been suggested to be associated with the initiation of autoimmune response. The accumulated apoptotic β cells due to defective clearance undergo a secondary necrosis, which results in the passive release of innate inflammatory molecules to trigger an autoimmune response (Erridge, 2010). On the other hand, auto-antigens can be released from those necrotic β cells and then uptaken by APCs resided in the pancreas. It is believed that PRR signaling can promote uptake of auto-antigens by APCs (West et al., 2006; Doyle et al., 2004; Blander & Medzhitov, 2004). In support of this notion, stimulation of TLRs enhances antigen processing by up-regulating scavenger receptors via MyD88-dependent pathway. Doyle et al. found that TLR signaling can increase both the percentage of macrophage uptake of microbes and the number of microbes uptaken by each macrophage (Doyle et al., 2004). In addition, actin cytoskeleton mobilization, which can facilitate antigen processing and presentation by DCs, is also enhanced by TLR signaling (West et al., 2006).

Danger signals sensed by PRRs determine the consequence of antigens after its endocytosis (Matzinger, 2002; van & Geijtenbeek, 2006). Danger signals include exogenous signal (such as pathogens and toxins) and endogenous signal (such as mammalian DNA, RNA, HSPs, HMGB1, and interferons). A recent report demonstrated that APCs discriminate self and
pathogenic antigens with the help of TLRs (Blander & Medzhitov, 2004). Blander and coworkers described that TLR signaling activated by bacteria regulates antigen internalization and phagosome maturation, and thus, promotes phagocytosis. Phagocytosis of bacteria but not apoptotic cells by macrophages was impaired in TLR2−/− and TLR4−/− or MyD88−/− mice (Blander & Medzhitov, 2004). Phagocytosis of bacterial induces DC maturation, whereas phagocytosis of apoptotic cells cannot. However, uptake of apoptotic cells along with LPS treatment can induce DC maturation, indicating that TLR signaling determines the fate of auto-antigen. Antigens derived from apoptotic cells cannot be efficiently presented by MHC class II. Nevertheless, co-administration with TLR ligand enhances antigen presentation and promotes antigen-specific CD4+ T cell response (West et al., 2006). Therefore, TLRs can sense danger signal and control the discrimination of self and non-self antigens. Normally, self antigens are excluded from antigen presentation due to the lack of TLR signaling. However, self antigens and TLR signals can co-exist under certain pathological circumstances. For example, defective clearance of apoptotic β cells, which is observed in diabetes-prone individuals, results in the release of both self antigen and endogenous danger signaling molecules (such as HMGB1, HSPs, and nucleic acids), while PRRs can bind to both exogenous and endogenous molecules (Matzinger, 2002). For example, TLR2 is the receptor for endogenous molecules HSP60 and HMGB1, as well as exogenous molecules of bacterial lipoproteins. Similarly, TLR4 is a receptor for HSP70, HMGB1, and LPS. Therefore, those endogenous danger signaling molecules (also called alarmins) released from necrotic β cells function as DAMPs to signal TLRs. With the presence of those TLR signals and auto-antigens, the tolerance to self antigens is broken down and an autoimmune response against β cells is then initiated. TLR2 has been suggested to be an important sensor for apoptotic or secondary necrotic β cells (Kim et al., 2007). Apoptotic β cells undergoing secondary necrosis can provoke a β-cell-specific autoimmune response in a TLR2-dependent manner. Therefore, autoimmune diabetes is significantly suppressed in TLR2−/− mice but not in TLR4−/− mice (Kim et al., 2007). Engagement of TLR3 by poly I:C is also reported to be able to accelerate diabetes in a dose-dependent manner (Sobel et al., 1992; Ewel et al., 1992).

