

**Type 1 Diabetes (T1D) In NOD Mouse Models: The Role of Toll-like Receptor 7
And An Enteric Bacterial Pathogen In Accelerating The Development of T1D**

by

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B.Sc(Hons)., Queen's University, 2006

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April 2009

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ABSTRACT

Enteric viruses, intestinal enteropathies and the subsequent activation of endosomal toll-like receptors (TLRs) have been implicated as triggers of type 1 diabetes (T1D). TLR7 detects single stranded RNA. TLR7 agonists can accelerate diabetes by enhancing islet expression of major histocompatibility complex (MHC) I restricted transgenic antigens but the role and source of TLR7 stimulation in promoting T1D (and reactivity to true self antigens) remains unclear. In addition, recent evidence has suggested that disruption of the intestinal barrier, a ‘leaky gut’, may provide an endogenous TLR source that drives the autoimmune response in T1D. We used non-obese diabetic (NOD) mouse models of human T1D to investigate the role of TLR7 activation and an enteric bacterial pathogen, *Citrobacter rodentium*, that disrupts the intestinal barrier integrity in the development of T1D.

TLR7 activation with the imidazoquinoline CL097 in NOD mice caused the activation of bone marrow derived dendritic cells *in vitro*, the general activation of T and B cells *in vivo*, and the production of proinflammatory cytokines. *In vivo* antigen-specific cytotoxicity studies revealed enhanced cytotoxicity against IGRP (islet autoantigen) peptide pulsed targets in NOD mice treated with CL097 and anti-CD40 compared to negative controls. This treatment combination accelerated the onset of T1D in NOD 8.3 T cell receptor (TCR) transgenic mice (8.3 NOD mice). This accelerated disease in 8.3 NOD mice was significantly delayed when TLR7 signaling was blocked using the oligodeoxynucleotide (ODN) inhibitor, IRS661.

Pre-diabetic (12-week) NOD mice displayed increased intestinal barrier permeability when compared to C57BL/6 and diabetes resistant NOR mice. Moreover, the development of invasive insulinitis is accelerated when young (4-week) NOD mice are infected with *C. rodentium*. *C. rodentium* infected NOD mice demonstrate increased colonic permeability, increased activation of polyclonal and diabetogenic cytotoxic T lymphocytes (CTLs) and increased *C. rodentium* counts in the mesenteric and pancreatic lymph nodes, compared to uninfected NOD mice.

Taken together, these findings demonstrate that TLR7 signaling can modulate the development of T1D and an enteric bacterial pathogen can modulate the development of invasive insulinitis. Thus, TLR7 antagonism and maintaining an intact intestinal barrier may provide distinct therapeutic approaches in preventing the development of T1D.

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ABBREVIATIONS

APC	allophycocyanin
BM	bone marrow
BMDC	bone marrow dendritic cells
CFSE	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester
CM	complete media
CpG	cytosine-phosphate-guanosine
CQ	chloroquine
CR	<i>Citrobacter rodentium</i>
CTLs	cytotoxic T lymphocytes
CTLA-4	cytotoxic T-lymphocyte antigen 4
CBV	coxsackie B viruses
DCs	dendritic cells
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FDA	Food and Drug Administration
GI	gastrointestinal
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP	glycoprotein
HLA	human leukocyte antigen
HMGB-1	high-mobility group box 1 protein
IBD	inflammatory bowel disease
IDDM	insulin-dependent diabetes mellitus
IECs	intestinal epithelial cells
IGRP	islet-specific glucose-6-phosphatase catalytic subunit-related protein
IgA	immunoglobulin A
IgG	immunoglobulin G

IL-1	interleukin-1
IL-12p70	interleukin-12 subunit 70
IL-10	interleukin-10
IL-2RA	interleukin-2 receptor α chain
IL-4	interleukin-4
IL-6	interleukin-6
IFIH1	interferon induced with helicase C domain
IFN	interferon
IFN- α	interferon alpha
IFN- γ	interferon gamma
IRF	interferon regulatory factor
IRM	immune response modifier
IRS661	immunoregulatory sequence 661
LCMV	lymphocytic choriomeningitis virus
LNs	lymph nodes
LOX	loxoribine
LRR	leucine-rich repeat
mAb	monoclonal antibodies
MadCAM-1	mucosal addressin cell adhesion molecule-1
MCP-1	monocyte chemotactic protein-1
MDA5	melanoma differentiation-associated protein 5
MHC I	major histocompatibility complex I
MS	multiple sclerosis
MyD88	myeloid differentiation primary response gene 88
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	non-obese diabetic
NOD.SCID	non-obese diabetic. severe combined immunodeficiency
NOR	non-obese diabetic resistant
ODNs	oligodeoxynucleotides
ORNs	oligoribonucleotides
PAMPs	pathogen associated molecular patterns

PBS	phosphate buffer solution
pDCs	plasmacytoid dendritic cells
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PRRs	pattern recognition receptors
PTPN22	protein tyrosine phosphatase non-receptor type 22
RA	rheumatoid arthritis
R-837	imiquimod
SC	subcutaneous
SLE	systemic lupus erythematosus
SPF	specific pathogen-free (SPF)
ssRNA	single stranded ribonucleic acid
T1D	type 1 diabetes
TCR	T cell receptor
TEDDY	The Environmental Determinants of Diabetes in the Young
TIR	toll / interleukin-1 receptor
TLRs	toll-like receptors
Th1	T helper cell 1
Th2	T helper cell 2
TNF- α	tumor necrosis factor alpha
Tregs	regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon- β
TRIGR	Trial to Reduce IDDM in the Genetically at Risk
Yaa	Y-chromosome autoimmune accelerator

ACKNOWLEDGEMENTS

I would like to sincerely thank all of the members of the Dutz and Vallance lab, and my CFRI and UBC colleagues for their encouragement and input throughout the duration of my degree. Particular thanks goes to Drs. YiQun Zhang and Deanna L. Gibson who brought me under their wings to teach me invaluable lab skills and guidance in my projects.

I am indebted to Dr. Jan Dutz for taking me into his lab and always providing insightful advice, expert guidance, encouragement to collaborate, explore new directions and an enthusiastic opinion.

I am also indebted to Dr. Bruce Vallance for taking me into his lab and providing me expert guidance and advice on the GI project.

Special thanks goes to Ms. Wing ki (Vicki) Cheng who provided encouragement and support during those long long long days in the lab.

Lastly, I would like to thank my parents who have supported me throughout my academic endeavors and my gratitude to them extends beyond words.

*To my Father,
Eun-Hyung (Peter) Lee*

CO-AUTHORSHIP STATEMENT

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Mr. Andrew S. Lee conducted all experimental designs, research, data analysis and manuscript preparation under the supervision of Dr. Jan P. Dutz.

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An Intestinal Barrier Disrupting Bacterial Pathogen Promotes Insulinitis And Activates Diabetogenic CD8⁺ T Cells In NOD Mice

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Dr. Jan P. Dutz supervised, mentored and provided guidance to Andrew S. Lee in the experimental designs, research and data analysis on the GI project. He also edited and made revisions to the GI manuscript.

1. INTRODUCTION

1.1 Type 1 Diabetes

Type 1 diabetes (T1D) is an organ-specific autoimmune disease in which insulin producing β -cells in the pancreas are destroyed by the immune system (1, 2). The destruction of β -cells is predominately mediated by self-reactive CD4⁺ and CD8⁺ T cells (3). T1D is a long-term consequence of this destruction and clinical manifestations of T1D do not develop until 80-90% of β -cells are destroyed (2). T1D accounts for about 10% of all patients with diabetes and requires constant blood glucose monitoring and administration of insulin several times a day to avoid organ damage, ketosis, coma and death (4). The etiology of T1D is complex and multi-factorial involving both genetic predisposition and environmental factors (2).

1.1.1 Genetics

Tremendous progress has been made in identifying the genes that play a pivotal role in the etiology of T1D. Linkage analysis and genetic association studies have identified more than 20 T1D susceptibility loci of which human leukocyte antigen (HLA) contributes up to 40-50% of the genetic risk (5). More than 90% of patients who develop T1D have either a DR3, DQ2, DR4 or DQ8 haplotype (6). These genes may be involved in presenting autoantigens that lead to the destruction of β -cells. In addition, recent studies (7) have focused on the role of non-HLA alleles in T1D susceptibility. Most of the non-HLA alleles identified to increase T1D risk have been found to be defects in regulating peripheral and central tolerance or in the immune response against viruses (7, 8). Defects in cytotoxic T-lymphocyte antigen 4 (CTLA-4) (9) and protein tyrosine phosphatase non-receptor type 22 (PTPN22) (7) which regulate T cell reactivity as well as defects in the IL-2 receptor α chain (IL-2RA/CD25) (10) which is essential to generate regulatory T cells (Tregs) have been reported to increase T1D susceptibility. Moreover, defects in the interferon induced with helicase C domain (IFIH1), also known as melanoma differentiation-associated protein 5 (MDA5), which is involved in the anti-viral immune response against RNA viruses have been associated to increase the risk of T1D susceptibility (8). However, a recent study by Nejentsev *et al.* (2009) (11) on IFIH1

has found the opposite in which IF1H1 variants may provide protection against the development of T1D. These susceptibility genes are not inclusive to T1D risk but have been linked to other autoimmune diseases. PTPN22 has been linked to the increase risk of thyroid disease, system lupus erythematosus (SLE), and rheumatoid arthritis (RA) (7). Thus, defects in immunoregulation or in anti-viral immune response may play an important role in triggering the development of T1D.

1.1.2 Environment

Although genes play an essential role in T1D susceptibility, there is mounting evidence that environmental factors are important contributors to the development of T1D. Twin studies have shown that the concordance rate among monozygotic twins is less than 40% (4, 12). Epidemiological studies indicate that the incidence of T1D has been increasing reaching 3% annually worldwide especially in developed countries (13, 14). This dramatic increase in global incidence of T1D cannot be solely attributed to gene interactions but indicate the influence of environmental factors.

Several environmental factors have been linked to causing T1D but the most investigated factors are viruses and dietary products (4). Viruses have been suspected of causing T1D in humans for over a century (4). It is thought that viral infections in early life lead to the subsequent development of T1D later in life. Viruses that have been implicated in the development of human T1D include mumps, rubella, cytomegalovirus, retroviruses and enteroviruses particularly the coxsackie B viruses (CBV) (12, 14). It remains unclear whether viruses are involved in triggering or even delaying the development of T1D. Animal studies have shown that certain viruses are capable of triggering T1D while others have shown the complete opposite – delay in the development of T1D (12, 14). The mechanisms that viruses use to trigger T1D remain unclear. Possible mechanisms include: direct β -cell attack, molecular mimicry, bystander activation of self-reactive T lymphocytes, or modulation of Tregs (14). Dietary products such as earlier exposure to cow's milk protein and gluten (ie. cereal) have been implicated in triggering the development of T1D (4). It remains unclear how dietary products modulate T1D but there are recent reports suggesting that a defect in the intestinal epithelial barrier integrity in

genetically susceptible individuals (15) may allow these dietary antigens to evoke an autoimmune response.

1.1.3 Non-obese diabetic (NOD) mouse

The NOD mouse has been the most extensively studied animal model of human T1D. It was originally developed in the 1980s as a cataract-prone strain derived from the outbred Jcl:ICR line of mice (16, 17). During this selection of the cataract prone strain, the NOD strain was established and was found to develop spontaneous diabetes (16, 17). The incidence of spontaneous diabetes in the NOD mouse is 60-80% in females and 20-30% in males (3, 16). The NOD mouse has provided a wealth of insight in understanding the etiology of human T1D. This model has characteristics similar to human T1D which has provided invaluable clues in identifying disease-causing genes (18), targets of self-reactive T and B lymphocytes (19), defects in cellular and immunoregulatory responses (20-25) and potential environmental triggers in the development of T1D (26). In addition, the NOD mouse has allowed researchers to investigate the different stages of disease progression which is similar to human T1D (3). In NOD mice, insulinitis begins at around 3-4 weeks and subsequently develops into severe invasive insulinitis at around 12 weeks. The clinical onset of T1D in NOD mice occurs at around 15-30 weeks of age in female mice and slightly later in male mice (3).

1.2 Toll-like Receptors (TLRs)

TLRs are evolutionary conserved transmembrane receptors that initiate the innate immune response. TLRs are a group of pattern recognition receptors (PRRs) that form one of the earliest warning systems against pathogens and are an integral part of the host defense system. TLRs function by recognizing a diverse repertoire of pathogen associated molecular patterns (PAMPs) on bacteria, fungi, protozoa and viruses (27, 28). Currently, there are at least 13 mammalian TLRs, each recognizing one or more specific PAMPs (27).

Structurally, TLRs are composed of a leucine-rich repeat (LRR) region on their extracellular domain and share the intracellular Toll/IL-1 receptor (TIR) domain with the

IL-1 receptor (27). Ligands that bind to TLRs can form either homo- or hetero- dimers that result in the recruitment of the signaling molecule MyD88. This initiates a signaling cascade that ultimately results in the production of proinflammatory cytokines, chemokines, and the maturation of DCs (27, 29). All TLRs signal through MyD88 except for TLR3 which signals through the MyD88 independent pathway, TRIF, and TLR4 which can signal through both MyD88 dependent and independent pathways (27, 29; Appendix A.1).

TLRs are predominately expressed on the cell surface but there is a class of TLRs, TLR3, 7, 8 and 9, that are expressed in endosomal compartments. These endosomal TLRs recognize nucleic acids, specifically double stranded (ds) RNA (TLR3 ligand), single stranded (ss) RNA (TLR 7/8 ligand) and unmethylated CpG DNA (TLR9 ligand). In humans, TLR7 and 9 are predominately expressed in plasmacytoid DCs (pDCs) and B cells whereas in mice myeloid DCs also express both TLR7 and 9 (30, 31). Endosomal TLR ligation triggers a signaling cascade that can result in the activation of NF κ B and production of pro-inflammatory cytokines or can result in a robust production of type 1 interferons (IFNs) through the activation of the IRF family of transcription factors (29).

1.2.1 TLRs and autoimmune diseases

TLRs represent one of the most ancient host defense mechanisms found in insects, plants and mammals (27). However, accumulating evidence has suggested that TLRs play a dominant role in the development of autoimmune diseases (31, 32). TLRs have been found to recognize endogenous ligands such as hyaluronate, heat shock proteins, high-mobility group box 1 protein (HMGB-1) and self- RNA and DNA complexes which may trigger the development of autoimmune diseases in humans (31). Animal models of autoimmune diseases (31) have shown that inappropriate TLR activation triggers the onset of rheumatoid arthritis (RA), multiple sclerosis (experimental autoimmune encephalomyelitis in mice), myocarditis, T1D, SLE and atherosclerosis. In addition, over-expression or deletion of TLR7 has been shown in animal models to enhance or reduce the severity of autoimmune diseases (32, 33). Moreover, studies in humans have shown that a decrease in TLR4 or 5 expression reduced autoimmunity in atherosclerosis

and SLE patients (32). Taken together, there is a strong indication that TLRs play an important role in the pathogenesis of autoimmune diseases.

The mechanisms involved in initiating autoimmune disease via TLR stimulation remains unclear. Inappropriate TLR stimulation has been postulated to break self-tolerance mediated by Tregs by skewing the immune response to either a Th1 or Th2 phenotype (31, 32). A Th1 phenotype results in the activation of DCs that produce pro-inflammatory cytokines, and prime and activate self-reactive T lymphocytes. On the other hand, a Th2 phenotype results in the activation of self-reactive B lymphocytes that lead to the production of autoantibodies.

1.2.2 Foreign versus self recognition by TLR7-9

Nucleic acid sensing TLRs are potent inducers of both proinflammatory cytokines and type 1 IFNs (29). The ability of these endosomal TLRs to distinguish self versus foreign nucleic acids and the potential to initiate autoimmune diseases remains unclear. Despite this lack of clarity, there are studies (34, 35) that are focusing on deciphering the molecular differences between self and foreign nucleic acids. These differences include structural modifications on self –DNA and –RNA that prevent or suppress endosomal TLR stimulation. For instance, self-RNA commonly incorporate modified nucleosides such as 5-methylcytosine (m5C), N6-methyladenosine (m6A), 5-methyluridine (m5U), 2-thiolated uridine (s2U) or pseudouridine (36, 37) that prevent or inhibit TLR7/8 stimulation. Moreover, other RNA modifications such as the 2'-O-methyl modification, commonly found in ribosomal RNA, have been found to suppress the activation of TLR7/8 (36, 38, 39). Similarly, self-DNA also contain modifications to avoid TLR9 stimulation. These modifications include the methylation of stimulatory CpG dinucleotides, low frequency of CpG sequences and the incorporation of inhibitory sequences (40).

1.2.3 TLR7-9 agonists

TLR7-9 agonists trigger a rapid immune response leading to burst of proinflammatory cytokines, type 1 IFNs, and the stimulation of both innate and adaptive immune cells (34). The robust immune response by TLR7-9 agonists has led to a broad spectrum of therapeutic applications such as adjuvant therapy, vaccinations against infectious

diseases, and the treatment for cancer (35, 41, 42). Moreover, TLR7 agonists such as Aldara (5% imiquimod cream) have been approved by the U.S. Food and Drug Administration (FDA) and are clinically used for the treatment of genital warts which is caused by the human papillomavirus, actinic keratosis and superficial basal cell carcinoma (42, 43). Despite these potential therapeutic benefits, concerns have been raised in which these agonists may increase the risk of TLR7-9 mediated autoimmune diseases (44). Thus, further studies are warranted to evaluate the risk of inducing autoimmune diseases with these agonists.

Although there are several different TLR7-9 agonists, these agonists can be grouped into three distinct categories: synthetic, natural, and endogenous ligands. Synthetic TLR7/8 ligands are composed of small molecular nucleoside analogs or derivatives of either adenosine, guanosine and pyrimidine (35). These synthetic TLR7/8 ligands are termed small molecular immune response modulators (IRMs) because of their relatively small molecular size and their potent ability to induce pro-inflammatory cytokines and type 1 IFNs (35, 43). The most commonly used IRMs are the imidazoquinolines such as imiquimod (R837), resiquimod (R848) and CL097 (35, 43). On the other hand, TLR9 agonists are oligodeoxynucleotides (ODNs) that contain CpG dinucleotides (45). The stimulatory effects of these large molecular CpG ODNs depend on its sequence and structural interaction with TLR9. There are three CpG ODN classes, termed class A/D, B/K and C, which differ in terms of their ability to stimulate pro-inflammatory cytokines, type 1 IFNs and immune cells (46-49). The natural ligands of TLR7/8 have been found to be distinct oligoribonucleotide (ORNs) sequences from ssRNA viruses. These ssRNA viruses include: human immunodeficiency virus (50), dengue virus (51), influenza virus (51-53), coxsackie B virus (54), parechovirus 1 (55), sendai virus (56), and vesicular stomatitis virus (53). The specificity of these viral ORNs that stimulate TLR7/8 remains unclear but studies (57, 58) have shown that uridine is an important ORN component in stimulating TLR7/8. In addition, viral ORNs rich in guanosine and uridine (57) or adenosine and uridine (57) are necessary to mediate the production of IFN- α or proinflammatory cytokines (TNF- α and IL-12), respectively. Similarly, natural TLR9 ligands consist of bacteria DNA, rich in unmethylated CpG dinucleotides, (47) and viral DNA. TLR9 has been shown to detect murine cytomegalovirus (MCMV) (59) and herpes

simplex virus 1 and 2 (60-62). Moreover, a recent study by Zucchini *et al.* (2008) (63) showed that TLR7 can detect the DNA virus, MCMV, which suggest an overlapping function between TLR7 and 9 in viral detection.

Despite the various mechanisms that prevent the detection of endogenous RNA and DNA by TLR7/8 and 9, respectively, there are instances where self-recognition still occurs. Studies have shown that self-RNA (64, 65) and short interfering RNA (58, 66-68) stimulate TLR7/8. The recognition and delivery of endogenous RNA and DNA is predominately mediated by antibody or immune complexes (34). However, there is accumulating evidence suggesting that other endogenous RNA or DNA transporters such as the ssDNA-binding protein, HMGB1 (69) and the anti-microbial cationic peptide LL-37 (70) are involved in exacerbating the onset of inflammatory mediated diseases. The delivery of endogenous DNA or RNA into pDCs or B cells which express TLR7 and 9 have been implicated to initiate and exacerbate disease onset in various autoimmune diseases (34).

1.2.4 TLR7-9 inhibitors

In the past decade, there has been an increasing commercial and scientific interest in manipulating TLR7-9 signaling using synthetic small molecular or ODN antagonists. Much of this interest stems from the research into the pathogenesis of SLE. SLE is a systemic autoimmune disease in which high IFN- α and autoantibody production correlate with disease severity (31, 34, 71). The inappropriate stimulation of TLR7/9 on pDCs - main producers of IFN- α - and B cells has been implicated as the key components that drive the autoimmune response in SLE (31, 34, 71). The inhibition of both receptors with either anti-malarials such as chloroquine (TLR7-9 inhibitor) or TLR7/9 specific ODN antagonists have been shown to be effective in alleviating the symptoms of SLE in various mouse models of SLE (42). Chloroquine has been used since the 1950s as a treatment option for SLE patients but is used sparingly due to its low safety margin and side effects such as cardiac muscle damage and retinopathy (42). In addition, recent studies have suggested that TLR9 (regulatory) and TLR7 (inflammatory) might have opposing roles in the pathogenesis of SLE (72, 73). Thus, further studies are required to determine the specific roles of TLR7 and/or 9 and their contribution to disease severity in TLR7/9 mediated autoimmune diseases.

The exact mechanism employed by small molecular or ODN inhibitors in exerting their effects remains unclear. Chloroquine is a small molecular weak base that interferes with endosomal acidification which is required for TLR7-9 activation (74, 75). The mechanisms exerted by ODN inhibitors are thought to be: competing for cellular uptake and sorting, specific TLR7 or 9 interaction, interfering with downstream TLR signaling or a combination of these mechanisms (42).

1.3 Gastrointestinal (GI) System As An Endogenous TLR Source

TLRs can recognize endogenous ligands which may trigger the onset of autoimmune diseases. A likely source of endogenous ligands is from dead or dying cells. The inefficient removal of these cells leads to an increase circulation of self-antigens that may be detected by TLRs (71, 76). O'Brien *et al.* (2006) (22) showed that there is a defective clearance of apoptotic cells by macrophages in NOD mice. This lag time may result in TLR detection resulting in the initiation of an overzealous immune response to host antigens. Another possible source of both endogenous and exogenous ligands may arise from the gastrointestinal system. Dysregulated interactions between commensal bacteria and TLRs have been reported to promote inflammatory bowel disease (IBD) and T1D (77, 78). Moreover, recent reports have shown in both humans and in animal models that an intrinsic or induced intestinal epithelial barrier defect may lead to the development of autoimmune diseases (77-83).

1.3.1 GI structure and the intestinal immune system

The gastrointestinal system (GI) represents the greatest surface area of the body that is in direct contact with the external environment. Its functions include the uptake of nutrients and fluids, co-existing with the commensal microbiota (10^{11} commensal bacteria in humans) and preventing the infiltration of both commensal and pathogenic bacteria (84). Invasive micro-organisms are prevented from infiltrating the gut by the formation of a physical barrier consisting of single monolayer of intestinal epithelial cells (IECs) which are firmly held together by tight junctions (84; Appendix A.2). On the apical side of each IEC are brush-boarder microvilli that further deter the attachment of bacteria on this

physical barrier. In addition, a thick mucus layer consisting of mucins, secretory IgA antibodies and glycocalyx reinforce this physical barrier (84). This continuous mucus layer is generated on the apical side of IECs and traps infiltrating bacteria or viruses which are eliminated via peristalsis. Moreover, Paneth cells in the gut release anti-microbial peptides into the apical side of this physical barrier (84). This provides a biological barrier that helps prevent both commensal and pathogenic bacteria from infiltrating the gut (84).

The intestinal immune system in the gut is divided into two distinctive regions: inductive and effector sites (84; Appendix A.2). The inductive sites are places in which antigens from the mucosal lumen are actively brought over by M cells or directly sampled by DCs. This may be a mechanism that allows the intestinal immune system to sense its external environment. On the other hand, the effector sites are places in which immune cells differentiate and exert their function of either maintaining tolerance or initiating a cellular or humoral immune response against invading micro-organisms. The inductive sites consist of the mesenteric lymph nodes, Peyer's patches in the small intestine, and colonic and isolated patches of lymphoid follicles. The effector sites consist of the intestinal epithelium and the lamina propria.

1.3.2 'Leaky gut' and T1D

The intestinal immune system, by default, is in a state of tolerance. However, when intestinal barrier disruption occurs an overzealous immune response may follow resulting in intestinal autoimmune diseases such as IBD and celiac disease (77, 79, 85). There is mounting evidence in both humans and in animal models that intestinal barrier disruption may be a prerequisite to the development of extra-intestinal autoimmune diseases such as T1D (15, 80-83). The development of an intrinsic or induced 'leaky gut' in a genetically susceptible individual may provide the appropriate inflammatory stimuli to initiate or drive the autoimmune response in the pathogenesis of T1D (Appendix A.3). Recent studies (15, 86) have suggested that the gut and its luminal contents might contribute to the initiation of T1D. Turley *et al.* (2005) (26) showed luminal antigens from the gut can be transported to the pancreatic lymph nodes (LNs) resulting in CTL proliferation.

Jaakkola *et al.* (2003) (87) found that diabetogenic CTLs were primed both in the pancreatic and gut-associated LNs in NOD mice. In addition, islet-infiltrating CTLs were found to express the gut-homing receptor $\alpha 4\beta 7$ integrin (15) and CTLs activated in the gastrointestinal tract of NOD mice were found to home into islets which express the mucosal homing receptor MadCAM-1 (15).

1.4 Thesis Objectives

My thesis objectives consist of two main goals. First, determine the role of TLR7 signaling in the pathogenesis of T1D in NOD mouse models. Second, determine if an induced intestinal barrier disruption by an enteric bacterial pathogen leads to an accelerated development of T1D in NOD mice.

1.4.1 TLR7 and T1D

Inappropriate endosomal TLR activation has been implicated as a trigger of T1D. The prolonged exposure of apoptotic cells, which turn necrotic, have been suggested as a prime source of endosomal TLR stimulation. There is a delayed clearance of apoptotic cells by macrophages in NOD mice (22, 88) and this defect may provide the appropriate stimuli to induce the activation of diabetogenic CD8⁺ T cells in NOD mice. In addition, our lab has shown that administration of chloroquine, a TLR7-9 inhibitor, delays the development of T1D in NOD mice (Zhang *et al.* submitted; Appendix B). Moreover, Lang *et al.* (2005) (89) showed that TLR3 or 7 stimulation by synthetic agonists were required with the lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) to induce T1D in a double transgenic model of T1D in which LCMV GP is expressed on β cells and also contain a large population of LCMV GP specific CD8⁺ T cells. Administration of the LCMV GP alone in this model was insufficient to induce autoimmune diabetes. Despite this finding, it remains unclear whether transgenically expressed antigens in this model differ in expression levels and location to endogenous autoantigens.

The goal of the first study was to further elucidate the role of TLR7 in the development of T1D in NOD mouse models of human T1D. Specifically, I investigated whether TLR7

signaling was involved in the activation of dendritic cells which may lead to the priming of functional diabetogenic CTLs. In addition, I investigated whether induction or blockade of TLR7 signaling enhances or delays T1D in the NOD 8.3 TCR transgenic mice (8.3 NOD mice) – mice with a large population of diabetogenic CD8+ T cells that are specific for IGRP (islet autoantigen).

1.4.2 GI and T1D

The intestinal epithelium plays a critical role in maintaining a protective barrier against both pathogenic and commensal bacteria. Disruption of this delicate barrier, resulting in severe inflammation, has been suggested to provide a source of inappropriate TLR stimulation which may act as a catalyst in the pathogenesis of autoimmune diseases (15). Recent studies (80-82, 90) have shown that pre-diabetic animals and humans at-risk for T1D develop an intrinsic barrier dysfunction that may precede the development of T1D. However, it remains unclear whether this intrinsic barrier dysfunction is a result of diabetes-related inflammation or from environmental factors.

The goal of the second study was to determine if an induced intestinal barrier disruption by an enteric bacterial pathogen, at a time when there is no intrinsic barrier dysfunction, could accelerate the onset of T1D in genetically susceptible individuals. To address this hypothesis, we infected pre-diabetic NOD mice (4-weeks) with *Citrobacter rodentium*, an enteric bacterial pathogen that induces intestinal barrier disruption, and assessed the activation of polyclonal and diabetogenic CTLs as well as their development of invasive insulinitis.

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2. Toll-like Receptor 7 Ligation Converts T-cell Autoreactivity Into Overt Autoimmune Diabetes¹

2.1 INTRODUCTION

Type 1 diabetes (T1D) is an organ-specific autoimmune disease in which β -cells are destroyed by the immune system (1, 2). Much of the β -cell destruction is mediated by self-reactive cytotoxic T lymphocytes (CTLs) (1, 2). Both genetic and environmental factors play a pivotal role in the development of T1D (3-6). Yet, the mechanism that initiates the disease remains relatively unknown. Recent evidence has suggested that toll-like receptors (TLRs) may trigger the pathogenesis of T1D (7, 8). TLRs act as pathogen sensors that recognize distinct patterns on pathogens and influence the production of proinflammatory cytokines and chemokines in dendritic cells and macrophages (9-12). Despite this role, there is evidence suggesting that TLRs can also recognize self-molecules such as endogenous RNA and DNA (13). The inappropriate activation of TLRs results in DC maturation which is thought to drive the priming and activation of self-reactive CTLs.

Endosomal TLRs (TLR3, 7, 8 and 9) that recognize RNA or DNA oligonucleotides have recently been linked to the development of autoimmune disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and T1D (14, 15). The prolonged exposure of apoptotic cells, which turn necrotic, have been suggested as a prime source of endosomal TLR stimulation. There is a delayed clearance of apoptotic cells by macrophages in NOD mice (16, 17). The presense of β cell death has been shown to prime the activation and proliferation of diabetogenic CTLs in NOD mice (18). In addition, environmental agents such as viruses have been found in both animal models and in humans to play an important role in the precipitation of T1D (19, 20). Coxsackie B viruses (CBV) are recognized through TLR7 and have been implicated as an initiator

¹ A version of this chapter will be submitted for publication. Andrew S. Lee, YiQun Zhang and Jan P. Dutz. Toll-like Receptor 7 Ligation Converts T-cell Autoreactivity Into Overt Autoimmune Diabetes.

of T1D development (21, 22). Interestingly, Lang *et al.* (2005) (23) showed that TLR3 or 7 stimulation by synthetic agonists with the lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) were necessary to induce T1D in a double transgenic mouse model of T1D in which LCMV GP is expressed on β -cells as well as having a large population of LCMV GP specific CD8+ T cells. Administration of the LCMV GP alone in this model was insufficient to induce autoimmune diabetes. As well, Deane *et al.*, (2007) (24) showed that over-expression of TLR7 alone led to an accelerated autoimmune phenotype in the Y-chromosome autoimmune accelerator (Yaa) mouse. TLR7 signaling may accelerate the development of T1D through the upregulated expression of transgenically expressed antigens on β cells, however it remains unclear whether transgenically expressed antigens in these models may differ in expression levels and location to endogenous autoantigens.

The aim of the present study was to further elucidate the role of TLR7 in the development of T1D in NOD mouse. We found that TLR7 signaling requires anti-CD40 agonist to induce priming of diabetogenic CTLs in NOD mice. This priming event led to an acceleration of T1D in 8.3 NOD mice – harboring a large population of IGRP-specific CD8+ T cells. The subsequent inhibition of TLR7 signaling with IRS661 led to a delay in accelerated diabetes in 8.3 NOD mice treated with CL097 and anti-CD40.

2.2 MATERIALS AND METHODS

2.2.1 Animals and reagents

NOD and NOD.SCID mice were obtained from Jackson Laboratory and bred in a specific pathogen-free environment. The 8.3 NOD mice, expressing the rearranged TCR genes of the diabetogenic CTL clone NY8.3, have been previously described (25). All animal studies were conducted at Child and Family Research Institute in Vancouver, Canada and were approved by the institutional animal ethics committee.

The TLR agonists and inhibitors used in both the *in vitro* and *in vivo* experiments were: TLR7 agonists: R-837 (imiquimod, 1-(2-methylpropyl)-1H-imidazo[4,5c]quinolin-4-amine; InvivoGen) and loxoribine (LOX, 7-Allyl-8-oxoguanosine; InvivoGen); TLR7/8

agonist: CL097 (InvivoGen); TLR9 agonists: CpG1826 (CpG, 5'-TCCATGACGTTTCCTGACGTT-3' (phosphothiorated backbone); IDT / UBC NAPS); TLR7-9 inhibitor: chloroquine (CQ; Sigma-Aldrich); and TLR7 inhibitor: IRS661 (described by (26); Integrated DNA Technology (IDT)). Monoclonal anti-CD40, agonistic antibody to CD40 was produced from a hybridoma secreting FGK45 cell line.

2.2.2 Generation and activation of mouse bone marrow dendritic cells (BMDCs)

BMDCs were generated from murine bone marrow (BM) precursors in complete media (CM) consisting of RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) as previously described (27). BM precursor cells were flushed out from extracted femur bones from the NOD mouse with RPMI 1640 medium with penicillin (100 U/mL) and streptomycin (100 µg/mL) and cultured for six days in CM. At day 1 and 3 of culturing, 0.5mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (supernatants from the X63 B cell line), 1uL IL-4 (10ug/mL, Sigma-Aldrich) and 9.5 mL 10% FBS RPMI were added to the cultures. At day 6, immature BMDCs were stimulated with R837 (5ug/mL; 20.8uM), LOX (170ug/mL; 500uM), CL097 (1ug/mL; 4.13uM) or CpG1826 (5ug/mL; 0.786uM) for 24-hours. TLR7/8 or 9 activated BMDCs were inhibited with either CQ (2ug/mL; 6.25uM) or IRS661 (9ug/mL; 1.4uM), respectively for 24-hours.

2.2.3 Detection of BMDC activation by cytokine release and surface markers

After 24-hours of incubation with each respective TLR agonists or inhibitors, culture supernatants were collected and frozen at -80°C for subsequent analysis and BMDCs were analyzed by fluorescence cytometry for CD11c⁺ dendritic cells (DCs). Cytokines were analyzed using the mouse inflammation cytometric bead array (CBA) kit (BD Pharmingen). The CBA assay allowed the simultaneous detection and quantification of soluble murine cytokines IL-6, IL-10, IFN-γ, TNF-α, IL-12p70, and MCP-1 in a single sample. The following monoclonal antibodies (mAbs) were used for BMDC activation: APC- or PE- conjugated anti-CD11c, and APC-conjugated anti-CD40 (all from BD Pharmingen).

2.2.4 Detection of TLR7/8 activation and inhibition in NOD mice by cytokine release and surface markers

Female NOD mice (6-8 weeks old) were injected subcutaneously (SC) with either CL097 (5mg/kg) or CpG (5mg/kg) for 24-hours. For TLR7 inhibition, IRS661 (266mg/kg) was administered SC 2-hours prior to CL097 treatment. Sera were collected after 0, 2, 6 and 12 hours post-injection with CL097 or CpG and frozen at -80°C for subsequent analysis. Cytokines were analyzed using the CBA kit (BD Pharmingen).

After 24-hours post-stimulation, the axillary, mesenteric, and pancreatic lymph nodes (LNs), and the spleen were extracted and analyzed by flow cytometry for CD8⁺ and CD4⁺ T cell and B220^{hi} B cell activation. CD11c⁺ DCs were isolated from the spleen by finely chopping the spleen and digesting with 1 mg/mL Collagenase D (Roche) supplemented with 40g/mL DNase (Boehringer Mannheim) in CM at 37°C for 60 min. The released cells were collected in CM with 0.1M EDTA buffer and incubated on ice with anti-FcR mAb (2.4G2; American Type Culture Collection) or with mouse IgG (Sigma) to block Fc-binding sites. DCs were enriched using either Ficol (Nycoprep) separation or anti-CD11c mAb-coated microbeads (MACS; Miltenyi Biotec). DCs were next stained on ice for 30 min with labeled antibodies prior to analysis with flow cytometry. The following mAbs were used: FITC-conjugated anti-CD4, FITC- or PE-conjugated anti-CD69, PerCP-conjugated anti-CD45R/B220, APC- or PE-conjugated anti-CD8, APC- or PE-conjugated anti-CD11c, and APC-conjugated anti-CD40 (all from BD Pharmingen).

2.2.5 Injections and in vivo cytotoxicity assay

Female NOD mice (6-8 week old) were injected with CL097 (5mg/kg; SC), CpG (5mg/kg; SC) or anti-CD40 (10mg/kg; intraperitoneally, IP) or in combination. The in vivo cytotoxicity assay was modified from (28). Briefly, splenocytes from wildtype NOD mice were differentially 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled with 0.3µM CFSE (CFSE LO) or 5µM CFSE (CFSE HI) for 10 min. These cells were pulsed with the target peptide, NRP-V7 (KYNKANVEL; islet cell mimotope of IGRP; 1ug/mL), or the control peptide, TUM (KYQAVTTTL; 1ug/mL), for 1-hour. A

1:1 mixture of CFSE-labeled and pulsed splenocytes (1×10^7 CFSE HI with NRP-V7 peptide : 1×10^7 CFSE LO with TUM peptide or vice versa) were adoptively transferred intravenously (IV) into mice injected 24-hours ago. On day 6 post-injection, the spleen was collected and analyzed through flow cytometry. Numbers of target (CFSE LO) versus control (CFSE HI) cells recovered were used to calculate the percentage of killing with the following formula: % specific lysis = $1 - [(\text{no. of targets}/\text{no. of control cells in injected animal}) / (\text{no. of targets}/\text{no. of control cells in control animal})] \times 100$.

2.2.6 Induction, acceleration and diagnosis of diabetes

Female 8.3 NOD mice (5-6 weeks old) were injected individually or in combination with: CL097 (5mg/kg; SC), anti-CD40 (10mg/kg; IP), or IRS661 (266mg/kg; SC). IRS661 was injected 2-hours prior to CL097 and anti-CD40 treatment in 8.3 NOD mice. Acceleration of diabetes was considered to occur within 14-days post-injection in 8.3 NOD mice. Blood glucose was measured in tail vein blood using Ascenia Contour glucostrips and glucometer (Bayer). Animals were considered to be diabetic when two consecutive blood glucose measurements exceeded 14mM.

2.2.7 Adoptive transfer of diabetogenic splenocytes into NOD.SCID mice

Adoptive transfers were performed as previously described (29). Recipient female NOD.SCID mice (6-8 wk of age) were injected IV with donor splenocytes (2×10^7 viable splenocytes) suspended in 200 μ l of PBS. Diabetic spleen donors were female NOD mice that displayed high blood glucose levels (30mM) for at least 1-week. Multiple diabetic donor spleens were pooled to yield a sufficient number of cells for each experiment. Chloroquine (20mg/kg) was administered to recipient female NOD.SCID mice for 5 consecutive days followed by 3 times a week.

2.2.8 Statistical analysis

A two-tailed Student's t test was used to calculate statistical significance where indicated, and a one-way ANOVA was used for multi-group comparisons. A log-rank test was applied to compare survival curves. All statistical analysis was conducted using Prism 3 (GraphPad software).

2.3 RESULTS

2.3.1 TLR7/9 agonists activate NOD derived bone marrow dendritic cells (BMDCs)

To understand the role of TLR7 in the pathogenesis of T1D, we first examined if small molecular TLR7 agonists could activate BMDCs derived from the NOD mouse. NOD derived BMDCs stimulated with the TLR7/8 agonist, CL097, or the TLR7 agonists, R837 and LOX, showed a robust upregulation of CD40, comparable to the TLR9 agonist, CpG, activated BMDCs (Figure 2.1A). A dose titration of each TLR7 agonists showed that CL097 was the most potent and effective in activating BMDCs (data not shown). Although CL097 can activate TLR8, in mice, TLR8's functionality remains controversial (30). In addition, supernatants collected from TLR7/9 stimulated BMDCs revealed a strong secretion of proinflammatory cytokines (IL-6, TNF- α and IL-12p70) and chemokine (MCP-1) (Figure 2.1B).

2.3.2 CL097 induces a general activation of T and B cells in NOD mice

We next examined the role of TLR7 activation *in vivo* by titrating CL097 in NOD mice. Subcutaneous or intraperitoneal administration of CL097 (5mg/kg) resulted in a general activation of CD4⁺ and CD8⁺ T cells, and B220^{hi}⁺ B cells in the axillary, mesenteric and pancreatic LNs as well as in the spleen (Figure 2.2). CL097 administration also activated splenic CD11c⁺ DCs (Figure 2.2). Furthermore, CL097 induced a substantial increase in serum levels of IL-6, TNF- α , IL-12p70 and MCP-1 compared to the negative control (Figure 2.3). The general activation of T and B cells and the increase in pro-inflammatory cytokines and chemokine were comparable to NOD mice injected with CpG (5mg/kg, SC or IP). This suggests that CL097 injection in NOD mice may not only increase the normal repertoire of CD4⁺ and CD8⁺ CTLs but also the diabetogenic CTL population.

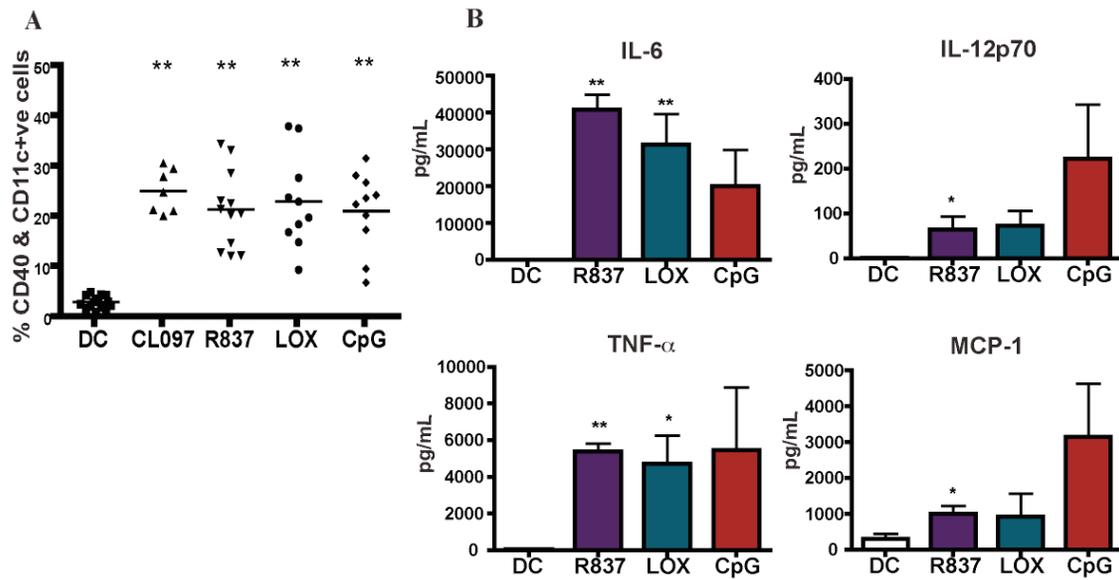


FIGURE 2.1. TLR7 or 9 activation of bone marrow derived dendritic cells (BMDCs) from the NOD mouse. A, NOD BMDCs were stimulated with optimal doses of different TLR7/8 and 9 agonists for 24-hours. TLR7 agonists: R837 (5ug/mL; 20.8uM) and LOX (170ug/mL; 500uM); TLR7/8 agonist: CL097 (1ug/mL; 4.13uM); and TLR9 agonist: CpG (5ug/mL; 0.786uM). Fluorescence cytometry was used to gate for CD11c+ve dendritic cells (DCs) and the expression of the activation marker CD40. B, Culture supernatants were collected (after 24-hours) from TLR7 or 9 stimulated BMDCs and analyzed by cytometric bead array for IL-6, IL-12p70, TNF- α and MCP-1. Data are representative of at least five independent experiments. Each TLR7, 7/8 or 9 agonist groups were compared to unstimulated control (DC). *, $p < 0.05$, **, $p < 0.006$ by one-way ANOVA.

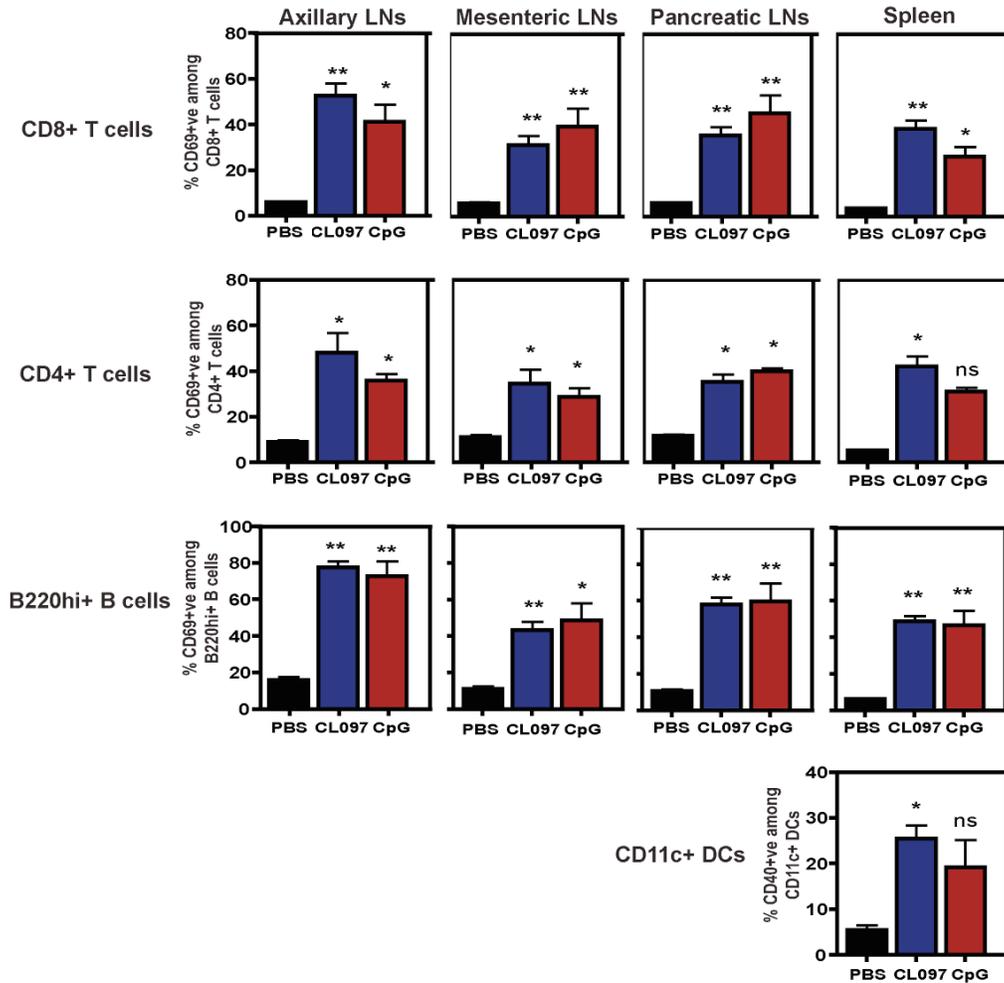


FIGURE 2.2. CL097 induces a general activation of T and B cells, and splenic DCs in the NOD mouse. CL097 (5mg/kg) or CpG (5mg/kg) were administered subcutaneously in the NOD mouse. After 24-hours, the axillary, mesenteric and pancreatic lymph nodes (LNs) and spleen were extracted and analyzed by fluorescence cytometry. The activation marker CD69 was used to determine activation of CD8+ and CD4+ T cells and B220hi+ B cells. The activation marker CD40 was used to determine activation of CD11c+ve DCs. Data are representative of at least three independent experiments. CL097 or CpG groups were compared to the PBS group. *, $p < 0.05$, **, $p < 0.001$ by one-way ANOVA.

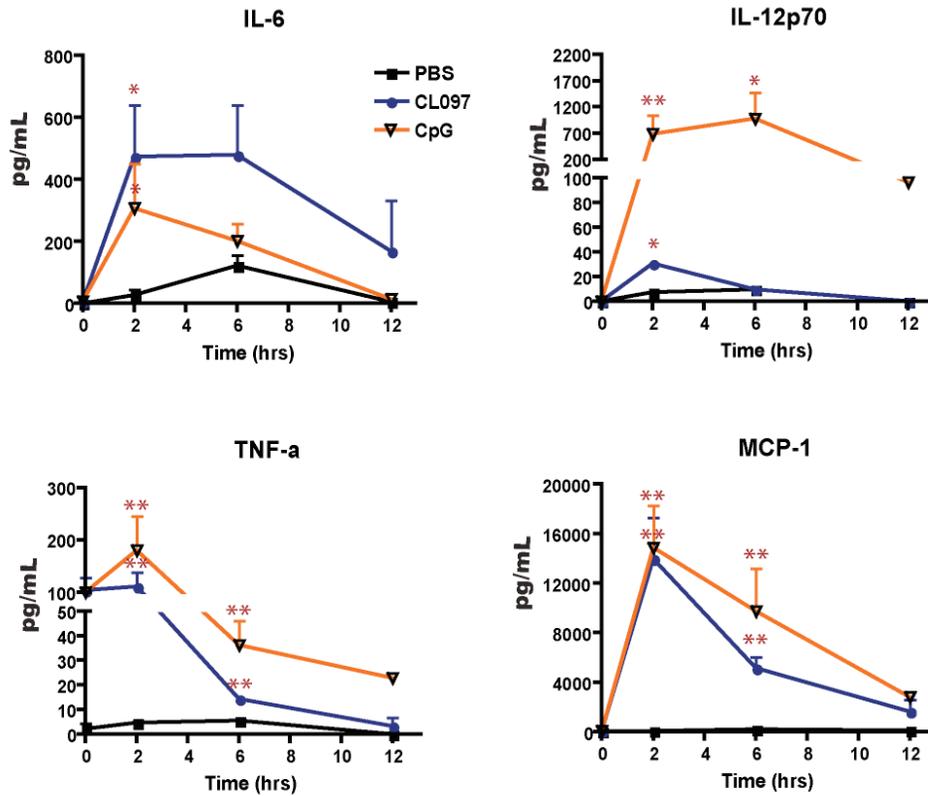


FIGURE 2.3. CL097 increases serum pro-inflammatory cytokines and chemokine in the NOD mouse. Serum levels of IL-6, IL-12p70, TNF- α , and MCP-1 were measured at 0, 2, 6 and 12 hours post-injection of CL097 (5mg/kg) or CpG (5mg/kg). Cytokines were measured by cytometric bead array. Data are representative of at least three independent experiments. CL097 or CpG groups were compared to the PBS group at each time point. *, $p < 0.05$, **, $p < 0.005$ by unpaired t-test.

2.3.3 CL097 and anti-CD40 stimulation induces efficient diabetogenic CTL function in NOD mice

We assessed if CL097 was able to induce the priming and activation of functional diabetogenic CTLs in NOD mice. An *in vivo* antigen specific cytotoxicity assay was used in which naïve splenocytes from a NOD mouse were CFSE-labeled (CFSE HI or CFSE LO) and pulsed with a target peptide, NRP-V7 (islet cell mimetope of IGRP), or a control peptide, TUM, into injected NOD mice. We found that CL097 (5mg/kg, SC) alone caused a modest specific lysis of the target peptide (~25%). However, the combination of CL097 and monoclonal anti-CD40 (10mg/kg, IP) resulted in about a two-fold increase in the specific lysis of the IGRP-peptide coated targets compared to CL097 treatment alone (Figure 2.4). Interestingly, the IGRP-specific lysis induced by the CL097 and anti-CD40 combination was even greater than the CpG and anti-CD40 stimulation in NOD mice. Anti-CD40 agonist was used because studies (31, 32) have shown that CD40 ligand is essential in generating functional CTLs. The main producers of CD40 ligand are thought to be activated CD4⁺ T cells (32). The efficient target lysis observed with combination therapy suggests that DCs may require both TLR7/9 and anti-CD40 stimulation for optimal priming and activation of diabetogenic CTLs in the NOD mouse.

2.3.4 CL097 and anti-CD40 induce the acceleration of T1D in 8.3 NOD mice

The generation of functional diabetogenic CTLs in NOD mice treated with CL097 and anti-CD40 suggest that this treatment combination could accelerate the development of T1D in 8.3 NOD mice. The combination of CL097 and anti-CD40 caused a profound acceleration of T1D compared to the negative control (Figure 2.5). The administration of CL097 resulted in a partial acceleration of T1D whereas no acceleration was observed with anti-CD40 stimulation alone.

2.3.5 IRS661 inhibits TLR7 but not TLR9 induced activation of NOD BMDCs and secretion of proinflammatory cytokines

We next assessed if the blockade of TLR7 signaling in 8.3 NOD mice treated with CL097 and anti-CD40 could delay the accelerated onset of T1D in these mice. To investigate TLR7 inhibition, we used the anti-malarial agent, chloroquine (TLR7-9 inhibitor), and

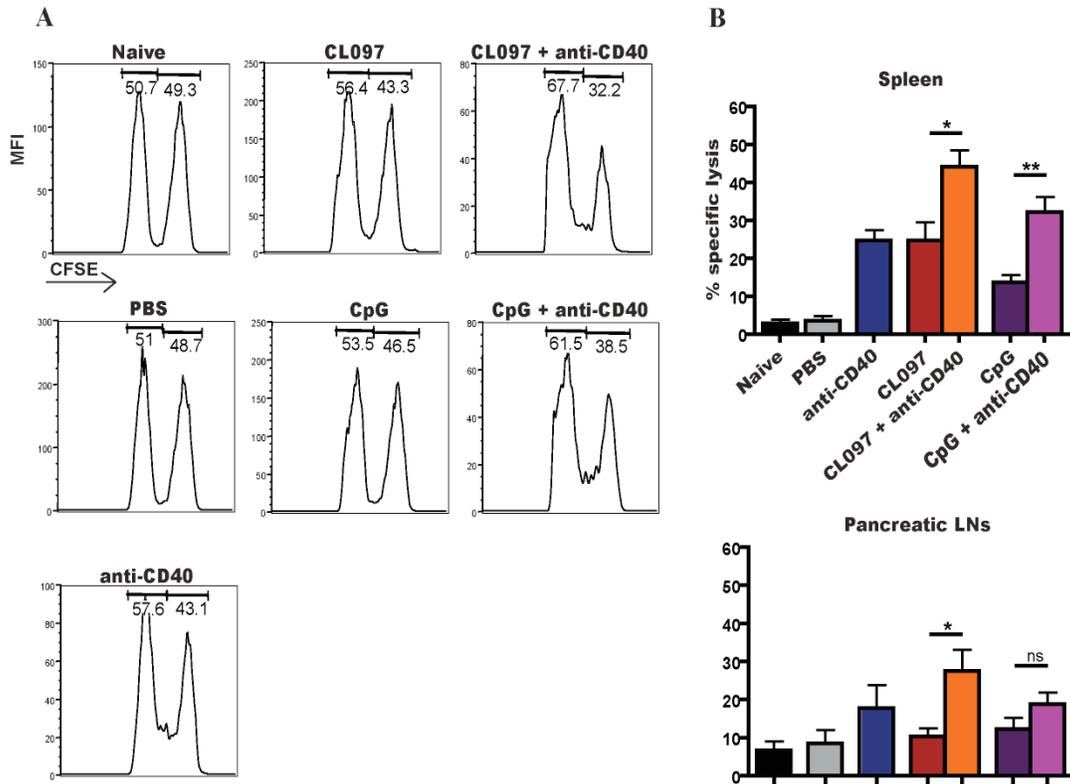


FIGURE 2.4. In vivo cytotoxicity assay of the spleen and pancreatic lymph nodes of CL097, CpG or anti-CD40 treated NOD mice. A, Representative histograms of the spleen showing the percent specific lysis of CFSE HI target peptide (NRP-V7 – islet mimotope of IGRP) over the CFSE LO control peptide (TUM). Splenocytes from a naïve NOD mouse were CFSE labeled (CFSE HI or CFSE LO) and pulsed with target or control peptide. This mixture was adoptively transferred by intravenous injection into NOD mice 24-hours after injection with either CL097 (5mg/kg, SC), CpG (5mg/kg, SC), anti-CD40 (10mg/kg, IP) or in combination. B, Summary of the percent specific lysis of each group in the spleen and in the pancreatic lymph nodes. Data are representative of three independent experiments. *, $p < 0.05$, **, $p < 0.005$ by unpaired t-test.

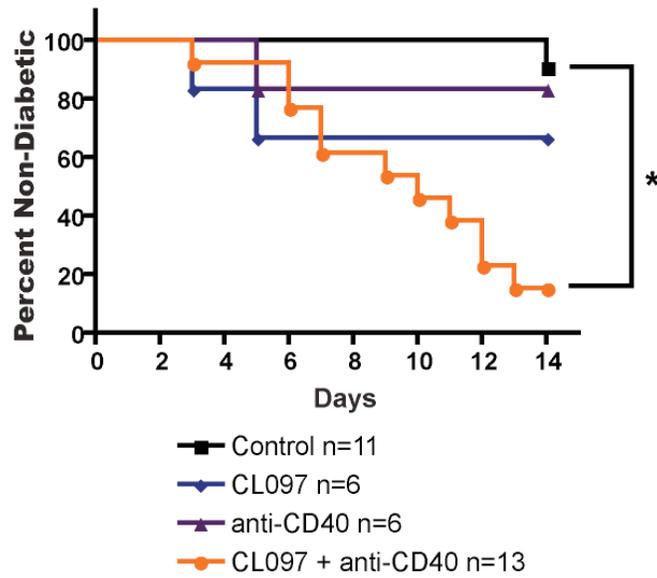


FIGURE 2.5. CL097 and anti-CD40 co-stimulation accelerate the onset of type 1 diabetes (T1D) in 8.3 NOD mice. 5-6 week old female 8.3 NOD mice were treated with either CL097 (5mg/kg, SC), anti-CD40 (10mg/kg, IP) or in combination once. Blood glucose was monitored twice weekly and mice were termed diabetic after two consecutive blood glucose measurements of > 14mM. The end point of accelerated diabetes was established to be within 14 days post-injection. *, $p < 0.005$ by log-rank test on survival curves.

the TLR7 ODN inhibitor, IRS661, on TLR7 or 9 stimulated NOD BMDCs. Chloroquine was found to be effective in blocking the upregulation of CD40 (Figure 2.6A) and the secretion of proinflammatory cytokines (Figure 2.6B) to CpG and R837 stimulated BMDCs. However, chloroquine was ineffective against LOX or CL097 stimulated BMDCs. Higher doses of chloroquine were not successful in inhibiting LOX or CL097 (data not shown). Chloroquine blocks TLR7-9 signaling by inhibiting endosomal acidification which is a necessary prerequisite to induce TLR7-9 activation (11). Similar results to our data has shown that chloroquine or bafilomycin A1 (inhibitor of endosomal acidification) are weak TLR7 but strong TLR9 inhibitors (33, 34). On the other hand, IRS661 was found to specifically inhibit TLR7 agonists only (Figure 2.6).

2.3.6 IRS661 inhibits the general activation of T and B cells, and proinflammatory cytokines in NOD mice treated with CL097 or CpG

Our *in vitro* observations showed that IRS661 specifically inhibits TLR7 only. We found that IRS661 (266mg/kg, SC) inhibits the general activation of CD4⁺ and CD8⁺ T cells and B220^{hi} B cells in the axillary, mesenteric and pancreatic LNs, and the spleen of both CL097 and CpG injected NOD mice (Figure 2.7). Furthermore, IRS661 inhibits the serum levels of IL-6, TNF- α , IL-12p70 and MCP-1 in NOD mice treated with either CL097 or CpG (Figure 2.8). A dose titration of IRS661, even at low concentrations, were found to inhibit both CL097 and CpG injected NOD mice (data not shown). This is the first report demonstrating that IRS661 is not TLR7 specific *in vivo*. The inhibition of both TLR7 and 9 *in vivo* by IRS661 which was not observed *in vitro* suggest a similar activation mechanism or interaction between TLR7 and 9 signaling. In addition, the mechanism of TLR7 inhibition by IRS661 remains unclear. This ODN inhibitor may act downstream of these receptors blocking adaptor molecules that are shared between TLR7 and 9 signaling.

2.3.7 IRS661 delays the acceleration of T1D in 8.3 NOD mice treated with CL097 and anti-CD40

We reasoned that blockade of TLR7 signaling may delay accelerated development of T1D induced by CL097 and anti-CD40 in 8.3 NOD mice. A single treatment of IRS661

in 8.3 NOD mice injected with CL097 and anti-CD40 delayed the acceleration of T1D (Figure 2.9A). The incidence of accelerated disease reverted back to the normal spontaneous development of T1D as mice aged (Figure 2.9B), suggesting that IRS661 treatments may be beneficial in blocking the initial as well as the late stages of type 1 diabetes in NOD mice. The initial priming of diabetogenic CTLs occurs at 2 weeks of age in NOD mice and is driven by high IFN- α production by plasmacytoid DCs (pDCs) (35) which express both TLR7 and 9 (36).

2.3.8 Chloroquine delays T1D transfer in NOD.SCID mice

Lastly, we wanted to determine if blockade of endogenous TLR7/9 signaling at a late stage of T1D would delay disease onset. Most therapeutics used in T1D research are started after the initial priming stage of T1D in NOD mice. To determine the effect of late stage T1D induced by previously activated T cells, we adoptively transferred 2×10^7 splenocytes from diabetic NOD mice into NOD.SCID mice treated with chloroquine. The systemic administration of chloroquine (20mg/kg, IP) caused a minor but significant delay (~one week) ($p < 0.05$) in the development of T1D compared to the negative control (Figure 2.10). This suggests that blockade of endogenous or exogenous TLR7/9 signaling delays the development of T1D induced by previously primed (memory) T cells in NOD mice.

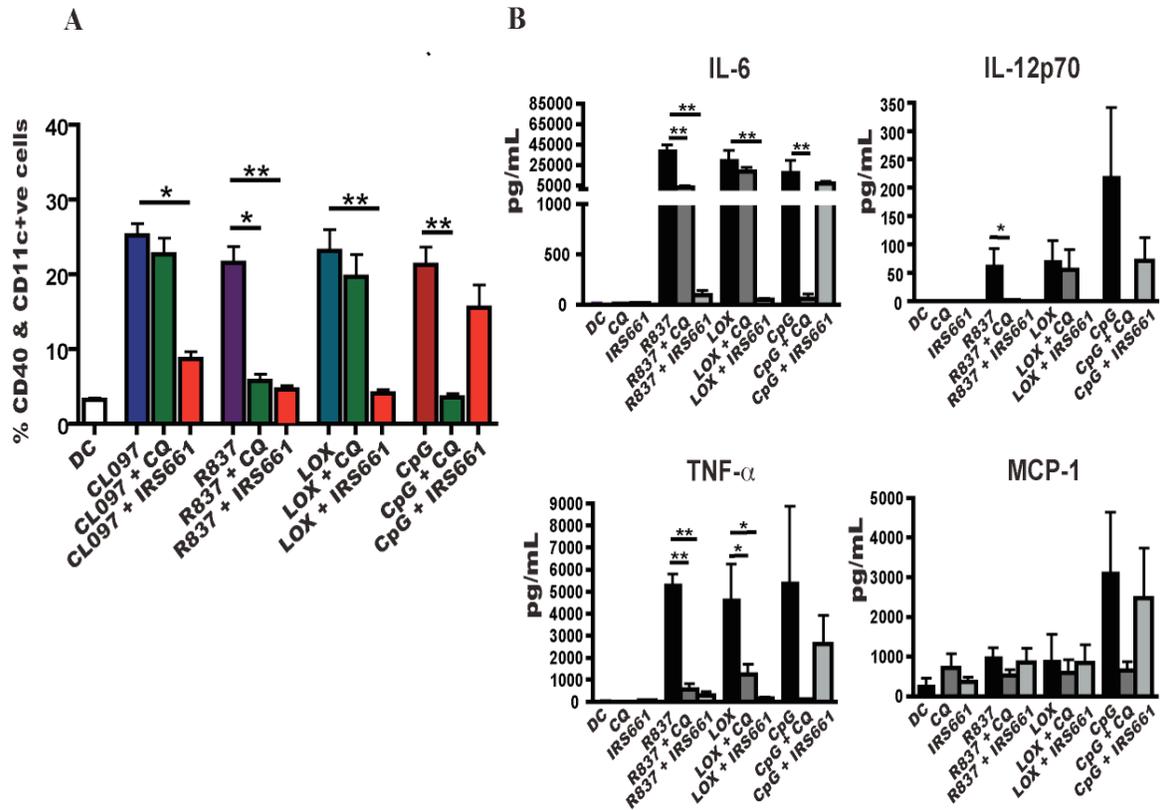


FIGURE 2.6. Chloroquine (CQ) inhibits TLR9 and some TLR7 ligands whereas IRS661 inhibits only the TLR7 ligands on NOD BMDCs. A, Chloroquine (2ug/mL; 6.25uM) or IRS661 (9ug/mL; 1.4uM) were used to inhibit different TLR7 or 9 ligands. TLR7 agonists: R837 (5ug/mL; 20.8uM) and LOX (170ug/mL; 500uM); TLR7/8 agonist: CL097 (1ug/mL; 4.13uM); and TLR9 agonist: CpG (5ug/mL; 0.786uM). Fluorescence cytometry was used to gate for CD11c+ve dendritic cells (DCs) and the expression of the activation marker CD40. B, The supernatants were collected (after 24-hours) from treated BMDCs and analyzed by cytometric bead array for IL-6, IL-12p70, TNF- α and MCP-1. Data are representative of at least three independent experiments. *, $p < 0.05$, **, $p < 0.006$ by one-way ANOVA.

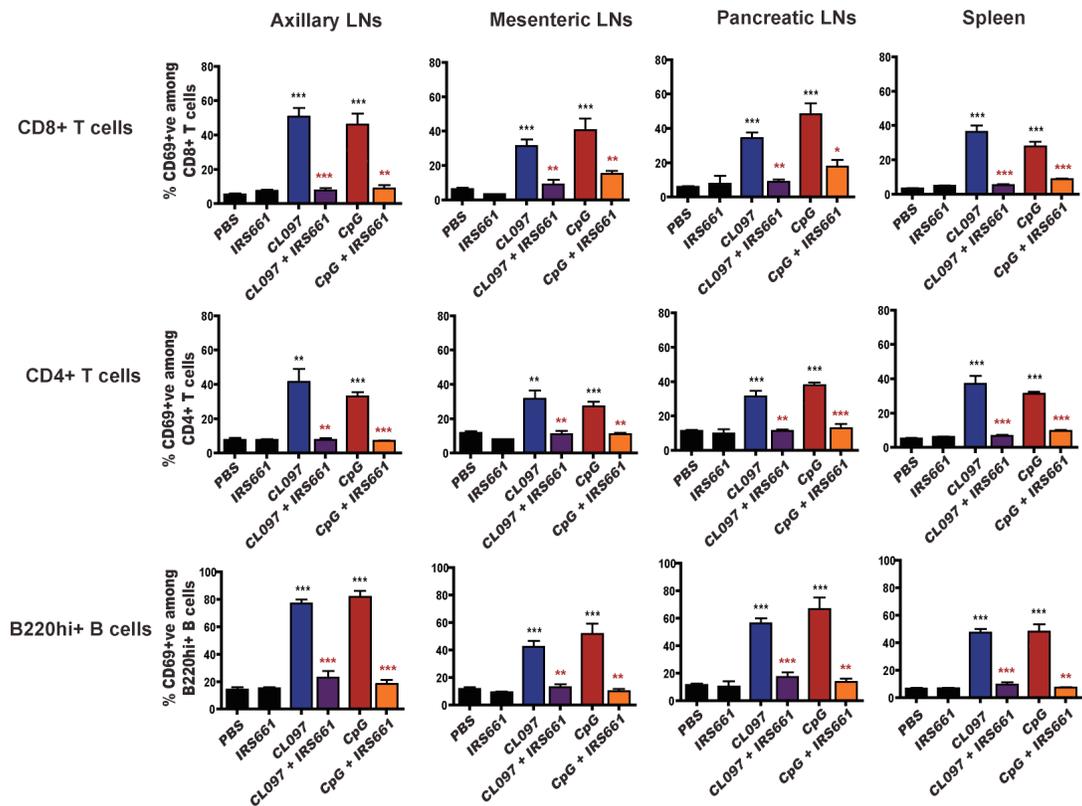


FIGURE 2.7. IRS661 inhibits both CL097 or CpG induced activation of T and B cells in NOD mice. IRS661 (266mg/kg, SC) was injected 2-hours prior to CL097 (5mg/kg, SC) or CpG (5mg/kg, SC) treatment in NOD mice. After 24-hours, the axillary, mesenteric and pancreatic lymph nodes and spleen were extracted and analyzed with fluorescence cytometry. The activation marker CD69 was used to determine activation of CD8+ and CD4+ T cells and B220hi+ B cells. Data are representative of at least three independent experiments. CL097 or CpG groups were compared to the PBS group. CL097 or CpG + IRS661 groups were compared with CL097 or CpG groups, respectively. *, $p < 0.05$, **, $p < 0.009$, ***, $p < 0.0001$ by unpaired t-test.

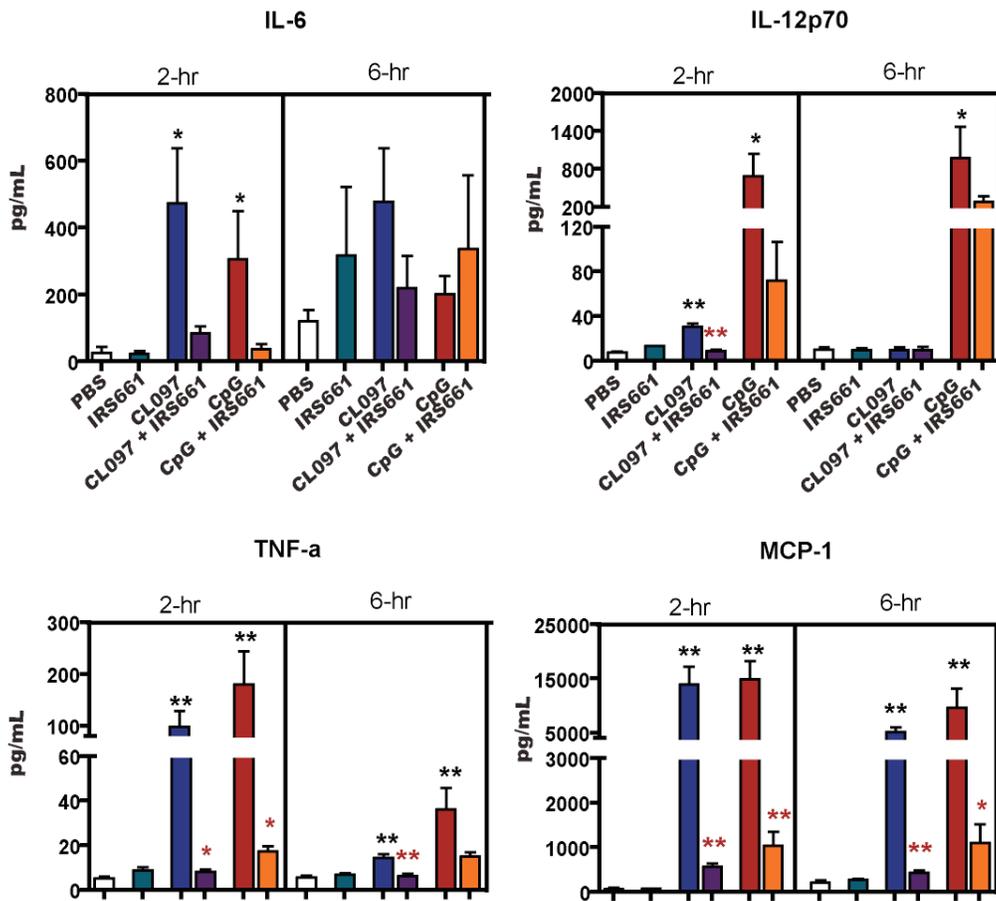


FIGURE 2.8. IRS661 inhibits the increase in pro-inflammatory cytokines and chemokine induced by CL097 or CpG treatment in NOD mice. IRS661 (266mg/kg, SC) was injected 2-hours prior to CL097 (5mg/kg, SC) or CpG (5mg/kg, SC) injection in NOD mice. Serum levels of IL-6, IL-12p70, TNF- α and MCP-1 were measure at 2- and 6- hours post-injection of CL097 or CpG. Cytokines and chemokine were measured by cytometric bead array. Data are representative of at least three independent experiments. CL097 or CpG groups were compared to the PBS group. CL097 or CpG + IRS661 were compared with CL097 or CpG groups, respectively. *, $p < 0.05$, **, $p < 0.009$ by unpaired t-test.

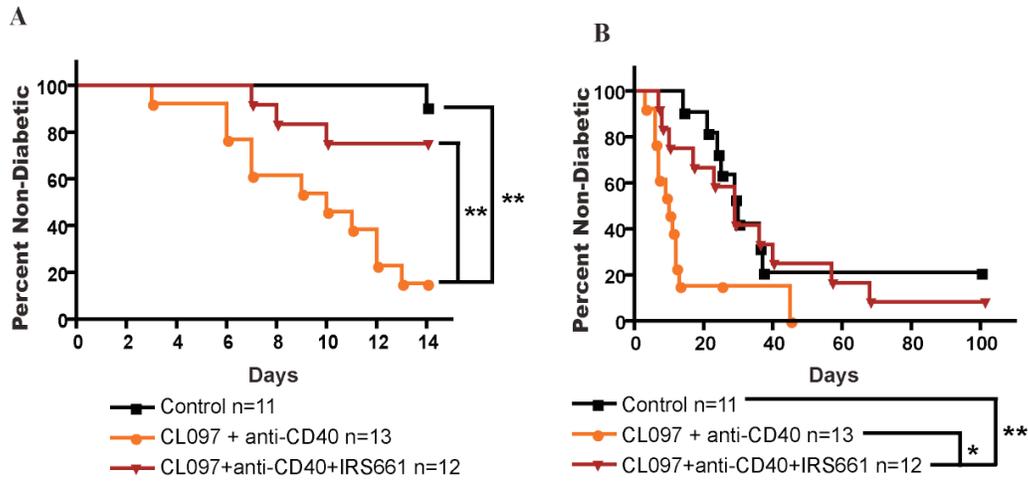


FIGURE 2.9. IRS661 delays the acceleration of type 1 diabetes (T1D) induced by CL097 and anti-CD40 treatment in 8.3 NOD mice. A, Diabetes survival curve after 14-days post-injection of 8.3 NOD mice. 5-6 week old female 8.3 NOD mice were injected with IRS661 (266mg/kg, SC) 2-hours prior to CL097 (5mg/kg, SC) and anti-CD40 (10mg/kg, IP) treatment. B, Diabetes survival curve over the long term in treated 8.3 NOD mice. Blood glucose was monitored twice weekly and mice were termed diabetic after two consecutive blood glucose measurements of > 14mM. The end point of accelerated diabetes was established to be within 14 days post-injection. *, $p < 0.05$, ** $p < 0.005$ by log-rank test on survival curves.

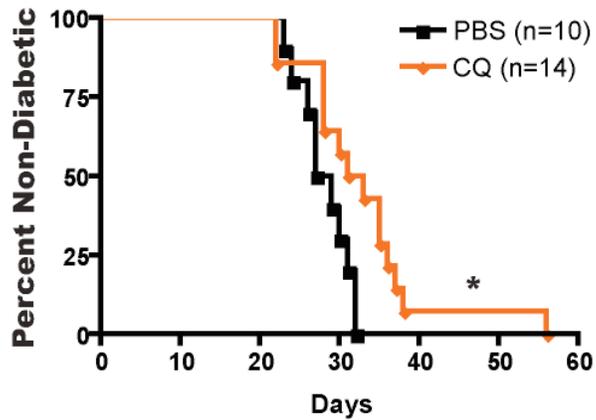


FIGURE 2.10 Chloroquine (CQ) inhibits the transfer of type 1 diabetes (T1D) in NOD.SCID mice. Chloroquine (20mg/kg, IP) was given for five consecutive days followed by twice per week until development of diabetes in NOD.SCID mice. 2×10^7 splenocytes from a diabetic NOD mouse were adoptively transferred intravenously into NOD.SCID mice treated with PBS or CQ. Blood glucose was monitored twice weekly and mice were termed diabetic after two consecutive blood glucose measurements of $> 14\text{mM}$. *, $p < 0.05$ by log-rank test on survival curves.

2.4 DISCUSSION

We have shown that TLR7 signaling requires CD40 agonist to induce the priming and activation of functional diabetogenic CTLs in the NOD mouse. This subsequent combination accelerated T1D onset in 8.3 NOD mice. Inhibition of TLR7 signaling with IRS661 delayed the acceleration of diabetes in 8.3 NOD mice treated with CL097 and anti-CD40. Chloroquine (TLR7-9 inhibitor) was also found to delay T1D transfer in NOD.SCID mice demonstrating that TLR stimulation may also accelerate diabetogenesis by (previously activated) memory T cells.

A single administration of CL097 in NOD mice induced a general activation of polyclonal CD4⁺ and CD8⁺ T cells as well as B220^{hi} B cells. This stimulation also induced high serum levels of IL-6, IL-12p70, TNF- α and MCP-1. Previous studies have shown that TLR7 can directly mature DCs (37, 38) to induce the production of proinflammatory cytokines, chemokines and type 1 interferons (IFNs) (37-41) as well as direct the activation of the adaptive immune response (37, 39, 42, 43). This supports our *in vitro* and *in vivo* data showing that TLR7 stimulation can lead to the activation of both the innate and adaptive immune responses in the NOD mouse.

The general activation of the immune system by CL097 may also generate diabetogenic CTLs in the NOD mouse. We have shown that CL097 treatment in NOD mice induces functional diabetogenic CTLs. A single treatment of CL097 was insufficient to accelerate T1D in 8.3 NOD mice. The co-administration of CD40 agonist with CL097 doubled the cytotoxic function of diabetogenic CTLs in the NOD mouse and led to an acceleration of T1D in 8.3 NOD mice. This finding is similar to what has been reported in transgenic models in which TLR signaling requires CD40 co-stimulation from activated CD4⁺ T cells to induce autoimmune disease (23, 44). These observations suggest that another signal besides TLR signaling is critical in breaking self-tolerance.

Studies (24, 45) have shown that TLR7 signaling alone generates an autoimmune phenotype in transgenic models but is insufficient to elicit T1D. We conclude that at least two triggers are required in succession or in combination in the NOD mouse to convert

autoimmunity into overt autoimmune diabetes. The actions of TLR7 and CD40 signaling are similar in that both play a role in generating an autoimmune phenotype but fail to initiate overt autoimmune diabetes in genetically susceptible individuals. In addition, TLR or CD40 signaling alone are able to prime diabetogenic CTLs but are insufficient to induce overt autoimmunity (23, 44). The subsequent combination of the two, as shown in our data, breaks self-tolerance to accelerate the development of T1D in 8.3 NOD mice. The inhibition of endogenous TLR signaling may provide a therapeutic avenue in maintaining self-tolerance in genetically susceptible individuals. Our TLR7 inhibition studies support this notion in which blockade of the TLR7 signaling component by IRS661 suppresses the accelerated development of T1D in 8.3 NOD mice treated with CL097 and anti-CD40. Further studies are required to determine if blockade of endogenous or exogenous TLR7 stimulation by self-ligands or viruses such as CVB, respectively can prevent the development of T1D in genetically susceptible individuals.

Although T1D is a T-cell mediated destruction of β cells, there are studies (35, 46) showing that self-reactive B cells and the IFN- α producing pDCs may play important roles in the pathogenesis of T1D. We show that TLR7 or 9 stimulation in NOD mice leads to the general activation of B cells as well as splenic DCs. This activation of DCs suggests a possible activation of pDCs in our model. In humans and mice, TLR7 and 9 are predominately expressed in pDCs and B cells. Mice also have these two receptors expressed on myeloid DCs (36). Moreover, the increased production of diabetes-related autoantibodies such as classical islet cell, insulin, glutamic acid decarboxylase (GAD), and tyrosine phosphatase-related IA-2 molecule (IA-2A) autoantibodies have been used to predict the clinical onset of T1D in genetically susceptible individuals (46). Furthermore, recent studies (35, 47) have shown that IFN- α initiates T1D in both NOD mice and in humans. Similarly, SLE is a B cell mediated autoimmune disease that has characteristics similar to the pathogenesis of T1D. In SLE, the increased production of autoantibodies and IFN- α by B cells and pDCs, respectively have been found to correlate with disease severity (36, 42). These two immune cells become activated in SLE through the directly uptake of antibody or immune complexes to self -DNA or -RNA through the Fc γ RIIa on pDCs (48) and through the B cell receptor on B cells (39, 42). The increased

production of IFN- α promotes self-reactive B cell survival, activation and differentiation into antibody producing plasma cells (39, 42). This leads to the increased formation of antibody or immune complexes which are potent activators of pDCs resulting in disease severity. A similar paradigm may arise in the pathogenesis of T1D in which inappropriate TLR7/9 stimulation results in the increased production of autoantibodies which may form antibody or immune complexes that stimulate the IFN- α producing pDCs. In T1D, the increased IFN- α may also contribute to the priming, activation and functional activity of diabetogenic CTLs. Moreover, viral infections in SLE patients which induces IFN- α production have been found to be associated with lupus flares (39). A similar mechanism may occur in T1D in which viral infections promote the production of IFN- α which in turn results in a wave of diabetogenic CTL activation and destruction of β cells. Further studies are required to determine the role of pDCs and B cells in the pathogenesis of T1D.

The use of the inhibitory ODN, IRS661 revealed a general suppression of both T and B cells in TLR7 or 9 stimulated NOD mice. It remains unclear whether TLR7 or 9 work independently, synergistically or even antagonistically in contributing to the pathogenesis of T1D in NOD mice. In mouse models of SLE, it has been suggested that TLR9 (regulatory) and TLR7 (inflammatory) have opposing roles in the development of this disease (49). However, we have studies suggesting that both TLR7 and 9 are important in modulating the development of T1D. TLR9 signaling plays a similar function to TLR7 signaling in the activation of diabetogenic CTLs and the induction of T1D in NOD and 8.3 NOD mice (Zhang *et al.*, submitted; see Appendix 1). In addition, administration of chloroquine delayed T1D (Zhang *et al.*, submitted; see Appendix 1) and the transfer of T1D in NOD and NOD.SCID mice, respectively. Further work will be required to clarify the relative importance of TLR7 and/or 9 signaling in the development of T1D.

Endosomal TLRs and innate immune activation play an important role in the development of T1D in NOD mice. Our data support the hypothesis that environmental factors such as viruses may contribute to the development of T1D (19, 22).

Understanding the role of endosomal TLR signaling in the pathogenesis of T1D in

genetically susceptible animal models may provide a therapeutic approach in preventing T1D in genetically susceptible individuals.

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3. Infection By An Enteric Bacterial Pathogen Accelerates Insulinitis And Activates Diabetogenic CD8⁺ T Cells In NOD Mice²

3.1 INTRODUCTION

There is evidence that changes in the intestine, induced by diet or enteric antigens, may alter the incidence and course of type 1 diabetes (T1D) (1, 2). In fact, impaired intestinal barrier function has been detected in type 1 diabetics and their relatives (3, 4). The mucosal surface of the intestine functions as the primary interface site between the host and its environment, including the myriad of microbes that make up the intestinal microbiota. As such, the intestine represents a major induction site for regulatory immune responses. Normally, the intestinal epithelium provides a critical protective barrier preventing both pathogenic and commensal bacteria from escaping the intestinal lumen and activating the systemic immune system. Disruption of this delicate barrier, and the resulting inflammation, promotes the initiation and development of intestinal and intestinal-related autoimmune diseases such as celiac disease (5) and inflammatory bowel disease (IBD) (6), however its impact on other autoimmune diseases such as T1D, is less clear. We hypothesize that impaired intestinal barrier function in diabetic-prone individuals may allow commensal or invading enteric bacterial pathogens to elicit inflammatory signals that promote T1D.

T1D is the result of an immune mediated destruction of insulin producing β cells in the pancreas where cytotoxic T lymphocytes (CTLs) are the predominant effectors of their targeted destruction (7). The initial factors that promote diabetogenic CTL activation in individuals at-risk for T1D are still unknown, however the primary activation of diabetogenic CTLs appears to occur principally within the pancreatic as well as in the gut-associated lymph nodes (LNs) (8, 9). This finding indicates that the lymphocytes

² A version of this chapter has been submitted for publication. Andrew S. Lee, Deanna L. Gibson, YiQun Zhang, Ho Pam Sham, Bruce A. Vallance and Jan P. Dutz. Infection By An Enteric Bacterial Pathogen Accelerates Insulinitis And Activates Diabetogenic CD8⁺ T Cells In NOD Mice.

within the pancreatic LNs connect and communicate with the intestinal immune system. In addition, other studies (2, 10) have found that the gut and its luminal contents might contribute to the initiation of T1D. Turley *et al.* (2005) (11) showed that luminal antigens from the gut are preferentially transported to the pancreatic LNs, rather than to the mesenteric LNs, resulting in the proliferation of antigen-specific CTLs. Additionally, diabetogenic CTLs were found to express the gut-homing receptor $\alpha 4\beta 7$ integrin (2) and CTLs activated in the gastrointestinal (GI) tract of NOD mice were found to home to islets which express the mucosal homing receptor MadCAM-1 (2).

Based on these findings, we hypothesized that impaired intestinal barrier function exposing the immune system to GI microbes and other luminal antigens might provide an inflammatory stimulus that drives the autoimmune response in T1D. In this study, we demonstrate that pre-diabetic NOD mice develop an intrinsic intestinal barrier dysfunction by 12-weeks of age. Moreover, colonic infection of young (4-week) NOD mice by *C. rodentium*, an enteric bacterial pathogen that disrupts intestinal barrier function, promotes the development of invasive insulinitis. These findings demonstrate for the first time a potential role for enteric bacterial pathogens in modifying islet inflammation in T1D and suggest that intestinal barrier function plays an important role in protecting against T1D.

3.2 MATERIALS AND METHODS

3.2.1 Mice and infection

NOD mice were obtained from the Jackson Laboratory and bred in a specific pathogen-free (SPF) environment. The 8.3 NOD mice, expressing the rearranged TCR genes of the diabetogenic CTL clone NY8.3, have been previously described (12). All animals were housed under SPF conditions at the Child and Family Research Institute in Vancouver, Canada and used for experiments between 4 and 12 weeks of age. Mice were infected by oral gavage with 0.1 ml of an overnight culture in Luria broth containing approximately 2.5×10^8 CFU of wild-type *Citrobacter rodentium* (formerly *C. freundii* biotype 4280 strain DBS100) (13). Experiments were conducted and approved by the institutional animal ethics committee.

3.2.2 FITC dextran assay

The FITC dextran assay has been previously described (13). Briefly, for assessment of barrier function within the entire GI tract, 150uL of 80mg/mL of FITC dextran (FD4, Sigma-Aldrich) in PBS was introduced to mice by oral gavage, while 100uL was introduced by enema for studies assessing intestinal barrier function following *C. rodentium* infection. After 2-hours (enema) or 4-hours (oral gavage), sera was collected by cardiac puncture and measured with a fluorimeter.

3.2.3 Insulinitis scoring

Paraffin-embedded sections of the pancreas were stained with hematoxylin and eosin, and examined by light microscopy. Assessment of insulinitis has been described (14). Insulinitis severity was assessed by an observer blinded to tissue source by counting at least 30 pancreatic islets per mouse from four parallel sections of different cut levels of the pancreas. The degree of insulinitis was classified into four categories: 0, no insulinitis; 1, peri-insulinitis with or without minimal lymphocytic infiltration in islets; 2, invasive insulinitis with <50% of lymphocytic infiltration of islets; 3, invasive insulinitis with $\geq 50\%$ of lymphocytic infiltration of islets.

3.2.4 Adoptive transfers, lymph node preparation and flow cytometry analysis

Naive 8.3 CD8⁺ T cells (IGRP-specific (islet antigen) CD8⁺ T cells) were prepared and purified from the peripheral LNs and spleen of transgenic mice and CFSE labeled as described (15). The labeled cells (1×10^7) were transferred to *C. rodentium* infected NOD recipients intravenously by tail vein at day 10 post-infection. LN single cell suspensions were prepared, stained and acquired on a FACSCalibur flow cytometer (BD Bioscience) as described (15).

3.2.5 Bacteria culturing and count

C. rodentium culturing and total bacteria counts have been previously described (13). Whole gut tissues including stools or LNs were collected from *C. rodentium* infected

mice, homogenized, plated on MacConkey plates, incubated at 37°C, and enumerated the following day.

3.2.6 Statistical analysis

A two-tailed Student's t test and non-parametric Mann–Whitney T-tests was used to calculate statistical significance where indicated. All statistical analysis was conducted using Prism 3 (GraphPad software).

3.3 RESULTS

3.3.1 Intrinsic intestinal barrier dysfunction in pre-diabetic (12-week) NOD mice

Recent evidence has suggested a intriguing putative link between intestinal barrier dysfunction and subsequent enteropathies, and an increased risk for T1D in both rat models and in humans (1, 2, 6). To explore the role of intestinal barrier permeability in the pathogenesis of T1D, we examined intestinal epithelial barrier function in the NOD mouse.

Pre-diabetic (12-week) NOD mice exhibited increased serum levels of FITC dextran compared to age matched C57BL/6 and non-obese diabetic resistant (NOR) mice, respectively following oral gavage (Figure 3.1). However, younger pre-diabetic (4 and 8 week) NOD mice did not exhibit differences in serum FITC dextran levels compared to age matched C57BL/6 and NOR mice, respectively (data not shown). This age-dependent dysfunction of the intestinal barrier in NOD mice shows that increased barrier permeability occurs at the time of insulinitis and precedes the development of T1D. The observed barrier disruption may either participate in diabetogenesis or may be a consequence of diabetogenic inflammation.

3.3.2 *C. rodentium* infection disrupts intestinal barrier function in NOD mice

To determine if intestinal barrier disruption may affect diabetogenic autoimmune inflammation, we assessed if damage to the barrier integrity at a young age (4-week) in NOD mice would alter inflammation within the pancreatic islets. *C. rodentium*, an enteric bacterial pathogen that disrupts the intestinal epithelium, was used to infect young pre-

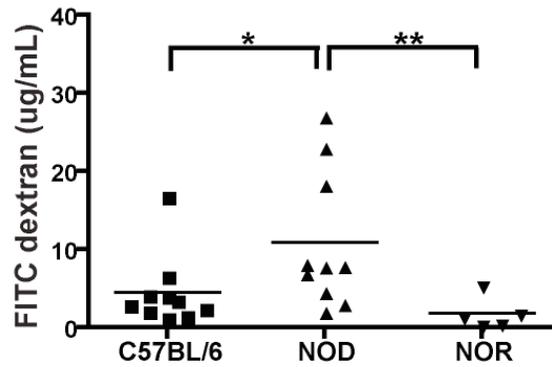


FIGURE 3.1. Intrinsic intestinal barrier dysfunction in pre-diabetic NOD mice.

Assessment of the intestinal barrier integrity with an orally administered FITC dextran in 12-week old C57BL/6, NOD and NOR mice. Data are representative of two independent experiments. *, $p < 0.05$; ** $p < 0.005$ by non-parametric Mann–Whitney t-test.

diabetic (4-week) NOD mice. *C. rodentium* is a gram-negative attaching/effacing mucosal pathogen that rapidly infects the colonic epithelium of mice in an identical fashion to that utilized by enteropathogenic and enterohemorrhagic *Escherichia coli* (16). In addition, *C. rodentium* infected mice develop a colitis characterized by inflammatory cell infiltration, crypt cell hyperplasia, goblet cell depletion and significant intestinal barrier disruption (13, 16). *C. rodentium* infection significantly increased intestinal barrier permeability in NOD mice as measured by the increased levels of FITC dextran recovered from the serum of infected mice following rectal instillation (Figure 3.2A).

3.3.3 *C. rodentium* translocates to the mesenteric and pancreatic LNs of infected NOD mice

We next assessed the *C. rodentium* colony forming units within the colon and cecum, and examined if the resulting breach of the intestinal barrier led to this pathogen reaching the mesenteric and pancreatic LNs. We found that *C. rodentium* counts were higher in the pancreatic and mesenteric LNs, and in the cecum and colon of infected NOD mice compared to infected C57BL/6 mice (Figure 3.2B). However, there were no differences in *C. rodentium* detected in the pancreas, spleen or liver in either mouse strain (data not shown). The *C. rodentium* recovered from the LNs of infected mice may reflect those microbes specifically transported to these lymphoid tissues by dendritic cells or macrophages, either as a result of sampling the intestinal lumen, or following phagocytosis of *C. rodentium* that passively leaked across a disrupted intestinal barrier.

3.3.4 *C. rodentium* accelerates invasive insulinitis in NOD mice

C. rodentium infection of young (4-week) pre-diabetic NOD mice accelerated the development of invasive insulinitis as assessed at 12 weeks of age (8 weeks post-infection). Infection was found to significantly increase the percentage of islets exhibiting $\geq 50\%$ lymphocytic infiltration as compared to age matched controls (Figure 3.3A). As expected, with more islets showing heavy lymphocytic infiltration in infected NOD mice, there were significantly fewer islets in these mice exhibiting no infiltration, compared to non-infected controls (Figure 3.3B). These results suggest that enteric infection with *C. rodentium* and subsequent inflammation may modulate the development of T1D.

3.3.5 C. rodentium increases the proliferation and activation of diabetogenic CD8⁺ T cells and the activation of polyclonal CD8⁺ and CD4⁺ T cells in NOD mice

The increase in lymphocytic infiltration into the islets in infected NOD mice suggest an increase in the priming and activation of diabetogenic or polyclonal CTLs. Adoptive transfer of IGRP-specific (islet antigen) CD8⁺ T cells (15) into infected NOD mice revealed an increase in the proliferation and activation of these cells in the pancreatic LNs (Figure 3.4A). In addition, infected NOD mice display increased expression of the early and late activation markers, CD69 and CD25, respectively, on polyclonal CD8⁺ and CD4⁺ T cells in the mesenteric and pancreatic LNs (Figure 3.4B). Thus, *C. rodentium* infection enhances the activation of polyclonal and diabetogenic CTLs in the early stages of T1D.

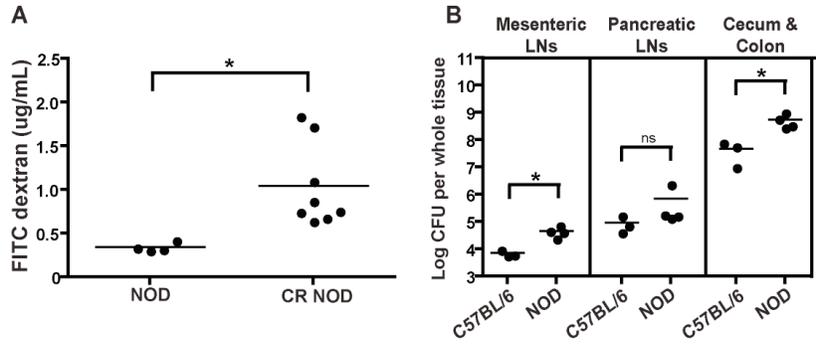


FIGURE 3.2. *C. rodentium* (CR) infection increases intestinal barrier dysfunction and increases translocation to the mesenteric and pancreatic LNs in NOD mice. A, Increased intestinal barrier permeability in 4-week old NOD mice infected with *C. rodentium* for 10 days versus non-infected mice. Assessment of the intestinal barrier integrity with an enema administered FITC dextran in infected versus non-infected mice. **B,** Increased *C. rodentium* colony forming units were found in the mesenteric and pancreatic LNs, and the cecum and colon of infected NOD versus infected C57BL/6 mice at day 8 post-infection. Data are representative of two independent experiments. *p <0.05; **p <0.009 by unpaired t-tests.

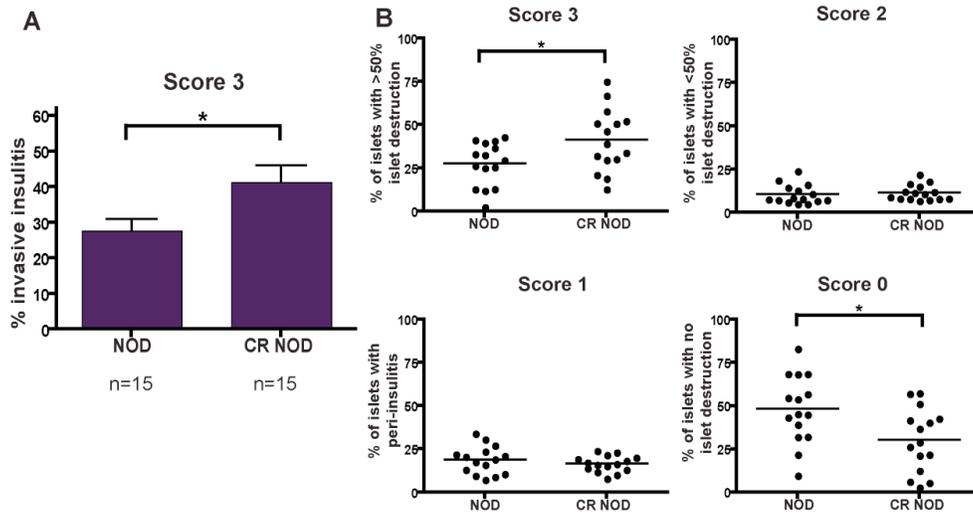


FIGURE 3.3. *C. rodentium* (CR) accelerates the development of invasive insulinitis in NOD mice. **A**, % invasive insulinitis: % of islets with $\geq 50\%$ lymphocytic infiltration in islets. **B**, Individual insulinitis score of the pancreas in infected and non-infected NOD mice at 12 weeks of age: 0, no insulinitis; 1, peri-insulinitis with or without minimal lymphocytic infiltration in islets; 2, invasive insulinitis with $< 50\%$ of lymphocytic infiltration of islets; 3, invasive insulinitis with $\geq 50\%$ of lymphocytic infiltration of islets. Data are representative of two independent experiments. * $p < 0.05$ by unpaired t-tests.

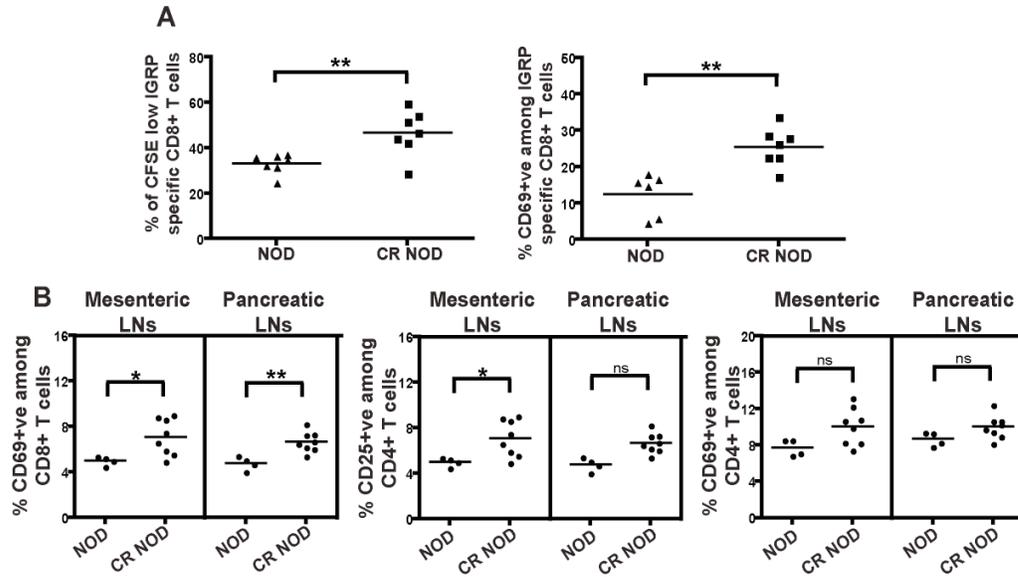


FIGURE 3.4. *C. rodentium* (CR) increases the proliferation and activation of diabetogenic CD8+ T cells and the activation of polyclonal CD8+ and CD4+ T cells. **A**, Increased proliferation and activation of IGRP specific CD8+ T cells in the pancreatic LNs of infected NOD mice. **B**, Increased upregulation of CD69 or CD25 in polyclonal CD8+ and CD4+ T cells in the mesenteric or pancreatic LNs of infected NOD mice. Data are representative of two independent experiments. *p <0.05; **p <0.009 by unpaired t-tests.

3.4 DISCUSSION

The present study demonstrates that alterations in GI barrier function modify the extra-intestinal autoimmune disease, T1D. NOD mice develop an overt intestinal barrier defect at the time of insulinitis and preceding the development of T1D. Moreover, infection of NOD mice with the enteric bacterial pathogen, *C. rodentium*, accelerates the development of invasive insulinitis. The increased lymphocytic infiltration within these islets is concurrent with increased activation of polyclonal and diabetogenic CTLs. These data suggest that exposure to gut antigens may increase the risk of activating diabetogenic CTLs and subsequently developing invasive insulinitis and T1D.

There is mounting evidence suggesting that intrinsic or induced intestinal barrier defects skew the intestinal immune environment or the commensal microbiota to promote autoimmune diseases (1, 2, 6). For example, T1D has been epidemiologically linked to celiac disease (1, 6). Both autoimmune diseases share common genetic susceptibility loci such as the HLA-DQ2 allele and similar environmental triggers such as wheat gluten (1, 6). In addition, T1D and celiac disease share similar autoimmune pathologies in which gut barrier dysfunction may be the prime initiator for each respective disease (1, 6). In contrast, relatively little is known about the potential role of enteric bacterial pathogens in modulating the occurrence of T1D. Recent studies have implicated innate recognition of enteric commensal microbes in the modulation of diabetes suffered by NOD mice (17).

We show an enhanced hematogenous FITC dextran uptake following oral gavage in pre-diabetic (12-week) NOD mice. This observation does not provide definitive anatomical location of barrier defect but Hadjiyanni *et al.* (2009) (18) have described a barrier defect in the jejunum of 7-10 week old NOD mice. It remains to be determined if this anomaly participates in the onset of diabetes or is the result of islet inflammation. In contrast, enteric infection with *C. rodentium* several weeks prior to the development of insulinitis induces significant intestinal barrier disruption in the large intestine (9) and accelerates invasive insulinitis suggesting that barrier dysfunction at different sites within the GI tract may modulate the development of T1D.

Interestingly, while *C. rodentium* infection of young NOD mice promotes invasive insulinitis, a report by Raine *et al.* (2006) (19) showed that intravenous infection with attenuated *Salmonella enteric* serovar Typhimurium (*Salmonella* Typhimurium) halted T1D onset in NOD mice. These differences in the impact on T1D development may reflect that *Salmonella* Typhimurium did not pass through an enteric phase or cause the intestinal barrier disruption seen with *C. rodentium* infection. Moreover, these differences suggest that the aggravation of insulinitis scores during *C. rodentium* infection is not merely due to the presence of bacteria within the host, but instead that disruption of the gut barrier is the predisposing factor. This hypothesis is supported by Turley *et al.* (2005) (11) which found chemical disruption of the intestine with 2% dextran sodium sulfate in the drinking water led to the proliferation of diabetogenic T cells and the accelerated development of invasive insulinitis.

It has recently become clear that innate and adaptive immune responses coordinated by epithelial toll-like receptor (TLR) signaling modulate the enteric flora and in turn are affected by gut microbes (20, 21). Epithelial TLR signaling has been shown to be critical in maintaining intestinal epithelial integrity (20) and promoting the generation of regulatory T cells (21) to provide a steady-state gut environment. Disruption of this homeostasis as a result of a dysregulated interaction between TLRs and enteric bacteria has been shown to promote IBD and T1D (2, 17, 22). Despite these findings, it remains unclear whether disease onset is initiated via epithelial and/or antigen presenting cell mediated TLR or other innate signaling.

Although there are no definitive clinical studies linking enteric bacterial infections to the development of human T1D, there are many examples of type 1 diabetics who also have colitis (23-25). Our results suggest that intestinal barrier dysfunction, in this case mediated by *C. rodentium*, is a catalyst in the accelerated development of invasive insulinitis by allowing enteric bacterial antigens to leak across and activate polyclonal and diabetogenic CTLs. Manipulation of the intestinal barrier integrity or the enteric flora may therefore offer therapeutic avenues for the treatment of T1D.

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4. CONCLUSION

This thesis addresses two potential triggers that are involved in modulating the development of T1D in NOD mouse models – TLR7 and an enteric bacterial infection. First, we demonstrated that TLR7 signaling plays an important role in the development of T1D. The TLR7/8 ligand, CL097, stimulates the activation and secretion of pro-inflammatory cytokines and chemokine from NOD derived BMDCs. *In vivo* TLR7 stimulation with CL097 in NOD mice induced a general activation of T and B cells as well as increased serum levels of pro-inflammatory cytokines and chemokine. The general activation of polyclonal T cells suggests that diabetogenic CTLs could also become activated in NOD mice. The treatment of CL097 and anti-CD40 led to an efficient specific lysis of IGRP targets which suggests that diabetogenic CTLs become functional with this treatment combination. This subsequent treatment combination accelerated the onset of T1D in 8.3 NOD mice. Inhibition of TLR7 signaling with an inhibitory ODN, IRS661, delayed the accelerated development of T1D in 8.3 NOD mice treated with CL097 and anti-CD40. This data suggests that TLR7 signaling plays an important role in generating functional diabetogenic CTLs that may accelerate the development of T1D in genetically susceptible individuals.

The second part of this thesis examined if the gastrointestinal (GI) system via a ‘leaky gut’ may provide an endogenous TLR source to initiate or fuel the development of T1D in NOD mice. We showed that pre-diabetic (12-week) NOD mice develop an intrinsic intestinal barrier dysfunction which precedes the development of T1D. This barrier dysfunction was not observed in young pre-diabetic (4 and 8 week) NOD mice suggesting that gut barrier impairment may be due to diabetes-related inflammation or environmental factors. To assess the impact of barrier impairment, an enteric bacterial pathogen, *Citrobacter rodentium*, that disrupts the intestinal barrier integrity was used in young pre-diabetic (4-week) NOD mice. *C. rodentium* infected NOD mice accelerated the development of severe invasive insulinitis compared to non-infected controls at 12 weeks of age. *C. rodentium* infection was found to induce barrier damage in young pre-diabetic (4 weeks) NOD mice compared to non-infected controls. Moreover, an increase in *C. rodentium* was detected specifically in the mesenteric and pancreatic LNs of

infected NOD versus infected C57BL/6 mice. The *C. rodentium* recovered from the LNs of infected mice likely reflect those microbes specifically transported to these lymphoid tissues by dendritic cells or macrophages, either as a result of sampling the intestinal lumen, or following phagocytosis of those *C. rodentium* that passively leaked across the disrupted intestinal barrier. In addition, *C. rodentium* infection was found to increase the activation of polyclonal CD4+ and CD8+ T cells as well as the activation and proliferation of diabetogenic CTLs. These results indicate that a physiological insult at an early age by an enteric bacterial pathogen activates diabetogenic and polyclonal CTLs and accelerates the development of invasive insulinitis in NOD mice.

Taken together, these findings have demonstrated that inappropriate TLR7, and temporal or induced changes in the intestinal barrier contribute to the pathogenesis of T1D. This suggests that a possible source of endogenous or exogenous TLR7 stimulation may arise from the GI tract. Studies have shown an association between enterovirus and rotavirus infections and the development of β -cell autoimmunity in humans at risk for T1D (1, 2). In addition, recent studies (3, 4) have shown an increase frequency of enteroviral-derived protein VP1 in small bowel biopsy samples and in the islets of pancreatic tissues from type 1 diabetic patients compared to non-diabetic patients. Moreover, enteroviruses such as the coxsackie B viruses (CBV) are recognized through TLR7 and have been implicated as a trigger of T1D development (5, 6). These findings suggest that enteric infections provide a potential source of inappropriate TLR stimulation resulting in an autoimmune process that leads to the development of T1D in genetically susceptible individuals.

4.1 Future Directions

This research showed that TLR7 and *C. rodentium* play an important role in modulating the development of T1D in NOD mouse models. TLR7 has been implicated in the development of several autoimmune and inflammatory diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjogren's syndrome, multiple sclerosis (MS), inflammatory bowel disease (IBD) and psoriasis (7). However, the mechanism involved in the activation and priming of functional diabetogenic and polyclonal CTLs

induced by TLR7 signaling as well as potential endogenous TLR7 sources that may mediate disease onset in T1D remains unclear. In addition, recent evidence (8-11) has suggested that the GI system via leakage of luminal contents may provide a potential source of exogenous and/or endogenous TLR stimulation. Moreover, the presence of an intrinsic or induced leaky gut has been linked to the development of intestinal autoimmune diseases such as celiac disease (12) and IBD (13), and more recently to extra-intestinal autoimmune diseases such as MS (14) and T1D (10, 11). In our enteric bacterial pathogen model, we demonstrated that an early intestinal epithelial barrier insult with *C. rodentium* activated polyclonal and diabetogenic CTLs and accelerated the development of severe invasive insulinitis in NOD mice. However, the mechanism involved in initiating this autoimmune process in *C. rodentium* infected NOD mice requires further examination.

4.1.1 TLR7 and T1D

We have shown that TLR7 signaling is involved in triggering the development of T1D in NOD mouse models, however there are several caveats that remain unclear and require further investigation. First, we show the efficient induction of functional diabetogenic CTLs in NOD mice stimulated with CL097 and anti-CD40. This subsequent treatment combination led to the accelerated disease onset of T1D in 8.3 NOD mice. The mechanism involved in promoting the priming and activation of functional diabetogenic CTLs remains unclear in our model. Recent studies (15-17) have shown that type 1 interferons (IFN) specifically IFN- α play an important pathogenic role in the development of several autoimmune diseases such as SLE, RA and T1D. The main producers of IFN- α are pDCs which also express TLR7 and 9 (18). In SLE, TLR7 and/or 9 stimulation activates pDCs to cause the production of IFN- α which has been shown to exacerbate disease onset (16, 17). In our model, a similar mechanism may occur in which pDCs become activated through TLR7 signaling to generate IFN- α which may promote the generation of functional diabetogenic CTLs.

Second, the single administration of CL097 induced the general activation of T and B cells in NOD mice but was ineffective in inducing the priming of functional diabetogenic

CTLs in NOD mice and the acceleration of T1D in 8.3 NOD mice. TLR stimulation alone is not able to convert autoimmunity into autoimmune diseases due to self-tolerance mechanisms (19). However, another possible explanation for a lack of TLR induced diabetogenesis may be due to the timing of the TLR agonist treatment. A recent study by Li *et al.* (2008) (15) demonstrated that there is sharp increase in IFN- α levels and IFN- α producing pDCs in NOD mice at 3-4 weeks of age compared older NOD mice. At 3-4 weeks, the pancreas begins to become infiltrated with mononuclear cells in NOD mice (15). The subsequent blockade of IFN- α receptor 1 with a neutralizing antibody in 2-3 week old NOD mice delayed the onset of T1D (15). This suggests that manipulation of the early autoimmune process in T1D may allow the prevention or even acceleration of T1D in NOD mice. Further studies are warranted to investigate if the treatment of 2-3 week old NOD mice with a TLR7/9 agonist or inhibitor accelerates or delays, respectively the development of T1D.

Third, TLR7 and 9 expression is not exclusive to pDCs but is also expressed on B cells (16). The generation of autoantibodies has been shown to correlate with disease severity in several autoimmune diseases such as SLE and T1D (16, 17). In our model, we did not address if autoantibody production contributes to the accelerated disease onset in 8.3 NOD mice treated with CL097 and anti-CD40. However, we have demonstrated that NOD mice treated with CL097 induced a general activation of polyclonal B cells. The subsequent treatment of NOD mice with CL097 and anti-CD40 may also induce functional self-reactive B cells that may lead to the increased production of autoantibodies.

Fourth, we have demonstrated that blockade of TLR7 signaling with an inhibitory ODN, IRS661, delays the induced acceleration of T1D in 8.3 NOD mice treated with CL097 and anti-CD40. However, we did not address how the blockade of TLR7 signaling prevented this accelerated development of T1D in 8.3 NOD mice. We postulate that blockade of TLR7 signaling prevents pDCs and/or conventional DCs to create an inflammatory environment that helps initiate the priming and activation of functional diabetogenic CTLs in our model.

Lastly, we have shown that IRS661 inhibited both TLR7 and 9 signaling *in vivo* but not *in vitro*. In our model, IRS661 inhibited both TLR7 and endogenous TLR9 signaling in 8.3 NOD mice treated with CL097 and anti-CD40. In addition, chloroquine administration in either NOD or NOD.SCID mice delays the development of T1D and diabetes transfer, respectively (Zhang *et al.*, submitted; Appendix B). Thus, it remains unclear whether TLR7 or 9 or both are involved in the pathogenesis of T1D. However, we have data suggesting that both TLR7 and 9 play an important role in the pathogenesis of T1D. We have data showing that TLR9 signaling also contributes to the accelerated disease onset of T1D in 8.3 NOD mice (Zhang *et al.*, submitted; Appendix B). Moreover, TLR9 deficient NOD mice have a delayed onset of T1D compared to NOD TLR9 heterozygote and NOD TLR9 sufficient mice (Zhang *et al.*, submitted; Appendix B). Further studies are required to determine if blockade of endogenous TLR7 and/or 9 signaling with IRS661 delays the onset of T1D in NOD and 8.3 NOD mice. To clarify the roles of TLR7 or 9, the use of specific TLR7 or 9 inhibitory ODNs or small molecules, or the use of TLR7 or 9 deficient NOD mice would be essential to help elucidate the role of these endosomal TLRs in the development of T1D.

4.1.2 GI and T1D

We have shown that an enteric bacterial pathogen, *C. rodentium*, that disrupts the intestinal barrier integrity results in the accelerated development of invasive insulinitis in NOD mice. This accelerated development of invasive insulinitis may be mediated by the increased activation of both polyclonal and diabetogenic CTLs in NOD mice infected with *C. rodentium*. Further studies are required to determine the mechanisms involved in this accelerated onset of invasive insulinitis in *C. rodentium* infected NOD mice.

First, we demonstrated that *C. rodentium* infection in NOD mice accelerates the development of invasive insulinitis. However, it remains unclear if the *C. rodentium* induced barrier disruption is a prerequisite to the increased development of invasive insulinitis. To address this, mutant *C. rodentium* strains such as Δ espF, that do not cause barrier disruption will be used to determine if barrier disruption is a requirement to the increased development of invasive insulinitis in NOD mice. Second, our *C. rodentium*

model did not address if T1D is accelerated in NOD mice infected with *C. rodentium*. As a result, long-term diabetes studies are required to determine if *C. rodentium* infection in NOD mice accelerates the development of T1D. Third, recent evidence has shown that commensal bacteria play an essential role in modulating the development of T1D in NOD mice (11). Thus, it remains unclear if commensal bacteria modulate the development of invasive insulinitis or T1D in our *C. rodentium* model. Lastly, there has been mounting evidence (20) showing that other barrier irritants such as 2% dextran sodium sulfate in the drinking water can lead to the increased development of invasive insulinitis in NOD mice. Further studies are required to determine if other barrier irritants, besides *C. rodentium*, are involved in modulating the development of insulinitis and/or T1D in genetically susceptible individuals.

4.2 Impact of Described Work

The identification of potential triggers in the pathogenesis of T1D is essential in creating and developing effective therapeutic interventions in the prevention and/or treatment of T1D in genetically susceptible individuals. By the time an individual at-risk for T1D is clinically diagnosed, 80-90% of the islets are destroyed resulting in a lifelong dependency on insulin. The creation of therapeutics that dampen or inhibit the autoimmune response in humans at-risk for T1D may prevent disease onset.

In our TLR7 project, we demonstrate that TLR7 signaling contributes to the activation of polyclonal and diabetogenic CTLs, and the accelerated development of T1D in NOD mouse models. The subsequent inhibition of TLR7 led to a delay in the accelerated development of T1D in 8.3 NOD mice treated with a TLR7 and CD40 agonist. In addition, administration of the anti-malarial, chloroquine (TLR7-9 inhibitor) delayed T1D transfer in NOD.SCID mice. Moreover, anti-malarials such as chloroquine have been used since the 1950s to treat or alleviate symptoms of patients who suffer from SLE, RA and Sjogren's syndrome – autoimmune diseases in which TLR7/9 activation exacerbate disease onset (7). However, the use of anti-malarials is limited due to their side effects and suboptimal efficacy. Thus, the use of suppressive ODNs or small molecule TLR7/9 antagonists with differentiated selectivity and a large safety window

may potentially have significant clinical utility in the prevention or treatment of TLR7/9 induced autoimmune diseases. Currently, the Coley Pharmaceutical Group Inc. are exploring this therapeutic avenue in the treatment of SLE (7).

In our GI project, we demonstrate that a *C. rodentium* induced intestinal epithelial disruption contributes to the activation of polyclonal and diabetogenic CTLs, and the accelerated development of invasive insulinitis in NOD mice. The data from our *C. rodentium* model helps confirm the hypothesis that an intrinsic or induced intestinal barrier dysfunction modulates the development of both intestinal and extra-intestinal autoimmune diseases. In addition, this research helps elucidate the relationship between TLR signaling, enteric bacteria and diabetes onset. Understanding the intricate relationship between the gut and T1D may allow for the rational design of gut-specific TLR agonists including enteric organisms or drugs that promote barrier integrity to prevent T1D in at-risk individuals.

The identification of TLR7 and enteric organisms in modulating the development of T1D helps in our understanding of the pathogenesis of T1D and in creating potential therapeutics in preventing such autoimmune diseases. In addition, identifying these T1D triggers may help clinical epidemiologists who are currently conducting large prospective studies such as the ‘Trial to Reduce IDDM in the Genetically at Risk’ (TRIGR) (10 year study) (21) and ‘The Environmental Determinants of Diabetes in the Young’ (TEDDY) (15 year study) (9) studies – follow newborns at-risk for T1D - to confirm or identify new and suspected diabetes-related environmental agents. Understanding the mechanisms involved in triggering T1D may have significant therapeutic potentials in preventing and treating T1D in at-risk individuals.

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APPENDICES

APPENDIX A

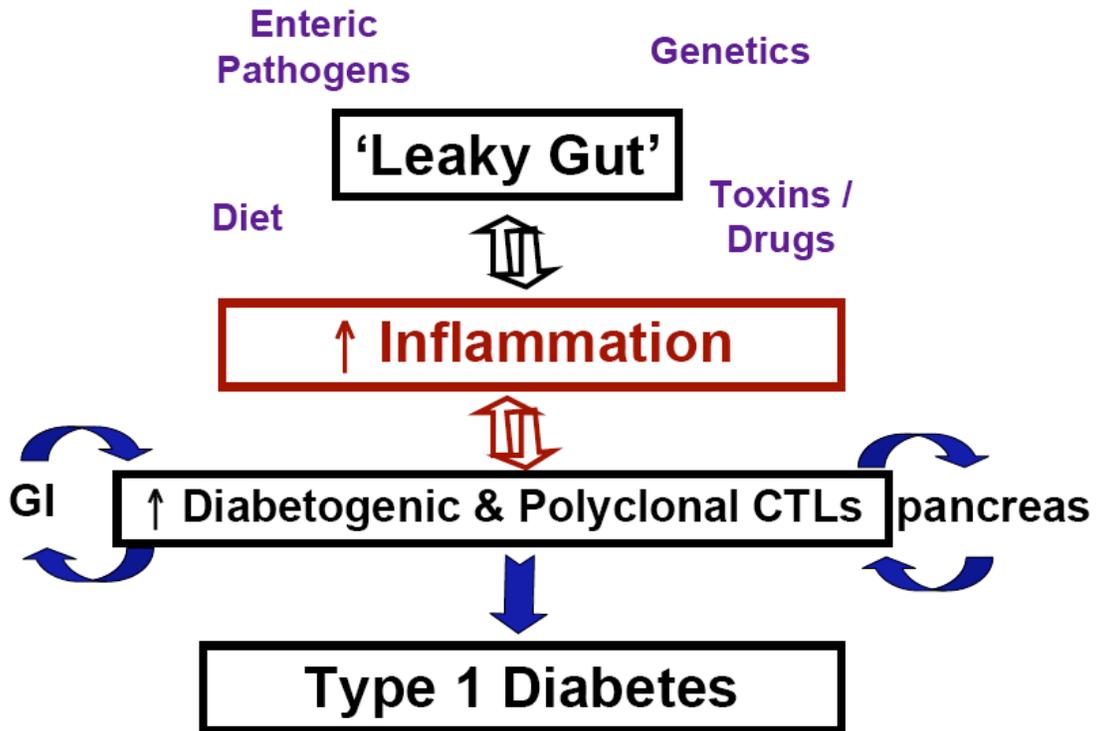
A.1 Toll-like receptor (TLR) signal transduction

Appendix A.1 has been removed due to copyright restrictions. The information removed is a figure summarizing the toll-like receptor signal transduction pathways. The original figure can be found here: Uematsu, S., and S. Akira. 2006. Toll-like receptors and innate immunity. *J Mol Med* 84:712-725.

A.2 Structure of the intestinal immune system

Appendix A.2 has been removed due to copyright restrictions. The information removed is a figure summarizing the structure of the intestinal immune system. The original figure can be found here: Magalhaes, J. G., I. Tattoli, and S. E. Girardin. 2007. The intestinal epithelial barrier: how to distinguish between the microbial flora and pathogens. *Semin Immunol* 19:106-115.

A.3 'Leaky gut' model of type 1 diabetes (T1D)



Appendix A.3 is a diagram of the proposed 'leaky gut' model of T1D adapted from our lab and from: Vaarala, O., M. A. Atkinson, and J. Neu. 2008. The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. *Diabetes* 57:2555-2562. Acronyms: CTL – cytotoxic T lymphocytes

APPENDIX B

B.1 TLR9 blockade inhibits activation of diabetogenic CD8⁺ T cells and delays autoimmune diabetes³

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Running title: TLR9 signal in type 1 diabetes

³ A version of this manuscript has been submitted for publication.

ABSTRACT

Objective: Diabetogenic CD8 T cells are primed in the pancreatic lymph nodes (PLN) by dendritic cells (DC) carrying islet cell antigens. The goal of this study was to determine the effect of Toll-like receptor 9 (TLR9) stimulation on diabetogenic CD8 T cell priming. *Research design and methods:* We explored the effects of CpG oligonucleotide, TLR9 antagonists and genetic TLR9 deficiency on the activation of diabetogenic CD8 T cells *in vitro* and *in vivo*. *Results:* NOD bone-marrow derived DCs (BMDC)s pulsed with freeze-thawed insulinoma cells in the presence of TLR9 agonist CpG and CD40 agonist induced diabetogenic CD8⁺ T cell activation. Addition of TLR9 antagonist oligonucleotide (ODN 2088) or chloroquine inhibited BMDC activation and CD8⁺ T cell priming in response to CpG. CpG alone or with CD40 agonist induced CTL activity that triggered diabetes development in 8.3 NOD TCR transgenic mice. ODN 2088 treatment of 8.3 NOD mice delayed spontaneous diabetes development. Chloroquine treatment delayed the onset of diabetes in NOD mice, co-incident with the decreased activation of PLN DC. Finally, TLR9^{-/-} NOD mice had delayed onset of diabetes when compared to TLR9^{+/-} NOD littermates. *Conclusion:* TLR9 activation contributes to the spontaneous onset of diabetes in NOD mice by promoting diabetogenic CD8 T cell activation and inhibition of TLR9 activation delays the onset of disease.

Keywords: CpG, TLR9, anti-CD40, BMDC, 8.3 CD8 T cell activation, diabetes, chloroquine.

Abbreviations: BMDC, bone marrow dendritic cells; NOD, nonobese diabetic; TLR, toll like receptor; FT NIT1, freeze thawed insolinoma cells; ODN, oligodeoxynucleotide; ELISA, enzyme-linked immune sorbet assay; CBA, cytometric bead array; CQ, chloroquine.

INTRODUCTION

Type 1 diabetes (T1D) is the result of immune-mediated damage to the pancreatic islets (1). A crucial pathogenic role has been ascribed to CD8⁺ T cells (2). The activation of CD8⁺ T cells to tissue antigen occurs by a process termed cross-presentation. Recent data, suggest that cross-presentation of apoptotic or necrotic antigen to CD8⁺ T cells can be either tolerogenic or immunogenic and that dendritic cell (DC) activation is crucial to this distinction (3). CD40 ligation on DC culminates in their activation to promote immunogenic cross presentation and to promote the onset of T1D (4-6).

Toll like receptors (TLRs) are a family of signaling molecules that bind TLR agonists released by infection or tissue injury. TLRs not only function as a key component of innate immune system, but also control multiple DC functions critically involved in the initiation of adaptive immune responses (7; 8). TLR9 is expressed in the endosome compartment of human plasmacytoid DC and B cells and in a broader range of DC in mice. TLR9 detects DNA with hypomethylated CpG motifs of microbial pathogens and their hosts (9). Ligation of TLR9 by CpG is synergistic with CD40 activation of DC and promotes the cross-presentation of antigens by DC (10; 11).

Cell death releases endogenous antigens and may also provide danger signals such as endogenous DNA or RNA which might bind to TLR9, TLR7/8 or TLR3, activate auto-reactive T cells, and predispose the host to autoimmune disease (12). A self-DNA-TLR9 interaction may promote B cell activation in systemic lupus erythematosus (13). We have previously observed that islet β -cell death promotes diabetogenic T cell priming *in situ* (14). Physiological β -cell death triggers the priming of self-reactive T cells by DC in the pancreatic lymph nodes (PLN) (15). How β -cell death promotes T cell activation and diabetes development is still unclear. The clearance of dying cells is deficient in NOD mice (16). Deficient clearance in dying cells may promote the presence of secondarily necrotic cells that may then activate DC via TLR2 (17). As self DNA liberated from dying cells may interact with TLR9 and promote immune responses (18), we explored the possibility that TLR9 signaling may contribute to the onset of T1D and that inhibition of this signaling could be used to delay the onset of diabetes.

MATERIALS AND METHODS

Mice and cells

8.3 NOD mice expressing the rearranged TCR genes of the diabetogenic CTL clone NY 8.3 have been described (19) and were bred both at the University of Calgary (Calgary, Alberta) and at the University of British Columbia. NOD mice (Jackson Laboratory, Bar Harbor ME) were bred in a specific pathogen-free environment. TLR9^{-/-} mice (20) were purchased from Oriental Bio-Service (Kyoto, Jp) and were bred onto the NOD genetic background using a speed congenic approach. NOD genetic background of offspring (6th backcross) was confirmed (99.51% NOD) using a panel of 104 micro-satellite markers (MAX BAX, Charles River Labs). Bone marrow derived dendritic cells (BMDC) were derived from the bone marrow of NOD mice, and cultured with GM-CSF (100 U/ml) and recombinant mouse IL-4 (1 ng/ml) in complete medium (CM - RPMI medium consisting of RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich, Oakville ON), penicillin (100 U/mL) and streptomycin (100 µg/mL) for 6 days prior to use in cellular assays. An insulinoma cell line (NIT-1, ATCC, Rockland Md), was used as source of islet antigens. Naïve 8.3 CD8 T cells were isolated from 4-6 weeks old 8.3 NOD mice. Single cell suspensions were prepared from peripheral lymph nodes and spleens. CD8⁺ T cells were purified by using CD8 microbeads and miniMac separation columns (Miltenyi Biotech. Auburn, CA).

Reagents and antibodies

Phosphothioate modified oligodeoxynucleotides CpG (TCC ATG ACG TTC CTG ACGTT), ODN 2088 (TCC TGG CGG GGA AGT) and CpG 1982 (TCC AGG ACT TCT CTC AGGTT) were synthesized by UBC biotechnology Lab, purified by HPLC, and were endotoxin free by Limulus assay. Recombinant mouse IL-4, biot-anti-CD40, biot-anti-CD69 and biot-anti-CD25 were obtained from Cedarlane (Burlington ON). Fluorescence-conjugated mAbs: Anti-IFN- γ -APC, streptavidin-APC, anti-CD11c-PE, anti-CD44-APC and anti-CD122-PE were from BD Pharmingen (San Diego, CA).

In vitro CD8⁺ T cell priming assay

BMDC 5×10^4 were incubated with 5×10^5 freeze-thawed NIT-1 cells (3 cycles of freezing and thaw using alternate immersion in liquid nitrogen and 37⁰ C water) with or without CpG (5 μ g/ml), LPS (1 μ g/ml, Sigma-Aldrich) or anti-CD40 monoclonal antibody (clone 3/23, BD Pharmingen - 15 μ g/ml) \pm ODN 2088 (12 μ g/ml) or 2 μ g/ml chloroquine (Sigma-Aldrich) overnight. 2×10^5 CFSE (5 μ M, Molecular Probes - Invitrogen, Burlington ON)-labeled 8.3 CD8 T cells were then added to the culture. After 4 days of culture, the cells were collected, labeled with fluorescent-conjugated mAbs, and analyzed by flow cytometry.

Flow cytometry

Cultured CD8 T cells, BMDC or PLN cells were harvested, washed with PBS with 0.5% BSA (Sigma-Aldrich), and stained with fluorescence-conjugated mAbs to CD25, CD69, CD40, CD44 and CD122 or with anti-IFN- γ -APC intra-cellularly for the co-expression of CD25, CD69, CD40, CD44 and CD122 and IFN- γ secretion. Intracellular cytokine secretion was determined after *in vitro* stimulation with PMA (50 ng/ml) and ionomycin (1 μ g/ml) for 5 hours. DC were purified from PLN cells using CD11c microbeads and MiniMacs separation (Miltenyi Biotech) prior to analysis. FACS analysis was performed on FACS calibur flow cytometer (BD biosciences, San Jose, CA) using Cell Quest software (BD Biosciences).

Cytokine assays

ELISA. 5×10^5 cells/ml BMDC were cultured in CM with or without CpG (5 μ g/ml) and LPS (1 μ g/ml) alone or in combination with anti-CD40 mAb (15 μ g/ml) \pm chloroquine (2 μ g/ml) or ODN 2088 (12 μ g/ml) in 5 ml polypropylene tubes. IL-12 p70 and IL-10 in 24-hour culture supernatants was measured by standard ELISA (BD Biosciences).

Cytometric Bead Array (CBA). Serum samples were assayed for IL-12 p70, IFN- γ , IL-6 and MCP1 by Mouse Inflammation Kit (BD Bioscience) and FACS.

In vivo cytotoxicity assay

8.3 NOD mice were injected (i.p.) with 100 µg CpG and/or 200 µg anti-CD40 mAb combined with 100 µg ODN 1982, 300 µg ODN 2088 or 200 µg rat IgG. Two days later, the mice were adoptively transferred (i.v.) with 1 µg/ml V7 peptide (KYNKANVFL) - pulsed and 5 µM CFSE labeled splenocytes or 1 µg/ml Tum (KYQAVTTTL)-pulsed and 0.5µM CFSE-labeled splenocytes. PLN cells were collected one day later for analysis.

Assessment of diabetes development

Blood glucose was measured using Elite glucostrips and glucometer (Bayer, Etobicoke, ON). Animals were considered to be diabetic when two consecutive measurements exceeded 14 mM.

Statistical analysis

Groups were compared using one or two-tailed Student's test, one or two-way ANOVA (Prism 4, GraphPad Software, La Jolla, CA). Survival was compared using Kaplan-Meier curves and logrank tests.

RESULTS

TLR9 agonism induces priming of diabetogenic 8.3 CD8⁺ T cells to self antigen in vitro

TLR9 stimulation promotes the cross presentation of antigens by BMDC resulting in the priming of CD8⁺ T cells (10). We investigated the effects of TLR9 signaling upon diabetogenic CD8⁺ T cell activation in response to a naturally occurring self-antigen. Immature BMDC from NOD mice were pulsed with freeze-thawed (FT) insulinoma cells (NIT-1) as a source of self-antigen in the presence or absence of TLR9 agonist (CpG) or TLR4 agonist (LPS) with or without agonist anti-CD40 antibody. 8.3-NOD CD8⁺ T cells recognize an immuno-dominant peptide from islet-specific glucose 6 phosphatase catalytic subunit-related protein (IGRP) termed IGRP₂₀₆₋₂₁₄, a protein expressed by pancreatic β cells and these cells are representative of a significant fraction of CD8⁺ T cells in pancreatic islets at the onset of inflammation (19; 21; 22). Naïve 8.3-NOD TCR transgenic CD8 cells were purified, labeled with CFSE, and added to the antigen-pulsed cultured BMDC. Three days later, we assessed CD8⁺ T cell activation using proliferation (CFSE dilution), intracellular IFN- γ production (Figure 1A) and CD25 expression (data not shown). 8.3-NOD CD8⁺ T cells proliferated weakly following culture with BMDC in the presence of FT-NIT1 cells alone. CpG, LPS or anti-CD40 induced partial CD8⁺ T cell activation. CD40 agonism had a synergistic effect with CpG or LPS in inducing 8.3 T CD8 cell activation, and the combination of CpG with anti-CD40 elicited highest 8.3-NOD CD8⁺ T cell activation.

Cell death provides a source of cellular antigens for cross-presentation, and may also release endogenous adjuvants that promote T cell responses (12; 23). To confirm that CpG-induced 8.3-NOD CD8⁺ T cell activation in our model is β -cell antigen specific, FT-NIT1 or FT-B16 cells (melanoma cells) were added to the culture of BMDC and CD8⁺ T cells with the combination of CpG and anti-CD40 (Figure 1 B). In contrast to BMDC pulsed with FT-NIT1 cells, BMDC pulsed with FT-B16 cells did not induce CD8⁺ T cell activation, as manifested by low CD25 expression (Figure 1B, $p = 0.037$). Similarly, FT-B16 cells did not elicit proliferation by 8.3 NOD CD8⁺ T cells (data not shown). Thus, despite the presence of CpG, CD40 co-stimulation and potential

endogenous adjuvant activity, the observed diabetogenic CD8⁺ T cell activation is β -cell antigen-specific.

ODN 2088 or chloroquine inhibit diabetogenic 8.3-NOD CD8⁺ T cell activation in response to β -cell derived antigens

TLR9 activation is blocked by inhibitory oligonucleotides such as ODN 2088 (24) or chloroquine (25). Chloroquine blocks CpG-TLR9 interaction by reducing endosomal acidification and is effective in treating B cell autoimmune diseases such as SLE (26) in addition to preventing CD8⁺ T cell activation and damage in murine GVDH (25). We examined the effect of ODN 2088 and chloroquine upon CpG-induced 8.3-NOD CD8⁺ T cell activation in response to β -cell antigen. CFSE-labeled 8.3 CD8 cells were co-cultured with BMDC pulsed with FT-NIT1 cells in the presence of CpG or LPS combined with anti-CD40, with or without ODN 2088 or chloroquine. The combination of CpG or LPS with anti-CD40 induced 8.3 CD8⁺ T cell proliferation and concomitant CD25 expression (Figure 2A). Addition of either ODN 2088 or chloroquine to the culture inhibited CD8⁺ T cell activation induced by CpG but not by LPS. CpG-induced CD25 expression by 8.3-NOD T cells correlated with proliferation and IFN- γ secretion (Figure 2B). CD40 stimulation enhanced CpG-induced CD8⁺ T cell activation. Antigen specific CD8⁺ T cell activation induced by CpG in the presence or absence of CD40 activation was abolished by the addition of chloroquine ($p < 0.05$, CpG+CD40 versus CpG+CD40+chloroquine).

ODN 2088 or chloroquine inhibit CpG-induced NOD BMDC maturation

TLR agonists promote CD8⁺ T cell immune responses by enhancing cross presentation of antigen and by the maturation and stimulation of DC to produce inflammatory cytokines. TLR expression and function may be abnormal in NOD DCs (27). We examined the effect of ODN 2088 or chloroquine upon CD40 expression on NOD BMDC. BMDC were cultured with or without CpG or LPS in the presence or absence of ODN 2088 or chloroquine and consequent CD40 expression by CD11c⁺ DC was assessed (Figure 3A). CpG and LPS increased CD40 expression. Either ODN 2088 or chloroquine prevented CpG-induced but not LPS-induced CD40 expression.

IL-12 promotes a Th1-type immune response and accelerates diabetes development (28). Conversely, local expression of IL-10 delays diabetes onset (29). To determine the effect of ODN 2088 or chloroquine on CpG-induced cytokine production from NOD BMDC, BMDCs were cultured with CpG or LPS either with or without anti-CD40 in the absence or presence of ODN 2088 or chloroquine. CpG alone induced higher IL-12 p70 secretion than LPS. The combination of CpG and anti-CD40 mAb induced highest IL-12 p70 production from BMDC, which was completely blocked by ODN 2088 or chloroquine ($p < 0.05$). In contrast, the combination of LPS and anti-CD40 induced low levels of IL-12 p70 and IL-10 secretion, an effect not modified by either ODN 2088 or chloroquine (Figure 3B). Thus ODN 2088 and chloroquine inhibit CpG-induced pro-inflammatory cytokine secretion and maturation of NOD DC.

CpG triggers diabetes, local CD8⁺ T cell activation and memory T cell expansion in 8.3 NOD mice

To determine whether TLR9 agonists promote the spontaneous onset of diabetes, we studied the onset of diabetes in 8.3 TCR transgenic NOD mice given CpG. Male and female 8.3 TCR-transgenic NOD mice develop spontaneous diabetes after 9 weeks of age (30). We treated 8.3 NOD mice with a single dose of either CpG, anti-CD40 or both at 5 weeks of age (Figure 4). Half of the 8.3 NOD mice treated with either CpG or anti-CD40 rapidly developed hyperglycemia ($p < 0.03$ compared to untreated). 8.3 NOD mice treated with a combination of CpG and anti-CD40 had the highest prevalence of diabetes within 10 days ($p < 0.01$). Notably, co-injection of CpG and the TLR9 inhibitory ODN 2088 abrogated diabetes acceleration, confirming a TLR9 mediated effect upon diabetes onset.

TLR9 agonists directly activate DC. To confirm that consequent diabetogenic T cell activation contributed to CpG mediated acceleration of diabetes in 8.3 NOD mice we examined CD8⁺ T cells within the PLN following treatment with CpG \pm ODN 2088 or chloroquine and compared this to animals treated with LPS \pm chloroquine (Figure 5A). CpG administration induced CD69 expression on 8.3-NOD CD8⁺ T cells and both ODN

2088 ($p=0.01$) and chloroquine ($p=0.02$) inhibited this expression *in vivo*. As expected, LPS-induced CD69 expression by CD8⁺ T cells was not inhibited by chloroquine.

CD44/CD122-positive CD8⁺ T cells represent a population of memory CD8⁺ T cells which are responsive to IL-15 for proliferation and survival (31). Incomplete CD8⁺ T cell activation results in abortive proliferation and absence of cytotoxicity (32). To confirm that CpG activation induces full maturation, we investigated the effect of CpG on the induction or expansion of a CD8⁺ T cell memory phenotype. CpG plus anti-CD40 treatment of 8.3 TCR NOD mice clearly increased the fraction of CD44^{hi} and CD122^{hi} 8.3 CD8⁺ T cells within the pancreatic DLN (Figure 5B). Full CD8⁺ T cell activation is associated with the development of cytotoxic potential. To assess the cytotoxic potential of 8.3 TCR CD8⁺ T cells *in vivo* following TLR9 agonist exposure, 8.3 NOD mice were treated with CpG and/or anti-CD40 combined with control ODN 1982 (which is neither stimulatory nor antagonistic) or rat IgG. These mice then received target splenocytes labeled with CFSE and loaded with the cognate L^d restricted peptide antigen of 8.3 CD8⁺ T cells or an irrelevant peptide. Target-peptide loaded splenocyte survival was diminished in CpG or anti-CD40-treated pancreatic lymph nodes and dramatically reduced in CpG plus anti-CD40 treated pancreatic LN (Figure 5C, $p=0.038$). In summary, CpG and/or CD40 stimulation induce diabetogenic 8.3 CD8⁺ T cell activation *in vivo*, memory phenotype, and CTL activity culminating in diabetes development. Thus NOD DCs process and present endogenous antigens to diabetogenic CD8⁺ T cells following TLR9 activation.

ODN 2088 delays diabetes development in 8.3 NOD mice

Our observations indicate that TLR9 stimulation promotes the onset of diabetes. Endogenous DNA, either liberated from damaged cells or from microbial commensals, may stimulate TLR9 (12). We therefore explored whether the TLR9 antagonist ODN 2088 would delay the onset of spontaneous diabetes. 8.3 NOD mice at 3-4 weeks of age were injected with ODN 2088 or controls (either PBS or non-stimulatory and non-inhibitory ODN 1982), 3 times weekly. Mice treated with ODN 2088 demonstrated a delayed diabetes onset when compared to the mice injected with PBS or ODN 1982

(Figure 6A, $p=0.037$). Thus endogenous TLR9 activation contributes to the onset of diabetes in 8.3 TCR NOD mice. We treated 3-4 week old 8.3 NOD mice with chloroquine (10mg/kg IP daily for 5 days as a loading dose and 2-3 times weekly thereafter) or PBS. Again, the spontaneous onset of diabetes was delayed (Figure 6B, $p=0.043$).

Chloroquine prevents diabetes development in NOD mice and inhibits DC maturation

8.3-NOD TCR mice have accelerated diabetes due to a greatly expanded population of islet specific CD8⁺ T cells. We sought to determine if chloroquine would inhibit diabetes development in NOD mice as these animals display a broader repertoire of islet-specific T cells than the transgenic mice. NOD female mice at 5-8 weeks of age were treated with 10 mg/kg chloroquine as described above. Whereas 19 of 29 PBS-treated mice became diabetic up to 30 weeks of age, only 11 of 29 chloroquine-treated mice developed diabetes at this age (Figure 7A, $p=0.035$). Thus, chloroquine administration delays spontaneous diabetes development in NOD mice.

To explore the mechanisms by which chloroquine prevents diabetes development in NOD mice, we assessed the effect of chloroquine treatment upon CD40 expression by pancreatic DCs. NOD mice at 5-7 weeks of ages were treated with chloroquine or PBS. The pancreatic lymph nodes were collected after 4 weeks of treatment (at an age when insulinitis is present). DCs from chloroquine-treated pancreatic lymph nodes demonstrated diminished levels of CD40 expression (Figure 7B, $p=0.029$). We next examined the effect of chloroquine on CpG-induced secretion of cytokines and chemokines *in vivo*. NOD mice were injected with CpG or LPS alone or combined with chloroquine at a dosage of 10 mg/kg. CpG treatment induced secretion of IL-12 p70, IFN- γ , IL-6 and MCP-1, and chloroquine significantly inhibited the production of these cytokines and MCP-1. In contrast, LPS-induced secretion of cytokines and chemokines were not inhibited by chloroquine (shown in Figure 7C). Thus, chloroquine treatment leads to diminished pancreatic DC activation and inhibition of cytokine release in response to TLR9 but not TLR4 agonists.

TLR9^{-/-} NOD mice demonstrate a delayed onset of diabetes

To confirm a role for TLR9 in the spontaneous onset of diabetes in NOD mice, TLR9^{-/-} NOD mice were generated by speed congenic mating of TLR deficient strains to NOD mice. The persistence of a full NOD background was confirmed using 104 micro-satellite markers. The spontaneous onset of diabetes in TLR9^{-/-} NOD females was then compared to heterozygote and wild type NOD littermates (Figure 8). TLR9^{-/-} NOD mice demonstrate a significant delay in onset of diabetes when compared to TLR9^{+/-} NOD or NOD littermates ($p= 0.042$). Thus TLR9 signaling contributes to the spontaneous onset of diabetes in NOD mice.

DISCUSSION

We have examined the role of TLR9 stimulation of DC in the development of T1D in NOD mice. Immature NOD BMDC pulsed with β -cell antigen in the form of necrotic insulinoma cells and stimulated by the TLR9 agonist CpG plus anti-CD40 prime diabetogenic CD8⁺ T cells resulting in proliferation, CD25 expression and IFN- γ production (Figure 1). Further, treatment of 8.3-NOD TCR transgenic mice with CpG induces diabetogenic CD8⁺ T cell activation, CTL activity and triggers diabetes (Figure 4). Using an *in vitro* assay of T cell activation, we demonstrate that either a TLR9 inhibitory oligonucleotide (ODN 2088) or chloroquine inhibit CpG-induced 8.3 CD8⁺ T cell activation *in vitro* (Figure 2). These agents inhibit CpG-induced diabetogenic CD8⁺ T cell activation *in vivo* (Figure 3) and delay the onset of diabetes in NOD mice (Figures 6 & 7). Finally, NOD mice deficient in TLR9 demonstrate delayed onset of diabetes when compared to TLR9-/+ littermates (Figure 8). Collectively this demonstrates that TLR9 signaling, albeit dispensable for diabetogenesis, contributes to diabetogenic CD8⁺ T cell activation and diabetes progression.

CD40 ligation activates DC and promotes insulinitis and diabetes in NOD mice (4; 6). Similarly, TLR ligation on DC causes DC maturation as manifested by an increase in display of MHC peptide ligands for T cell recognition and up-regulation of co-stimulatory molecules including CD40 (7; 8). TLR signaling has a synergistic effect with CD40 ligation upon the activation of DCs permitting the priming and expansion of CD8⁺ T cells to foreign antigen (33) and the priming of auto-reactive CD4 T cells in myocarditis (34). We demonstrate that TLR9 agonism combined with CD40 ligation induces NOD DC to produce high levels of IL-12, in contrast to LPS stimulation (Figure 3). This combined agonism rapidly induces diabetes in 8.3 NOD TCR mice (Figure 4). As 8.3 NOD CD8⁺ T cells respond to the islet self-antigen IGRP, TLR9 signaling and CD40 activation induce CD8⁺ T cell activation to physiologic levels of this self-antigen and promote the onset of diabetes. Recent data indicate that islet inflammation can substitute for pro-insulin specific CD4⁺ T cell help to activate IGRP-specific CD8⁺ T cells (35). Our work suggests that TLR9 stimulation may induce such inflammation.

Antimalarials have been used for decades for the treatment and prophylaxis of malaria and, more recently, for the treatment of autoimmune diseases and inflammatory skin disease (36). The immunologic effect of chloroquine was previously thought to relate to inhibition of lysosomal function resulting in decreased presentation of class II MHC antigen to CD4 T cells (37). However, nanomolar rather than micromolar concentrations (required for effects upon Class II MHC antigen presentation) prevent bacterial DNA-induced IL-6 production by human PBMCs, an effect now known to be due to inhibition of TLR9 signaling (38). Chloroquine inhibits rheumatoid factor production by B cells induced by chromatin-immune complexes that signal through TLR9 (13). In SLE, DNA and DNA-associated auto-antigens activate auto-reactive B cells via sequential engagement of the B cell antigen receptor (BCR) and TLR9. We demonstrate that chloroquine inhibits diabetogenic CD8⁺ T cell activation and prevents autoimmune diabetes development. DC activate memory cells as well as naïve T cells (39). Chloroquine also delays the onset of diabetes following the adoptive transfer of splenocytes from diabetic NOD into NOD-SCID mice (data not shown), suggesting a beneficial effect of this drug upon memory cell activation in established disease. Antimalarials have other potentially beneficial effects. In SLE patients, these drugs lower lipid levels and are cardioprotective (40). Antimalarials improve glucose tolerance in type 2 diabetes (41). All these observations suggest that chloroquine therapy may be beneficial in the treatment of T1D.

Aminoquinoline antimalarials, through their effects on lysosomal function, have multiple effects on cellular physiology. Endosomal TLRs potentially inhibited by antimalarial therapy include TLR-3, TLR-7/8 and TLR-9. To provide more specific TLR inhibition, suppressive oligodeoxynucleotides have been screened and developed. ODN 2088 is one such suppressive oligodeoxynucleotide (24) that blocks the stimulatory effect of CpG upon B cell activation. Such G rich ODNs are the most effective inhibitors of TLR9 activation identified to date (42) and they have been used to directly block the binding of CpG to TLR9 (43). We demonstrate that TLR9 agonist induced activation of diabetogenic CD8⁺ T cells *in vivo* is inhibited by the systemic administration of a TLR9 ODN antagonist (Figures 4 & 5). This suggests that development of TLR9 antagonists for

the prevention and treatment of T1D, as is currently being pursued for other autoimmune diseases (44), is worthwhile.

The generation of specific TLR deficient autoimmune prone animal models has confirmed the role of TLR signaling in the onset of spontaneous and infection-associated autoimmunity. TLR2 deficient NOD mice have delayed onset of diabetes possibly related to decreased sensing of apoptotic cell induced particles (17). Viral mediated TLR9 activation participates in the acceleration of diabetes in the Biobreeding rat (45). Interestingly, TLR9 deficiency in mouse models of SLE has been reported to accelerate disease (46). TLR9 deficiency paradoxically resulted in increased activation of plasmacytoid DC and IFN- α production. Possibly, this was the consequence of tonic TLR9 ligation providing a negative signal for immune responses through the induction of IDO production (47) and the subsequent induction of T regulatory cell function (46). Whereas murine myeloid and plasmacytoid DC express TLR9, in humans TLR9 induces IFN- α production by plasmacytoid DC. Consistent with the consequences of TLR9 activation shown here, IFN- α has recently been shown to initiate diabetes in the NOD mouse (48). IFN- α administration has also been reported to trigger autoimmune diabetes in man (49). A further exploration of type I interferon pathways in antimalarial treated NOD mice and TLR9^{-/-} NOD mice would be of interest. The therapeutic effects of ODN-2088 and chloroquine as well as the delayed onset of diabetes in TLR9^{-/-} NOD clearly point to TLR9 agonism as contributory to diabetes progression. We demonstrate that TLR9 agonists promote the activation of diabetogenic CD8⁺ T cells through the stimulation of pancreatic draining LN DC. TLR3 and TLR7 stimulation accelerate diabetes and immunity towards transgene-expressed islet antigens via IFN- α production and increased local class I MHC expression (50). TLR9 activation may also affect islet function directly and promote endothelial cell dysfunction. The source of TLR9 agonists mediating the spontaneous onset to diabetes in NOD mice remains unclear but could include gut bacteria-derived DNA, viral DNA, or endogenous DNA (derived from dying cells in the islet). Future identification of self versus foreign (commensal or pathogen) TLR9 agonists and their role in the onset of diabetes will allow the design of more specific interventions.

ACKNOWLEDGEMENTS

This work was supported by the JDRFI grant # 4-2004-783 as part of the β -Cell Apoptosis and Autoimmunity Network (β -CAAN) Program. (J.D.) and by the Canadian Institutes of Health Research (P.S.). A.S. is supported by a studentship from the Alberta Heritage Foundation for Medical Research (AHFMR). P.S. is a Scientist of the AHFMR. The JMDRC is supported by the Diabetes Association (Foothills). We are grateful to members β -CAAN Program and of the Dutz laboratory for their comments and support.

FIGURE LEGENDS:

Figure 1. *Effect of CpG, LPS and anti-CD40 on the activation of 8.3 CD8⁺ T cells in response to β -cell antigens in vitro.*

A. NOD BMDC were incubated with freeze-thawed (FT)-NIT-1 cells in the presence or absence of 5 μ g/ml CpG or 1 μ g/ml LPS \pm 15 μ g/ml anti-CD40 antibody overnight, CFSE-labeled 8.3 CD8⁺ T cells were added to the culture next day. After four 4 days of incubation, the cells were stained with anti-IFN- γ -APC intracellularly, and CFSE expression of CD8 cells was determined by FACS. B. NOD BMDC were incubated with either FT-NIT-1 or FT-B16 cells in the presence of CpG and anti-CD40. CFSE-labeled 8.3 CD8⁺ T cells were added to the culture next day. Four days later, CD25 expression of CD8⁺ T cells was determined. Bars represent average of 4 independent experiments with SEM. $p=0.037$ by Student T test.

Figure 2. *Effect of ODN 2088 or chloroquine on the induction of 8.3 CD8⁺ T cell activation.*

NOD BMDC were incubated with FT-NIT-1 cells with or without CpG or LPS and anti-CD40 in the presence or absence of 12 μ g/ml ODN 2088 or 2 μ g/ml chloroquine overnight. CFSE-labeled 8.3 CD8⁺ T cells were then added to the culture. After four days of incubation, the cells were assayed for proliferation, CD25 expression and IFN γ secretion. A: Dot-plots of one representative experiment. B: Mean of triplicate experiments. Bars represent SEM.

Figure 3. *Effect of ODN 2088 or chloroquine on the induction of NOD BMDC maturation.*

A. NOD BMDC were cultured with CpG or LPS in the presence or absence of ODN 2088 or chloroquine overnight. CD11c-positive cells were analyzed for CD40 expression. Representative histograms of 3 independent experiments are shown. B. NOD BMDC were cultured with or without CpG, LPS 1 μ g/ml and anti-CD40 in the presence or absence of ODN 2088 or chloroquine for 24 hours. IL-12 and IL-10 in the culture supernatants was determined by ELISA. Bars represent the means of triplicates with SEM.

Figure 4. *Effect of CpG, anti-CD40 and ODN 2088 on diabetes development in 8.3 NOD mice.*

5 week old 8.3 NOD mice were given 100 µg CpG and / or 200 µg anti-CD40 intraperitoneally, combined with 100 µg ODN 1982, or 200 µg rat IgG or with 300 µg ODN 2088. Blood glucose was determined every other day for 10 days. 4 female and 6 male mice in each group. p values represent logrank test results compared to untreated mice.

Figure 5. *Effect of CpG alone or with anti-CD40 on 8.3-NOD CD8⁺ T cell activation in vivo.*

A. 8.3 NOD mice were given 100 µg CpG or LPS alone or combined with 100 µg ODN 2088 or chloroquine at 10mg/kg or 20mg/kg IP. One day later, pancreatic lymph node cells were assayed for CD69 expression on CD8 cells. The left panel is a representative of four experiments. The summary of four experiments is shown in right panel. B. 8.3 NOD mice were given CpG plus anti-CD40 or PBS as control. After 4 days, blood circulating CD8⁺ T cells were assayed for CD44 and CD122 expression. The mean of triplicate experiments is shown in right panel. C. In vivo cytotoxicity assay. Five week old 8.3 NOD mice were given CpG and rat IgG, anti-CD40 and ODN 1982 (control ODN), or CpG and anti-CD40. After two days, the mice were adoptively transferred with self IGRP-derived V7 peptide (CFSE high) or irrelevant peptide (Tum)-pulsed (CFSE low) labeled splenocytes. The pancreatic lymph node cells were assayed for CFSE profile. The mean of triplicate experiments is shown in right panel.

Figure 6. *Effect of ODN 2088 or chloroquine on the induction of diabetes in 8.3 NOD mice.*

A. Female 3 to 4 week old 8.3 NOD mice were given either ODN 2088 or control PBS or ODN 1982 three times weekly. B. 8.3 NOD mice were given chloroquine or PBS (9 female and 9 male mice in each group). Blood glucose was measured weekly. The results represent the aggregate of 5 independent experiments for A and B. Differences were determined by logrank statistic.

Figure 7. *Effect of chloroquine on the induction of diabetes and BMDC maturation in NOD mice.*

A. Female 5 to 8 week old NOD mice were given chloroquine or PBS daily for 5 days and 2-3 times weekly thereafter. Blood glucose was measured weekly. The results represent the aggregate of 4 independent experiments. B. NOD mice were given chloroquine or PBS. After 4 weeks, the pancreatic lymph node cells were analyzed for CD40 expression on CD11c-positive cells. One representative experiment is shown in left panel. The right panel shows the mean of four experiments. C. NOD mice were given chloroquine or PBS, and then CPG or LPS. Cytokine and chemokine were detected from plasma by cytometry bead array.

Figure 8. *NOD TLR9 deficient mice delay diabetes development.*

Female NOD or NOD TLR9 knockout heterozygous (+/-) or homozygous (-/-) mice were followed for diabetes development. Differences were assessed using logrank analysis.

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B.2 TLR9 manuscript figures

Figure 1

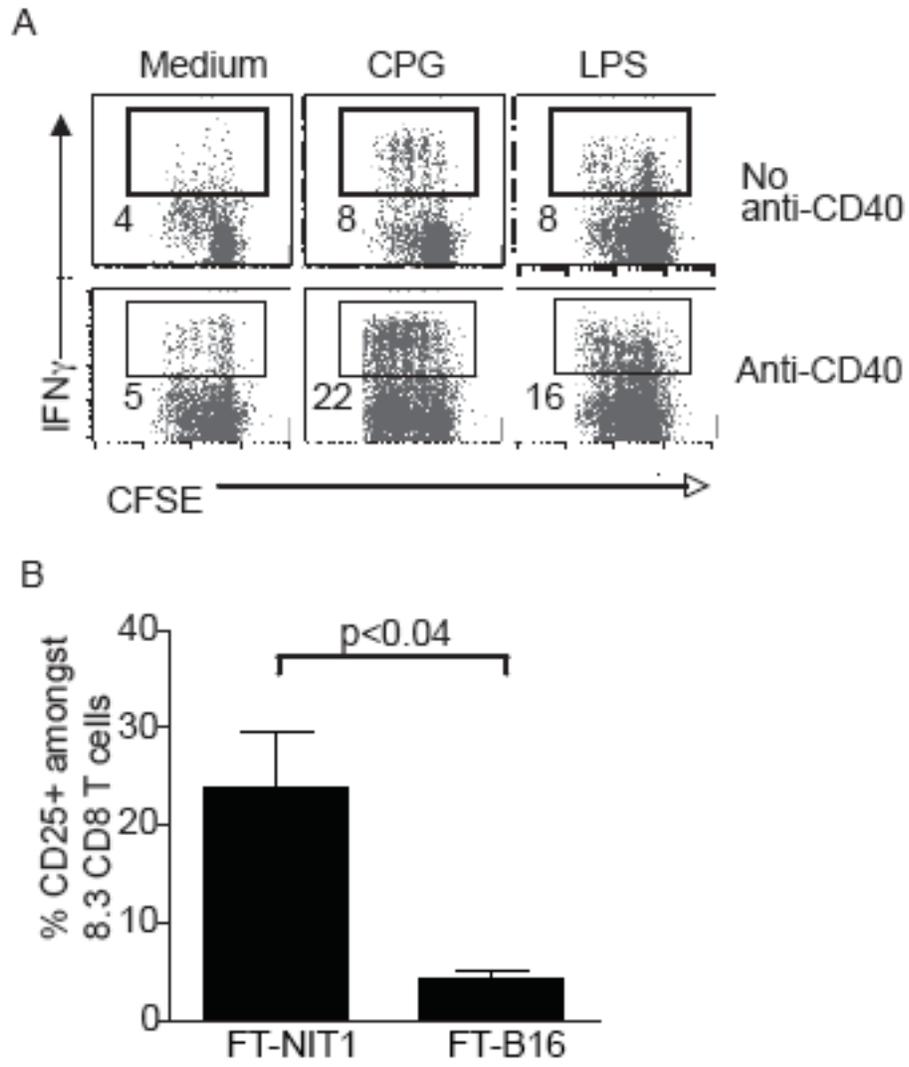


Figure 2

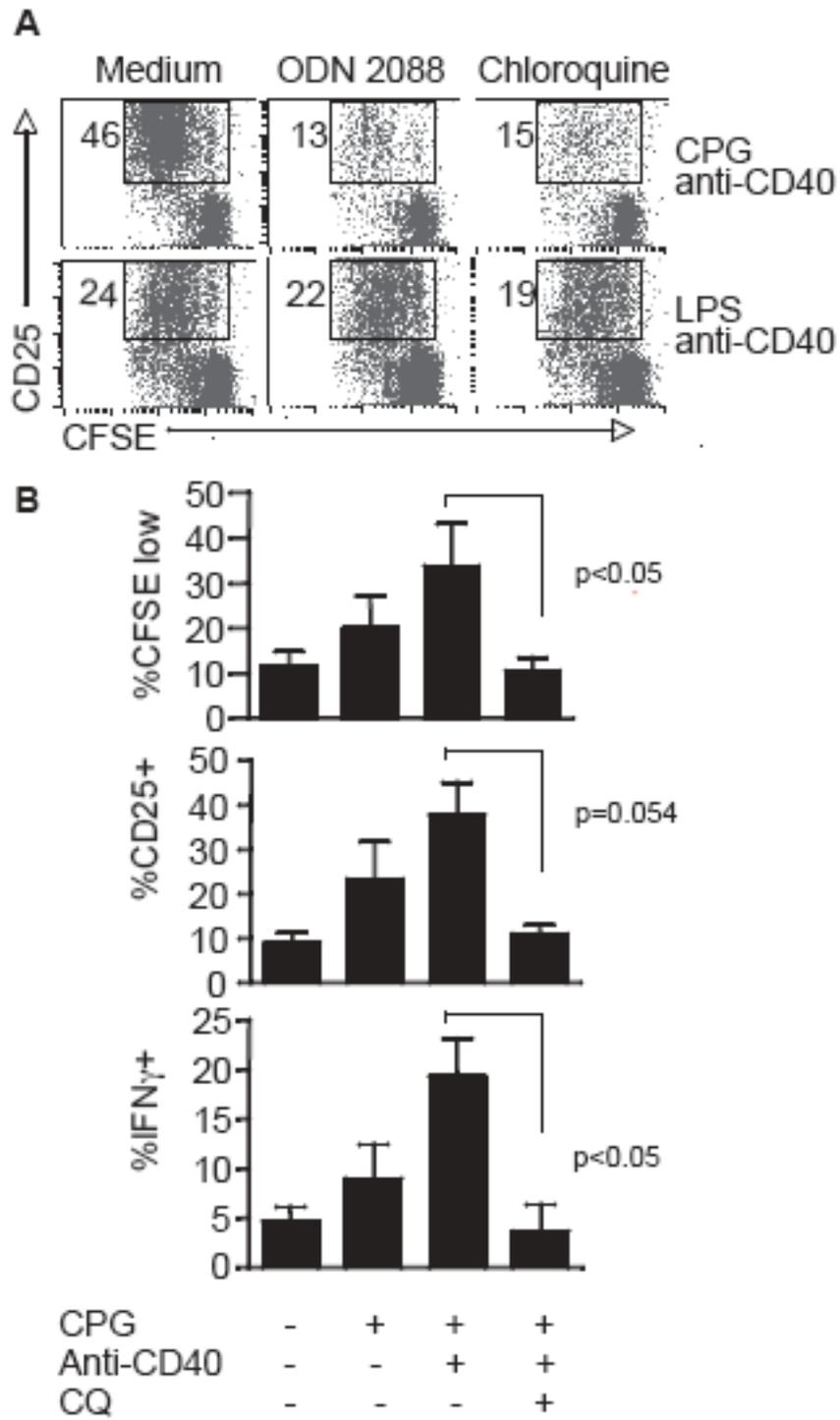


Figure 3

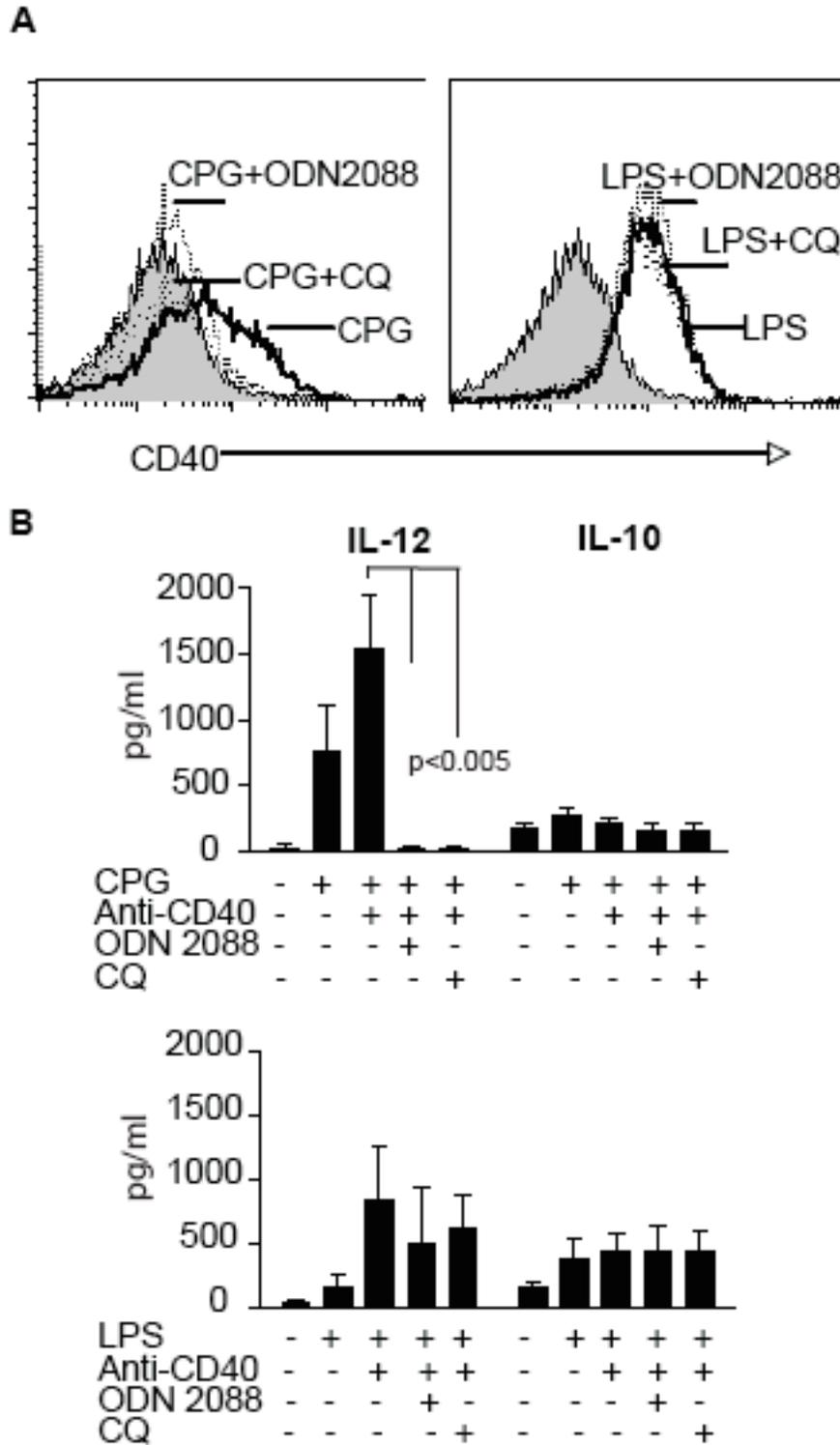


Figure 4

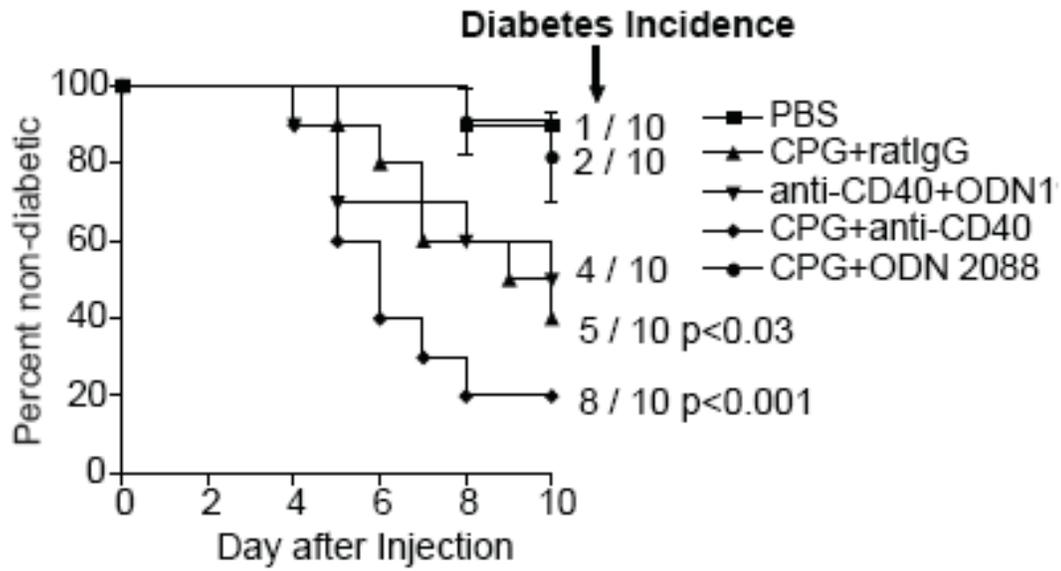


Figure 5

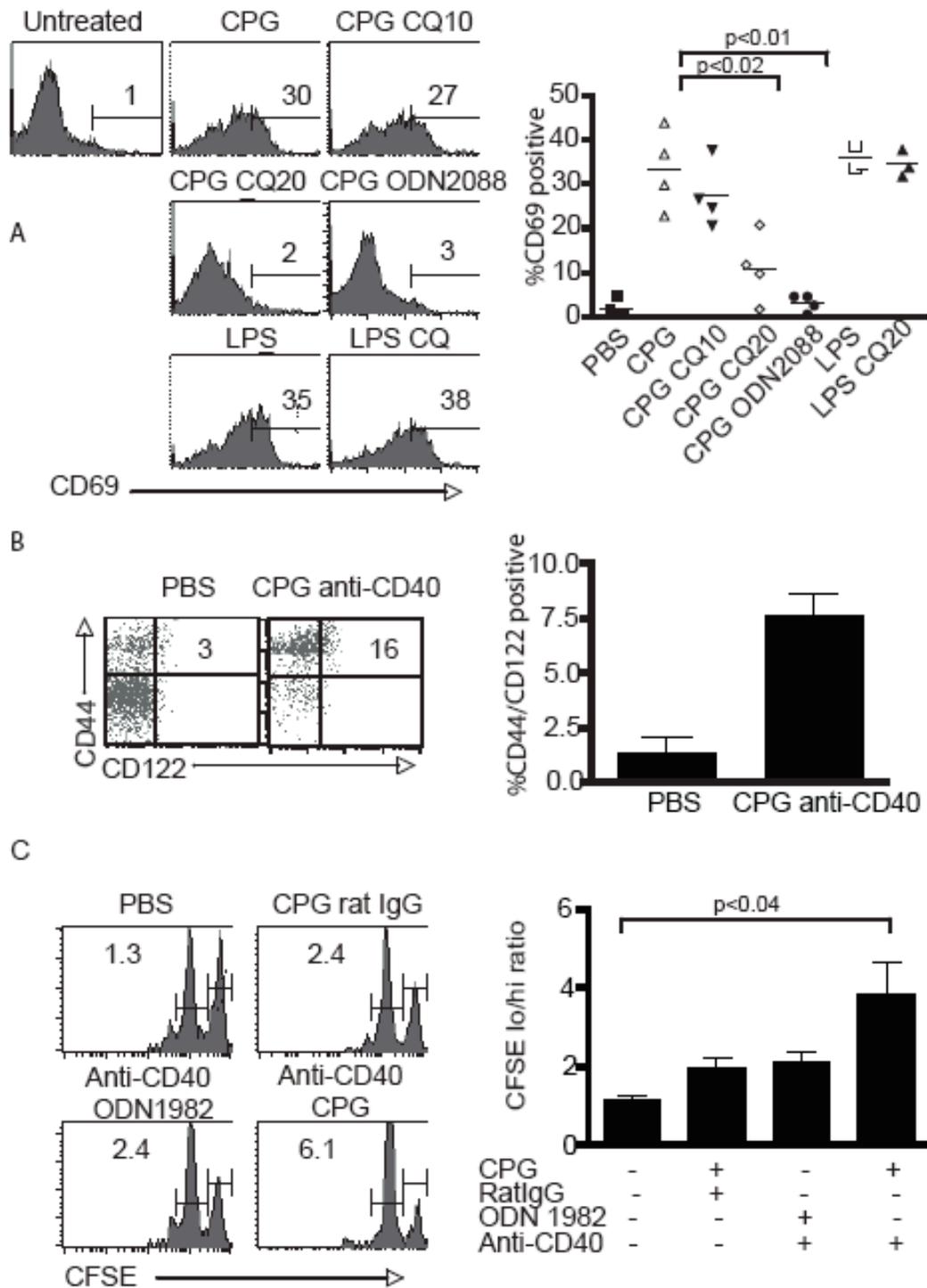


Figure 6

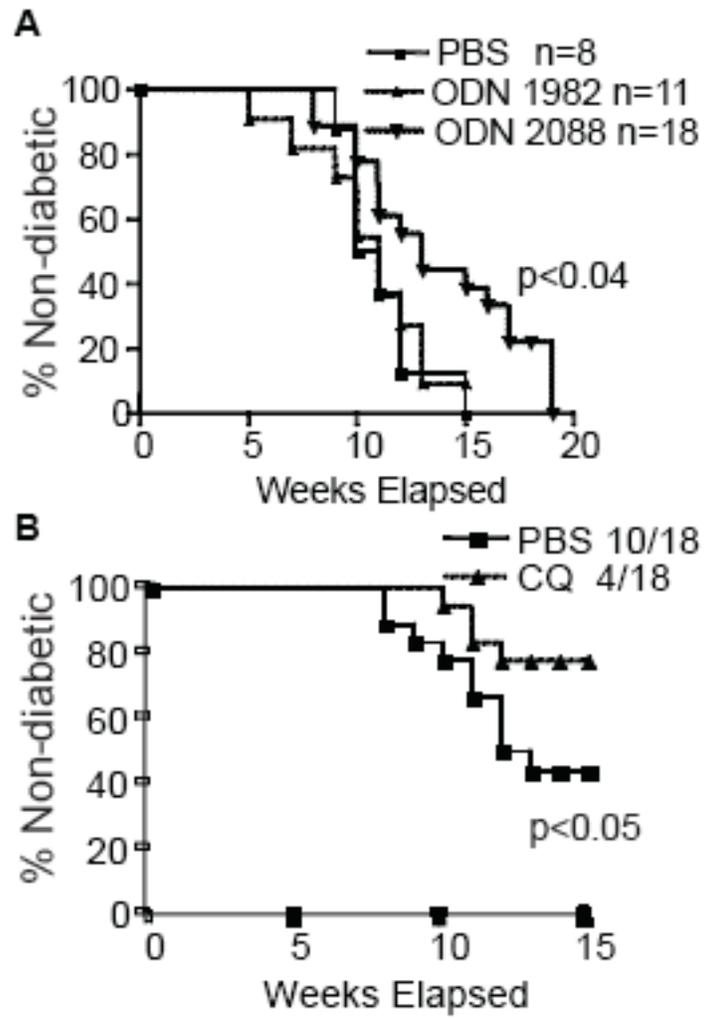


Figure 7

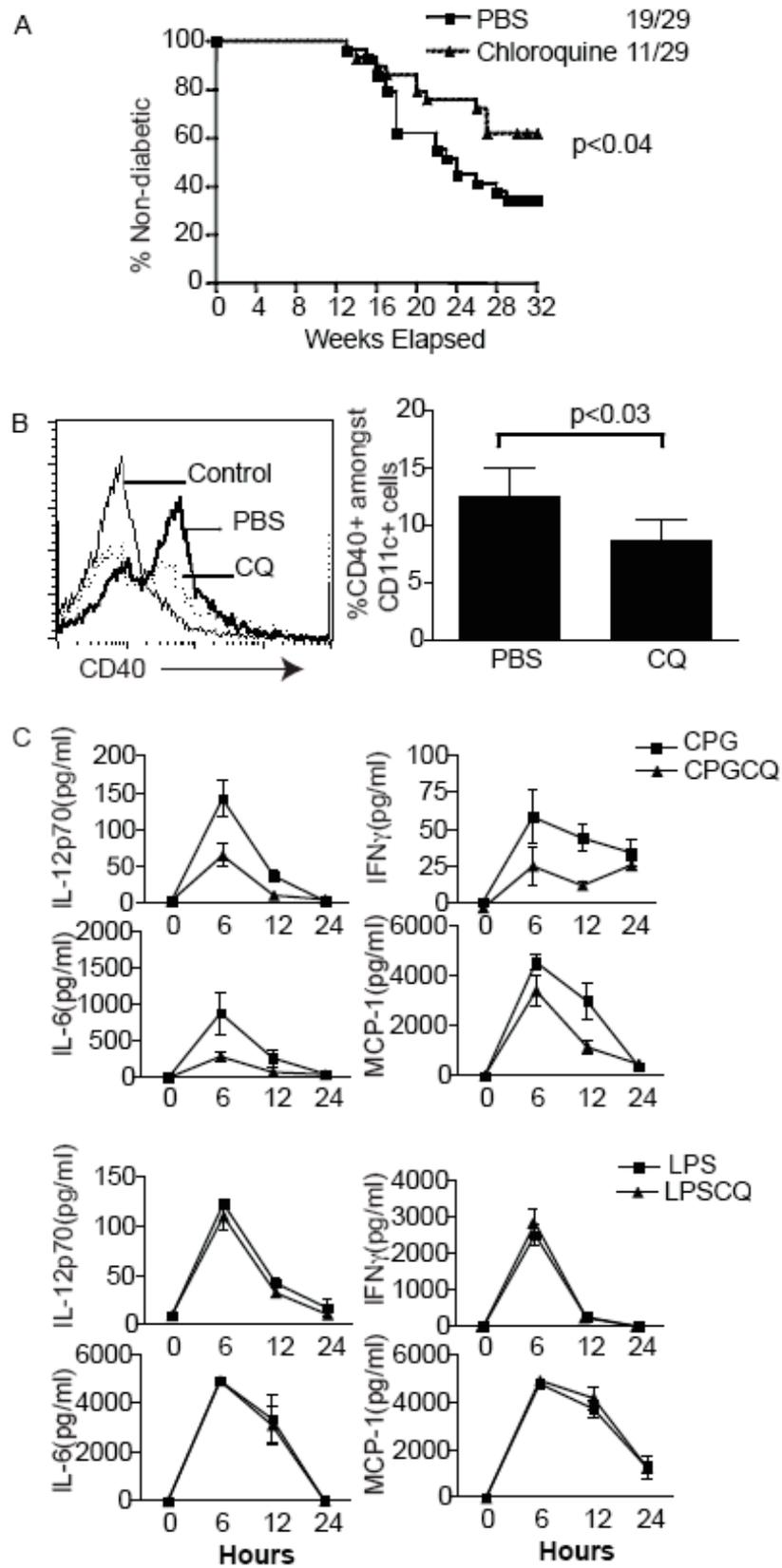


Figure 8