As a multifactorial autoimmune disease, T1D is affected by both genetic and environmental factors. Viral infection, as an environmental perturbant, is believed to be the most common trigger for T1D development (Akerblom et al., 2002). TLRs have been shown to be implicated in the process of virus-induced diabetes. Kilham rat virus (KRV) can induce autoimmune diabetes on BioBreeding diabetes-resistant (BBDR) rats (Nair et al., 2008). KRV infection has been shown to be able to induce the production of pro-inflammatory cytokines such as IL-6 and IL-12 in BBDR rats, which can be abolished by TLR9 antagonists (Zipris et al., 2007). TLR9 blockade on KRV-infected BBDR rats decreased diabetes incidence (Zipris et al., 2007). Furthermore, engagement of TLR3, 4, 6, 7, and 8 was also found to significantly increase the incidence of KRV-induced diabetes on BBDR rats (Zipris et al., 2005). A lymphochoriomeningitis virus (LCMV)-induced diabetes model with RIP-GP transgenic mice was employed to dissect the role of virus infection in triggering diabetes (Ohashi et al., 1991). The islet cells of RIP-GP mice express LCMV-GP protein under the control of RIP promoter. Unlike LCMV, viral peptide failed to induce diabetes in RIP-GP mice (Lang et al., 2005; Ohashi et al., 1993). However, co-administration of TLR3 and 7 ligands with a viral peptide successfully induces diabetes in RIP-GP mice (Lang et al., 2005), indicating that TLR signals play a critical role in virus-induced autoimmune diabetes.
HMGB1, an evolutionarily conserved nuclear protein, has recently been found to be a “danger signal” to alert the immune system of tissue damage. It can be passively released from damaged cells during various pathogenic processes. For example, HMGB1 released from damaged cells during liver ischemia-reperfusion plays a critical role in mediating hepatic injury (Tsung et al., 2005). In line with this result, we demonstrated that HMGB1 can be passively released during cardiac cold ischemic injury as well as in graft with acute rejection (Huang et al., 2007). In a model for syngenic heart transplantation, HMGB1 increased for a few days after surgery and dropped back to normal level thereafter, while HMGB1 steadily increased in allografts after transplantation along with acute allograft rejection, suggesting that HMGB1 is implicated in the pathogenesis of allograft rejection. In support of this notion, we characterized that allograft infiltrated immune cells actively secrete HMGB1. Therefore, administration of recombinant A box, a specific antagonist for the endogenous HMGB1, reduced pro-inflammatory cytokine production and Th1 response, and thus, prolonged cardiac allograft survival (Huang et al., 2007). Together, these data support a critical role for HMGB1 in mediating allo-immune response. Given the similarity between allograft rejection and autoimmune destruction of the pancreatic β cells in type 1 diabetes, we next proposed that HMGB1 might serve as an innate mediator implicated in β-cell specific autoimmune response during T1D development. By studies in NOD neonates, we demonstrated the accumulation of apoptotic β cells during neonate β mass turnover, which is associated with secondary β cell necrosis and passive release of HMGB1 into the extracellular milieu in the pancreatic islets (Zhang et al., 2009). The passively released HMGB1 could then serve as an innate alarmin for the initiation of autoimmune response in genetic predisposed subjects. To address the role of HMGB1 in T1D progression, we next confirmed that HMGB1 can be released from apoptotic β cells and thus served as a danger signal to enhance autoimmune response in T1D (Han et al., 2008). In addition to being passively released by the damaged β cells, HMGB1 can also be actively secreted by DCs and other islet-infiltrating immune cells. Upon LPS or TNFα/IFN-γ stimulation, HMGB1 translocated from the nucleus into the cytosol in DCs and then secreted into the extracellular matrix. In consistent with these results, in situ immunostaining confirmed the co-localization of HMGB1 and CD11c, a specific surface marker for DCs, in the pancreatic sections originated from diabetic NOD mice (Han et al., 2008). Therefore, with the presence of extracellular HMGB1, APCs efficiently uptake apoptotic β cells and become matured. Our subsequent studies further revealed that extracellular HMGB1 released by damaged cells or secreted by activated immune cells is potent in promoting inflammatory response. For example, treatment of DCs with HMGB1 significantly increased their pro-inflammatory cytokine production and allo-stimulatory capacity along with higher expression of MHC class II and costimulatory molecules. More important, this stimulatory effect can be abolished by the administration of HMGB1 blockades such as HMGB1 neutralizing antibodies. Therefore, treatment of NOD mice with a neutralizing HMGB1 Ab dramatically reduced insulitis progression and diabetes onset (Han et al., 2008). Consistent with our observations, Dumitriu et al. found that HMGB1 can be released from plasmacytoid DCs (pDCs) following TLR9 stimuli. Furthermore, pDCs express RAGE, a receptor for HMGB1. Disruption of HMGB1/RAGE signaling suppressed the maturation of pDCs (Dumitriu et al., 2005). In addition, HMGB1 was found to promote inflammatory response by enhancing DC migration, and as such, blockade of HMGB1 or RAGE, suppressed homing receptor expression on monocyte-derived DCs and inhibited their migration which is required for T-cell priming (Yang et al., 2007; Dumitriu et al., 2007).
7. Conclusion

Innate immune response, as one of the two pillars of the immune system and the mediator of adaptive immune response, plays an essential role in the pathogenesis of T1D. Pattern recognition receptors expressed on the innate immune cells sense the conserved pathogen specific molecules (PAMPs) or alarmins released by host cells (DAMPs) to initiate an immune response. In the normal condition, self antigens can be distinguished from foreign antigens and do not provoke an immune response. However, under certain circumstances, self antigens released from damaged host cells could be processed and presented to autoreactive T cells with the presence of PRR signaling. For the case of type 1 diabetes, defective clearance of apoptotic β cells during neonate β mass turnover by phagocytes results in the accumulation of apoptotic β cells in the pancreas, which then undergo a secondary necrosis along with the release of β-cell-derived autoantigens and danger signals (e.g., HMGB1, HSPs, and DNA). Danger signals subsequently activate PRRs and promote self antigen presentation by APCs. Furthermore, PRR signals also induce maturation and migration of APCs, which facilitate both innate and adaptive immune response to mediate the destruction of β cells. The effect of current therapeutic approaches for T1D is unsatisfied. A variety of severe complications developed in the relatively large proportion of T1D patients. Therefore, a clear understanding of the recognition of β-cell antigens and the initiation of autoreactive immune response against β cells is essential to the development of better effective therapeutic approaches for this devastating disorder.

8. Acknowledgements

Jixin Zhong and Jun-Fa Xu contributed equally to this work. Correspondence should be addressed to Dr. Cong-Yi Wang (cwang@georgiahealth.edu). Our research is supported by grants from the Juvenile Diabetes Research Foundation International (JDRFI) and the EFSD/CDC/Lilly Program for Collaborative Diabetes Research between China and Europe, and by the Bridge Funding from Tongji Hospital of HUST to CYW. The authors declare that they have no competing financial interest.

9. References


Innate Immunity in the Recognition of β-Cell Antigens in Type 1 Diabetes

immunological self shadow within the thymus by the aire protein. Science 298, 1395-1401.


Innate Immunity in the Recognition of β-Cell Antigens in Type 1 Diabetes


Hysi, P., Kabesch, M., Moffatt, M.F., Schedel, M., Carr, D., Zhang, Y., Boardman, B., von, M.E., Weiland, S.K., Leupold, W., Fritzsch, C., Klopp, N., Musk, A.W., James, A.,


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.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following: