UNIVERSITY OF CALIFORNIA, SAN DIEGO

Application of a Toll-Like Receptor 7 Agonist in Immunotherapy of Type 1 Diabetes

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Caroline Sheng is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

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2013

DEDICATION

To my father Jinsong Sheng, for his enduring example of innovation and creativity. To my mother Hong Sheng, for her enduring example of diligence and self-sacrifice. In recognition of their hard work and dedication for my sake, as well as of the loving care of my friends and Redeemer's Grace Church, whose gracious oversight and patient prayers are indispensible.

EPIGRAPH

We must learn to regard people less in the light of what they do or omit to do, and more in the light of what they suffer.

-Dietrich Bonhoeffer

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LIST OF ABBREVIATIONS

1V136	9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine
1Z1	3-(1-(1-(4-((6-amino-8-hydroxy-2-(2-methoxyethoxy)-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8,11,14,17,20-hexaoxa-2-azadocosan-22-yl)-1H-1,2,3-triazol-4-yl)propanoic acid
Ab	antibody
ANOVA	analysis of variance
BMDC	bone marrow-derived dendritic cell
BMDM	bone marrow-derived macrophage
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
DC	denritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC50	half maximal effective concentration
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
Foxp3	forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	hemotoxylin and eosin
IC50	half-maximal inhibitory concentration
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of κB
IL	interleukin
i.p.	intraperitoneal
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
i.v.	intravenous
JNK	c-Jun N-terminal kinase
KC	keratinocyte-derived cytokine
LN	lymph node
LPS	lipopolysaccharide
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MyD88	myeloid differentiation primary-response protein 88

NFkB	nuclear factor κ- light chain-enhancer of activated B cells
NOD	non-obese diabetic
OVA	ovalbumin
pDC	plasmacytoid dendritic cell
P/S	penicillin:streptomycin
PAMP	pathogen-associate molecular pattern
PD-1	programmed death-1
PD-L1	programmed death-1 ligand 1
PLN	pancreatic lymph node
pre-tx	pre-treatment
PRR	pattern recognition receptor
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
s.c.	subcutaneous
SEM	standard error of measurement
Treg	regulatory T cell
TCR	T cell receptor
Th	T helper
T1D	Type 1 Diabetes
TIR	Toll/interleukin-1 receptor homology
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRIF	TIR domain-containing adaptor-inducing interferon-β
TRAM	TRIF-related adaptor molecule
UNC93B1	unc-3 homolog B1
veh	vehicle

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ABSTRACT OF THE THESIS

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Type 1 Diabetes (T1D) is an autoimmune disease characterized by T-cell mediated destruction of the insulin-producing pancreatic islets. The activation of dendritic cells (DCs) contributes to initiating and perpetuating this inflammatory process as well as in other autoimmune disorders. It has been shown previously that low dose stimulation with a synthetic TLR7 ligand (1V136) led to TLR hyporesponsiveness and suppressed DC activation. A new TLR7 ligand was synthesized through conjugation to

six units of polyethylene glycol (1Z1) and the effects of 1Z1 on DC phenotype and function were characterized. 1Z1 did not stimulate proinflammatory cytokine production in comparison to the parent compound, 1V136, but still induced refractory status to subsequent TLR7 stimulation. DCs treated with 1Z1 exhibited a semi-matured phenotype and had decreased $CD4^+$ T cell activation function. Daily 1Z1 administration to prediabetic non-obese diabetic (NOD) mice significantly delayed the onset of disease and reduced immune cell infiltration into the pancreatic islets. 1Z1 increased programmed death-1 ligand 1 (PD-L1) expression on $CD11c^+$ DCs in the local lymph nodes. 1Z1 treatment neither caused any adverse effects such as weight loss or cytokine storm in vivo nor induced B cell proliferation in vitro. In summary, chronic 1Z1 treatment induced suppressive phenotype and functionality in DCs which reduced pancreatic T cell activation in prediabetic NOD mice. 1Z1 may be a novel treatment for DC-mediated organ-specific inflammatory diseases.

INTRODUCTION

1. Toll-Like Receptors

The discovery and study of Toll-like receptors (TLRs) has greatly improved our understanding of the role of innate immunity in autoimmune disease. Named as such for homology to *Drosophila* Toll, which was first noted for its role in embryonic dorso-ventral axis formation then discovered to be essential in the fly innate immune response (Akira et al., 2006, Janeway and Medzhitov, 2002), the mammalian TLRs are germline-encoded pattern recognition receptors (PRRs) that are specialized to recognize distinct molecular structures originating from pathogenic microbes or self components known as pathogen associated molecular patterns (PAMPs). Each TLR is characterized by an ectodomain of leucine-rich repeats, which mediate PAMP recognition, a transmembrane domain, and a cytoplasmic TIR domain, which mediates initiation of downstream signaling through interaction with and recruitment of various adaptor proteins (Kumar et al., 2009). The resulting signal transduction cascades induce and shape innate and adaptive immune responses (Lien and Zipris, 2009).

Thirteen mammalian TLRs have thus far been characterized, distinguished by expression, localization, and recognized PAMP (Table 1). TLRs 1, 2, 4, 5, 6, and 11 are expressed on cell surfaces and are specialized for the recognition of protein or lipid components of pathogens; TLRs 7, 8, and 9 are expressed intracellularly and recognize nucleic acids. The intracellular endosomal localization of these receptors is important for antiviral immunity without inducing response to self nucleic acid components. These receptors are synthesized and sequestered in the endoplasmic reticulum (ER) and must be trafficked to endolysosomes, a process that requires UNC93B1, a protein expressed in the

endolysosome (Kim et al., 2009). Proteolytic processing within the endolysosome is necessary for signaling functionality (Maschalidi et al., 2012).

Upon ligand binding and activation, TIR-domain containing adaptor proteins such as MyD88, TIRAP, TRIF, and TRAM are recruited. All TLRs except TLR3 signal through MyD88 (Akira et al., 2006). Activation of a signaling TLR recruits MyD88, which recruits IL-1 receptor-associated kinases (IRAKs) promoting downstream activation of mitogen-activated protein kinases (MAPKs) and nuclear translocation of the transcription factor nuclear factor kappa B (NFκB) which regulates the inflammatory response and the production of proinflammatory cytokines (Kawai and Akira, 2007). In addition, signaling through MyD88 or TRIF induces translocation of IRF3 or IRF7 into the nucleus (Kawai and Akira, 2010) which promotes expression of type I IFN, contributing to the antiviral immune response (Kaisho 2008). Activating these pathways also regulates expression of costimulatory molecules that promote antigen presentation. Thus, TLR-mediated activation of the innate immune system in response to PAMP recognition is an important mechanism in the body's defense against infection, and TLRs function as initiators of the innate and adaptive immune responses.

1.1 TLR tolerance

Detection of PAMPs through PRRs induces a robust inflammatory response characterized by cytokine release. Since an uncontrolled inflammation can result in tissue damage, this response must be under tight regulation. One such method of protection is endotoxin tolerance, a negative feedback regulation in response to TLR4 stimulation (Fan and Cook, 2004). Described as early as 1946, endotoxin tolerance is a phenomenon in which repeated exposure to low concentrations of endotoxin, the TLR4 agonist

lipopolysaccharide (LPS), results in a desensitization of the host to further endotoxin stimulation. Mice exposed to a sublethal dose of LPS are protected against a usually lethal dose of LPS, and exhibit reduction of proinflammatory cytokine production including TNF-α, IL-6, and IL-12, as well as downregulated activation of signaling cascades including the MAP kinases and IκB kinases, through TLR4-meditated mechanisms (Sato et al., 2002). This downregulation corresponds to increased expression of negative regulators such as IRAK-M, A20, and SOCS3 (Xiong and Medvedev, 2011). Refractory immune response after initial exposure has been characterized with other TLR stimuli (Dalpke et al., 2005; Kim et al., 2011; Tsukada et. al., 2007, Hayashi et al., 2009).

1.2 TLRs in autoimmune disease

Although TLRs are crucial in protecting against infection, hyperresponsiveness or misregulation of TLR to endogenous self-ligands is known to contribute to many inflammatory and autoimmune diseases (Zipris 2010). Autoreactivity results from activation of TLRs by self-antigens resulting from a breach of tolerance. Central tolerance is maintained by thymic clonal deletion (Hoqquist et al., 2005); peripheral tolerance, by costimulatory molecule expression (Jeker et al., 2012). When these protective measures are dysregulated or compromised by factors such as genetic predisposition, self-antigenic load, immature self T-cells, and absence of Treg functions, these developments can lead to the production of autoreactive T cells, autoantibodies, and inflammatory cytokines, causing autoimmune disease. Lack of restriction of TLR expression or negative regulation of signaling has been shown to initiate and aggravate these effects (Deane et al., 2007, Kondo et al., 2012); because of this, pharmacological suppression of TLR responses has been suggested as a method of treating such diseases

(Kawai and Akira, 2010). Notably, dysregulated TLR7 signaling has been shown to have roles in Type I Diabetes (Zipris 2010, Lee 2011).

2. Type 1 Diabetes

Type 1 diabetes (T1D), also known as insulin-dependent diabetes mellitus, is a T cell-mediated autoimmune disease in which autoreactive T cells target antigens expressed on pancreatic β cells, resulting in a progressive loss of insulin-producing cells in pancreatic islets (Bach, 1994). Though islet destruction is attributed to T cell infiltration, innate immune cell types such as B lymphocytes, neutrophils, and DCs are also involved in disease pathogenesis (Zipris 2010, Diana et al., 2013). Long-term disease progression leads to complete destruction of β cells, causing insufficient insulin production and submaintenance of glucose homeostasis (Katz et al., 1993).

A general overview of the natural history of T1D suggests that genetically susceptible individuals exposed to an environmental trigger develop β-cell autoimmunity, which precipitates the activation of autoreactive T cells, leading to a loss of β-cell mass and insulin production (Atkinson 2012). The genetic factors that influence T1D are complex and multifactorial, but susceptibility loci such as the MHC locus and IDDM locus have been identified (Concannon et al., 2009). Though the triggering events for the disease are yet to be made clear, functional abnormalities in antigen-presenting cells, such as DCs, have been implicated in initiating and perpetuating disease through studies in various models (Raine et al., 2006, Zipris, 2010, Diana et al., 2013).

2.1 NOD mouse

In particular, the NOD mouse (Non-Obese-Diabetic) model, in which disease develops spontaneously in a manner similar to humans, has been commonly used to

elucidate these processes. Developed in Japan in the 1970s, the NOD mouse exhibits multiple genetic susceptibility loci, including a defective MHC haplotype and impaired function in multiple subsets of leukocytes (Makino et al., 1980, Hanafusa et al., 1994). The progression of autoimmunity includes two checkpoints: insulitis, which is completely penetrant, and overt diabetes, which is partially penetrant. Peri-insulitis, or the beginnings of mononuclear infiltrates around the pancreatic islet, can be observed as soon as 3 to 4 weeks of age, and most mice exhibit severe insulitis by 10 weeks of age (Bach, 1994). Diabetes onset is usually observed from 12 to 14 weeks of age at a 60-80% rate of incidence for female mice. Percentage and timeframe differ in the male mouse (Anderson and Bluestone, 2005). It has been noted that disease incidence increases when the mice are kept in "cleaner" housing environments and decreases in "dirtier" caging, which indicates the possible role of environment in disease development (Bach and Chatenoud, 2012). Studies in this model have demonstrated that disease prevention can result from upregulation of Toll-like receptor signaling (Lien and Zipris, 2009).

2.2 Role of Dendritic Cells in T1D Pathogenesis

Signaling through TLRs induces DC maturation. DCs are antigen presenting cells (APCs) that are central in shaping T cell responses and coordinating the outcomes of immune recognition events. TLR-triggered activation of DCs results in activation, beginning a complex maturation process that involves the upregulation of surface expression of MHC class II, maturation markers such as CD83, and costimulatory molecules such as CD40, CD80, and CD86 (Steinman et al., 2003). The phenotypic and functional status of DCs determine whether the DCs will promote tolerance or present an antigen in an immunogenic manner (Jeker et al., 2012)

T1D is a T-cell mediated disease: transfer of $CD4^+$ and $CD8^+$ T cells from diseased NOD donors results in disease incidence in naïve recipients. Even so, DCs are also responsible for initiating islet-specific autoimmunity, as evidence points to the involvement of innate cells such as pDCs in the initiation of NOD T1D (Diana et al., 2013). β islet cells in the pancreas are the source of the autoantigens that drive disease, but they cannot directly prime the T cell response. DCs in the pancreatic lymph nodes (PLNs) prime naïve T cells, which then proliferate and differentiate to perform effector functions. The ability of DCs to take up and present pancreatic β cell antigens to autoreactive T cells in the pancreatic lymph nodes is essential for T1D pathogenesis (Turley et al., 2003; Ganguly et al., 2013; Diana et al., 2013). It has been reported that adoptively transferred immunomodulatory DCs prepared in vitro prevent T1D in NOD mice (Ma et al., 2003; Raine et al., 2006).

2.3 PD-L1 in T1D

Programmed Death-1 (PD-1), an inhibitory receptor of the B7/CD28 superfamily that is inducibly expressed on T cells, macrophages, and B cells, has been implicated to have a crucial role in peripheral tolerance (Keir et al., 2006; Fife et al., 2009). It has two differentially expressed ligands: PD-1 Ligand 1 (PD-L1; B7-H1; CD274), which is constitutively expressed on resting T cells, B cells, macrophages, and DCs, as well as inducibly on parenchymal cells, and PD-1 Ligand 2 (PD-L2; B7-H2), which is only inducibly expressed on DCs and macrophages. Due to the broad expression of PD-1 and PD-L1 on hematopoietic and non-hematopoietic cells, bidirectional inhibition is possible—PD-1 can inhibit T or B cell activation through engagement with TCR or BCR; additionally, PD-1 can inhibit macrophage and DC responses to TLR stimulation

(Francisco et al., 2011). TLR7 stimulation from viral infection has been shown to upregulate expression of PD-L1 on monocytes and DCs (Meier et al., 2008). The expression pattern of PD-L1 implies a direct role in regulating T-cell responses and prevention of organ-specific autoimmunity (Keir et al., 2006).

PD-L1/PD-1 ligation is an important negative regulatory pathway in T cell activation. Generally, T cell activation is initiated through the interaction of the TCR with the peptide/MHC complex on an APC and requires a costimulatory signal such as CD3/CD28 ligation—if signaling occurs through the TCR alone, the T cell enters a state of anergy or undergoes apoptosis (Mueller et al., 1989). T cell activation is negatively regulated through inhibitory costimulatory receptors such as PD-1; ligation of PD-1 to its PD-L1 suppresses CD28-mediated signaling cascades (Parry et al., 2005). Binding of PD-L1 to PD-1 during TCR activation inhibits secretion of cytokines and cytolytic function while negatively influencing T cell survival (Gianchecchi et al., 2013).

NOD mice have lower PD-L1 expression with respect to non-diabetes prone strains (Yadav et al., 2009). Studies conducted in NOD mice in which blocking antibodies against PD-L1 exacerbated disease onset (Won et al., 2010; Fife et al., 2009), and in which transgenic overexpression of PD-L1 in pancreatic islet cells decreased disease incidence (Wang et al., 2008), demonstrate the importance of this negative regulator of diabetes autoimmunity. Interestingly, though PD-L1 is also expressed on B cells, B cells are not necessary for PD-L1-blockade-mediated acceleration of disease (Guleria et al., 2007). SNPs in the *PD-1* gene have been associated with higher susceptibility to T1D in humans (Nielson et al., 2003).

2.4 Current Treatment

Insulin therapy has been the primary method of alleviating symptoms of T1D; before the discovery of insulin in 1921, patients with T1D died very soon after the initial onset of hyperglycemia. Since then, insulin therapy has developed and improved in increasing patient longevity, but it does not reverse or prevent degenerative complications typical of the disease. Despite insulin supplementation, acute and chronic complications along with mortality still occur (Atkinson and Eisenbarth, 2001). Alternative therapies that restore the body's regulation of endogenous insulin production have since been developed, including: islet or whole pancreas transplantation, artificial pancreas, regeneration of β-cells, and immunotherapy (Bach and Chatenoud, 2011)

Since insulin dependence is not immediate after disease diagnosis— it has been shown that 20% of patience within 5 years of diagnosis still produced insulin — using immunotherapy intervention to prevent or stop the progression of disease could potentially save existing β cell function and reduce reliance on exogenous insulin (Michels and Eisenbarth, 2011). Cyclosporin immunosuppressive therapy was tested in the mid 1980s and successfully induced prolonged disease remission, but was discontinued due to renal toxicity (Bach and Chatenoud, 2011). Other problems were observed with cyclosporine treatment: chronically administered chemical immunosuppressants such as cyclosporin can lead to permanent functional impairment of T cells, which can lead to secondary infections or tumorgenesis; additionally, after cessation of treatment, endogenous insulin production decreased, showing that tolerance to the islet antigens was not induced (Michels and Eisenbarth, 2011; Bach and Chatenoud, 2011).

Since then, other therapeutic strategies have been proposed and are under study, but the model of cyclosporine treatment demonstrates key concepts in immunotherapy for T1D: First, treatments must be strictly monitored for toxicological and immunologicak safety; second, especially in the case of children, the treatment should have lifelong efficacy without compromising the body's ability to ward off infection.

Because of the central role of Toll-like receptors (TLR) in the induction of immune responses, TLR agonists have been put forth as possible immunotherapy for T1D (Bach and Chatenoud, 2012).

3. TLR7 immunomodulation with 1Z1 in NOD mice

Various TLR agonists prevent disease in NOD mice when administered at 6 weeks of age (Quintana et al. 2000; Aumeunier et al. 2010). TLR7 stimulation has been shown to promote T1D in NOD mice, while inhibition prevented disease onset (Lee et al., 2011). Modulation of the immune response through TLR7 is a possible means to treat autoimmunity in this disease model.

Though the natural ligand for TLR7 is single-stranded RNA, imidazoquinolines and purine-like molecules also activate TLR7 (Lee et al., 2003), offering opportunity for pharmacological modulation of this pathway. In particular, it has been demonstrated that chronic, low-dose treatment with the purine-like molecule, 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (Figure 2, designated 1V136), results in reduced inflammation through specific and potent suppression of the TLR7-MyD88- NFκB signaling pathway in several mouse models of autoimmune disease (Hayashi et al., 2009). To develop this immunosuppressive effect, modifications of 1V136 were synthesized in order to minimize toxicity and to modify potency (Chan et al., 2011). From these modified

compounds, a 6-unit polyethylene glycol (PEG) chain modification (Figure 2, designated 1Z1) drew attention because of its reduced levels of inflammatory cytokine induction compared to the parent compound. This lowered agonistic potency indicated 1Z1 for antagonistic applications (Chan et al., 2011).

Preliminary studies characterized 1Z1 as a TLR7 agonist of low potency that blocked TLR7 activation and non-specific inflammation in thioglycolate peritonitis and DSS-induced colitis models of human autoimmunity (Hayashi et al., 2012). This reduction of inflammation by administration of 1Z1 was exhibited by DCs and not T cells, suggesting that 1Z1 diminished autoreactive T cell responses through a DC-targeted mechanism. Based on the efficacy of 1Z1 in these models, the involvement of TLR7 and DCs in the onset and perpetuation of T1D, and the ability of 1Z1 to induce a refractory state through TLR7, we hypothesized 1Z1 to be a good candidate a safe and effective immunotherapy of T1D. Further elucidating the mechanism of action, efficacy, and safety of this drug will aid in further pharmacological development of this ligand and in identifying further targets and strategies in immunotherapy of autoimmune disease. In this study, we characterized the function and phenotype of 1Z1-treated DCs in vitro and in vivo.

METHODS

Mice

Six to eight-week-old female NOD/ShiLtJ mice and C57BL/6 mice were purchased from Jackson laboratory (Bar Harbor, MA) and maintained in the University of Calfornia, San Diego Animal facility under standard conditions including food (PicoLab Rodent Diet 20) and water *ad libitum* and a 12 hour light and 12 hour dark cycle. All mice were studied according to the NIH Guidelines for the Care and Use of Laboratory animals, and all protocols were approved by the UCSD Institutional Animal Care and Use Committee.

Reagents

The PEGylated TLR7 agonist, 1Z1, and reference TLR7 agonist, 1V136, were synthesized in our laboratory (synthesis described in Chan et al., 2011; Chan et al., 2009) and dissolved in DMSO as a 100 mM stock solution and kept at -20 ºC until use. The stock solution was diluted in normal saline with a final DMSO concentration of 0.5 %, which was also used as the vehicle control. Endotoxin levels were determined by Endosafe® (Charles River laboratory, Wilmington, MA). Endotoxin levels of the drugs were <10 EU/μmol.

Cells

To generate bone-marrow derived DCs (BMDC), bone marrow was flushed from femurs and tibia of C57BL/6 mice and cultured in DC medium: RPMI (RPMI (Irvine Scientific, Irvine, CA) supplemented with 10% heat-inactivated FCS (Life Technologies, Gaithersburg, MD), 2 mM L-glutamine (Cellgro, Natham, VA), and 100 U/ml penicillin/100 μg/ml streptomycin (P/S; Cellgro) containing 5 ng/ml recombinant murine

GM-CSF (BD Bioscience). On day 3 and 6, the cultures were supplemented with additional DC medium. The nonadherent cells were harvested on day 7.

To prepare mouse bone marrow-derived macrophages (BMDM), bone marrow was flushed from femurs and tibia of C57BL/6 mice and cultured in macrophage medium, which consisted of supplemented RPMI containing 30% L929 cell conditioned media. L929 media was prepared by harvesting the supernatant from L929 cells cultured for 7 days. The adherent cell population was harvested between days 7 and 10.

Induction of TLR7 tolerance in BMDM

BMDM were seeded in flat-bottom 96-well plates (5×10^4/well) in complete DMEM and pre-treated by incubation in graded concentrations of 1Z1 or vehicle (0.05% DMSO) overnight. The next day, cells were washed and restimulated by incubation with 1μM 1V136 for an additional 18 h. Culture supernatants after pre-treatment and restimulation were collected and IL-6 concentration was determined by ELISA: antibody for IL-6 (BD Pharmingen, San Jose, CA) was used to coat half-area 96-well plates. Samples were diluted 1:2 and biotinylated rat anti-mouse antibodies (BD Biopharmigen and R&D Systems), horseradish peroxidase-conjugated streptavidin (BD Biopharmingen and R&D Systems) and tetramethylbenzidine (KPL, Gaithersburg, MD) were used to detect cytokine concentration. The absorbance was measured at 450nm-650nm using a microtiter plate reader (TECAN Infinite 200 PRO).

Immunoblot and quantitative RT-PCR

Bone marrow derived macrophages were replated after 7 days in 6-well plates at $2x10^6$ cells per well and tested on day 10. After removal of medium, cells were lysed and proteins were electrophoresed under reducing conditions and transferred to a membrane.

Antibodies to IRAK-M, phospho-JNK, phospho-ERK, phospho-p38 (Cell Signaling, Danvers, MA) and IκBα (Santa Cruz, Dallas, TX) were used. Horseradish peroxidase-conjugated anti-IgG (Santa Cruz) was used as the secondary antibody. The membranes were developed using a chemiluminescence system (ECL detection reagent: Amersham Life Science).

A portion of cell lysate was treated with DNase, after which total RNA from BMDMs was isolated using RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA). Quantity and purity were determined by absorbance using a NanoDrop spectrophotometer. cDNA was prepared from total RNA (1 μg) using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). DNA sequence for IRAK-M was amplified by real-time PCR in a 25-μl reaction volume using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) using an iCycler IQ (Bio-Rad). All samples were normalized to the housekeeping reference gene 18s RNA (ΔCt) or GAPDH. The comparative ΔΔCt method was employed to measure fold changes in expression of RNA transcript levels between untreated (t = 0 h) and drug-treated cells.

Flow cytometry

C57BL/6 BMDCs were cultured with 1Z1 or vehicle overnight and stained for CD40, CD80, CD86, or MHC class II (BD Biosciences, San Jose, CA). Expression of CD40 (clone 3/23), CD80 (clone 16-10A1), CD86 (clone GL1), or MHC class II in the gated $CD11c^+$ (clone HL3) population was studied using FACS Calibur cytometer (BD Biosiences), and the data were analyzed using FlowJo software (Ashland, OR). In another experiment, DCs were isolated from NOD mice treated daily with 1Z1 (400nmol) or vehicle for a week. Further purification of the cells using anti-CD11c magnetic beads

and a MACS column per manufacturer's instructions (Miltenyi Biotec, Auburn, CA) was performed for selected studies.

T cell isolation and co-culture with BMDC 1Z1 treatment in vitro

Ovalbumin (OVA) primed $CD4^+$ T cells were isolated from C57BL/6 mice immunized with 100 µg of OVA (Worthington Biochemical Corporation, Lakewood, NJ) mixed with ODN1860 (50 µg/mouse) day 0 and 14. $CD4^+$ T cells were isolated from splenocytes, using EasySep® Mouse CD4 Positive Selection Kit (Stemcell Technologies, Vancouver, BC. Canada). T cells (2×10^5/well of a 96-well plate) were labeled with CFSE (1 µM) for 30 min at 37°C, washed and then cultured with 10 µg/mL OVA(323-339) (Anaspec, Fremont, CA) and BMDC (2×10^5/well of a 96-well plate) in the presence of 1Z1 (1 to 5 µM) or vehicle for 3 days. $CD4^+$ T cell proliferation was monitored by CFSE dilution.

1Z1 treatment in NOD mice and assessment of diabetes status

Female NOD mice (n=5 to 10 /group) were given daily subcutaneous (s.c.) treatment of saline or 1Z1 (400nmol) beginning from 9 weeks of age for 18 weeks. Diabetes status was evaluated weekly by urine glucose levels assessed by Diastix (Bayer, Elkhart, IN). Upon detection of glucosuria (glucose levels in urine>250 mh/dL), blood glucose levels were determined by OnetouchUltra (Lifescan, Milpitas, CA). The hyperglycemia (>300 mg/dL) was confirmed by additional evaluations of blood glucose levels 24 h later. The first day of glucosuria (glucose levels in urine>250 m/dL) and hyperglycemia (blood glucose >300 mg/dL) detection, followed by an additional positive evaluation of blood glucose level 24 hours later, was defined as the date of diabetes onset.

In a separate experiment, mice were sacrificed after 4 weeks of treatment (8-12 weeks of age) and harvested pancreata were fixed in 4% paraformaldehyde and embedded in paraffin, then stained with hematoxylin and eosin (H&E). Ten to forty islets from each pancreas were scored for inflammation according to established criteria (Table 4). Sera were collected 2 h after the 1^{st} and 7^{th} injections and serum levels of IL-1β, IL-6, and TNF-α expression were determined by Luminex beads assay.

To evaluate DC phenotype after 1Z1 treatment, female NOD mice were treated for 7 days with 1Z1 or vehicle, after which buffy coat, pancreatic lymph nodes, and spleen were harvested. One group received LPS (100μg/animal) intraperitoneally five hours prior to sacrifice. Pancreatic lymph node cells and splenocytes were prepared using collagenase/DNase digestion (Inaba et al., 2011). The cells were stained for CD11c, CD86, B220, and PD-L1. Expression levels of CD86 or PD-L1 in $CD11c^+$ or $B220^+$ population were determined by flow cytometry.

Ex vivo immunological assay of T cells from NOD mice

$CD4^+$ T cells were isolated from vehicle or 1Z1-treated mice and labeled with carboxyfluorescein succinimidyl ester (CFSE). The cells (2×10^5/ well of a 96-well plate) were cultured with naïve (untreated) DCs isolated from 8 week old non-diabetic NOD mice (2×10^5/ well of a 96-well plate) in the presence of 10 μM β cell peptide NRP-V7 (KYNKANVFL, Genemed Synthesis, San Antonio, TX) for 3 days. T cell proliferation was monitored by CFSE dilution using flow cytometric assay.

In vitro cytokine induction after 1V136 or 1Z1 administration

C57BL/6 mice were injected with 200, 400, or 600 nmol of 1V136 or 1Z1. Two hours after the injection, mice were bled. Serum levels of IL-6, IL-12, and TNF-α were

detected by Luminex assays using the corresponding beads (Invitrogen, Carlsbad, CA). The plates were read using a Luminex IS 100 plate reader (Luminex Corporation, Austin, TX), and data were analyzed using Upstate BeadView v.1.0.4.15303 software (Upstate Biotech, Temecula, CA).

In vitro B cell proliferative activity

To evaluate the B cell proliferation induced by 1Z1 treatment, splenocytes isolated from C57BL/6 mice were labeled with 10μM CFSE in saline for 10 min (Lyons, 2000). The CFSE-labeled cells were incubated with 2 or 10 μM 1Z1 or 1V136 for three days. The cells were then surface stained for B220 to identify B cells. B cell proliferation was analyzed by flow cytometry.

Assessment of maximum tolerated dose

6 to 7 week old male and female CD-1 mice (n=5) were dosed with a single i.v. injection of 6, 60, 150, 200, or 1000 mg/kg 1Z1 and observed over a period of 14 days. During this period, clinical signs (reduced activity, semi-closed eyes, ataxia, hunched posture, piloerection) were scored daily, and body weight was measured daily. After 14 days, the animals were sacrificed and a necropsy examination was performed on all animals.

Statistical analysis

Prism5.0 (GraphPad, San Diego CA) was used for statistical analyses including regression analyses. Data were plotted and fitted by nonlinear regression assuming a Gaussian distribution with uniform standard deviations between groups and EC50 and IC50 were calculated. The data are represented as means ± standard errors of the mean (SEM). The one-way ANOVA with Bonferroni or Dunnett's *post hoc* test was used for

multiple comparisons to a control group. Kaplan-Meier Survival Analysis was used for analysis of diabetes incidence.

RESULTS

PEGylated TLR7 compound 1Z1 exhibits attenuated immunostimulatory profile but maintains immunosuppressive ability.

The PEGylated TLR7 agonist, 1Z1, was synthesized in our lab for the purpose of improving the systemic bioavailability of the water insoluble 1V136 (Chan et al., 2011). 1V136 is a potent TLR7 agonist that has been demonstrated to induce a hyporesponsive immune status following chronic low dose administration (Hayashi et al., 2009). The addition of a short (6-unit) PEG chain in 1Z1 resulted in reduced proinflammatory agonistic potency in comparison to the parent compound: subcutaneous administration of 1Z1 of C57BL/6 mice up to 600nmol did not induce TNF-α, IL-6, IL-12 in vivo, whereas 1V136 administration resulted in high levels of cytokine release in sera (Figure 3). Though 1Z1exhibits reduced proinflammatory activity, it maintains the ability to induce hyporesponsiveness to subsequent TLR7 stimulation: BMDC pre-treated with 1Z1 exhibited less proinflammatory response (IL-6 production) to subsequent TLR7 stimulation with 1V136 (Figure 4). To compare the dose response action of the proinflammatory and inhibitory effect of 1Z1, EC50 (50% maximum stimulatory dose) and IC50 (50% maximum inhibitory dose) of 1Z1 were calculated. Since the EC50 was calculated to be 10.6 μM and 1C50 to be 0.5 μM, the data suggested that 1Z1 is twenty times more potent as an inhibitor than an activator.

The ability of 1Z1 to induce hyporesponsiveness to subsequent TLR7 stimulation was further studied in the effect of the treatment on the MyD88-NFκB signaling pathway. BMDC pretreated with 1Z1 (Figure 5A right panel) showed suppressed phosphorylation of MAPK (ERK and JNK) and reduced degradation of IκBα in response to TLR7

stimulation in comparison to cells that received the vehicle (Figure 4A). In addition, we investigated the effect of 1Z1 treatment on the expression of IRAK-M (IRAK-3, interleukin-1 receptor-associated kinase 3), which is known to be a negative regulator of NFκB activation and plays an important role in countering inflammation in endotoxin tolerance (Kobayashi et al., 2002; Fan and Cook, 2004, Zhou et al., 2013). 1Z1 treatment induced IRAK-M expression in a dose dependent manner assessed by quantitative RT PCR (Figure 5B, EC50 4.8 μM) and the protein xpression of IRAK-M was confirmed by immunoblotting (Figure 5C). This upregulation of IRAK-M corresponded to the suppressed phosphorylation of ERK and JNK (Figure 5A).

In vitro 1Z1 treatment induces phenotypically semi-mature DCs with suppressive function.

The stage of DC maturation determines the balance of DC function between preserving tolerance and activating inflammation (Torres-Aguilar et al., 2010, Lutz and Schuler, 2002). Published data show that TLR ligands and the cytokine milieu can affect this DC differentiation—under the control of specific TLR activation or cytokine environment, a DC subset can be slanted towards tolergenic rather than immunogenic function, distinguished by a specific phenotypic maturation pattern (Frick et al., 2010, Lutz 2012). To define the effect of 1Z1 treatment on DC phenotype, BMDCs from NOD mice were incubated overnight with 1Z1 and flow cytometry was used to evaluate surface costimulatory molecule expression (Figure 6). 1Z1-treated BMDCs exhibited modestly increased levels of CD86, CD80, CD40 and MHC class II when compared to LPS-treatment as a positive control, suggesting 1Z1 induces a semi-mature phenotype of DCs in vitro (Steinman et al., 2003).

1Z1 induces functionally tolergenic DC

To determine the effect of 1Z1 treatment on the T cell activation function of DCs, 1Z1-treated DCs were cultured together with T cells in vitro (Figure 7). Ovalbumin (OVA) primed $CD4^+$ T cells were isolated from OVA immunized mice. OVA-primed $CD4^+$ T cells were incubated with WT or TLR7 null BMDC in presence and absence of 1Z1 and resultant T cell proliferation was monitored by CFSE dilution. Co-culture with 1Z1 treatment showed reduced $CD4^+$ T cells in WT DCs (Figure 7, upper panel). This inhibition was diminished in the co-culture with *Tlr7* $^{-/-}$ BMDC (Figure 7, lower panel). These data indicated that 1Z1 suppressed T cell proliferation through TLR7 on DCs.

Systemic 1Z1 administration induces phenotypically semi-mature DCs that affect T cell activation in the local lymph nodes.

To investigate the influence of 1Z1 treatment on DCs in vivo, pancreatic lymph nodes (PLNs) were harvested from 1Z1- or vehicle-treated NOD mice and expression of a maturation marker, CD86, was evaluated by flow cytometry. $CD11c^+$-gated DC population in 1Z1-treated mice exhibited a modest increase in levels of CD86 as compared to the LPS-treated groups as a positive control (Figure 8). To determine whether this semi-mature phenotype exerted a suppressive effect on T cell activation, $CD69^+$ expression in the $CD4^+$ gated population was compared. We observed a reduced proportion of activated ($CD69^+$) $CD4^+$ T cells in the pancreatic lymph nodes of 1Z1-treated mice (vehicle- vs. 1Z1-treated; 21 % vs. 11% respectively), but not in the spleen (5.3% vs. 4.9%, respectively) (Figure 9A). When $CD4^+$ T cells isolated from 1Z1-treated mice were incubated with BMDC from non-diabetic, naïve NOD mice and pulsed with pancreatic peptides, the $CD4^+$ T cells were capable of proliferating at similar levels as

vehicle treated mice (Figure 9B), suggesting that 1Z1 treatment suppressed T cell activation though attenuating DC function in a reversible manner.

Daily administration of 1Z1 inhibits development of diabetes and insulitis in NOD mice

Previous studies have indicated the efficacy of daily low dose treatment with the parent (unconjugated) TLR7 ligands in attenuating the severity T cell-mediated autoimmune disease models (Table 3).Because of the demonstrated efficacy of these models and the central role of DCs in T1D disease initiation (Turley et al., 2003; Ganguly et al., 2013; Diana et al., 2013), we asked whether 1Z1 treatment affects diabetic incidence in NOD mice in vivo. NOD mice were treated subcutaneously with daily dose of 1Z1 starting at 9 weeks of age for 18 weeks. 9 weeks is known to be after onset of insulitis and prior to the clinical diabetes (Anderson 2005). 1Z1-treated mice were significantly ($p=0.01$) protected from the onset of diabetes and the treated mice maintained diabetes-free status up to 14 weeks after withdrawal of the treatment (Figure 10A). While the average age of disease onset was 24 weeks, there was a >90% decrease in disease incidence in 1Z1-treated mice at 32 weeks of age in comparison to the vehicle-treated group.

To evaluate whether the disease-preventative effect of 1Z1 treatment continued after treatment was withdrawn, 8 week old NOD mice were given a daily dose of 1Z1 for 4 weeks (8 to 12 weeks) and diabetic status continued to be monitored after the discontinuation of treatment (Figure 10B). 1Z1-treated NOD mice maintained diabetic-free status 6 weeks after the withdrawal of treatment, while vehicle-treated mice developed hyperglycemia rapidly ($p=0.03$).

Since 1Z1 treatment was initiated after the onset of insulitis, we asked whether the treatment affected islet inflammation. Histological examination of pancreases isolated from treated mice showed decreased insulitis scores in the islets of 1Z1-treated mice in which less immune cell infiltration in the pancreatic islets was observed (Figure 11A and 11B).

Involvement of PD-L1 in diabetes onset of NOD mice

Since PD-L1 is involved in the onset and progression of diabetes in NOD mice (Wang et al., 2008; Yadav et al., 2009; Paterson et al., 2011), we hypothesized that the PD-L1 expression induced by 1Z1 could be involved in the mechanism of action of anti-diabetic effects of this treatment. In vitro, 1Z1 induced the upregulation of PD-L1 expression on DCs in a dose dependent manner (Figure 12A), with an EC50 in $CD11c^+$ cells of 1.6 μM (Figure 12B). In vivo, NOD mice treated with 1Z1 for 7 days showed elevated PD-L1 expression in $CD11c^+$-gated populations in the PLNs (Figure 13). This elevation of PD-L1 expression was also seen in the $CD11c^+$ population in the buffy coat, but not in B cells (Figure 14).

1Z1 lacks pro inflammatory activity in mice

Subcutaneous administration of 1Z1 to C57BL/6 mice up to 600 nmol did not induce TNF-α, IL-6 or IL-12 in vivo, whereas the administration of reference TLR7 ligand resulted in a vast cytokine release in sera (Figure 3). To evaluate the safety of long term administration of 1Z1, NOD mice were treated daily with 1Z1 (400nmol s.c.) and cytokine levels after the 1^{st} and 7^{th} treatment were assessed (Figure 15A). Concurrently, body weight was recorded daily (Figure 15B). Daily treatment with 1Z1 for 7 days did not induce inflammatory cytokines in the sera nor cause body weight loss. In an acute

toxicity study, mice administrated with a single i.v. injection of up to 1000 mg/kg 1Z1 did not exhibit any significant toxic effects, indicating that the maximum feasible dose of 1Z1 in mice was >1000mg/kg. TLR7 is expressed on B cells; stimulation of TLR7 on B cells causes B cell proliferation that is known as an adverse effect by TLR7 ligand treatment in clinical studies (Fidock et al., 2011). To evaluate whether 1Z1 influences mouse splenic B cell proliferation, splenocytes were incubated with 1Z1 and B cell proliferation was determined in the gated $B220^{+}$ population. Only a minimum proliferation was observed at 10 μM 1Z1 (Figure 15C).

DISCUSSION

DCs are professional antigen presenting cells that are involved both in activating and suppressing the immune response. Upon receiving danger signals and exogenous antigens, DCs initiate inflammatory responses to induce adequate immune protection. Simultaneously DCs suppress excessive and unnecessary immune responses to prevent harmful reactions in steady states in healthy host (Steinman et al., 2003). In individuals with certain predisposed genetic and environmental backgrounds, however, the balance between immunogenic and suppressive function of DCs is dysregulated and abnormal activation of autoreactive T cells causes chronic inflammation (Torres-Aguilar et al., 2010). Autoimmunity can develop from this dysregulation of the balance between DC tolergenic and immunogenic functionality (Frick et al., 2010, Lutz and Schuler, 2002, Jeker et al., 2012). This study demonstrates that 1Z1, a PEGylated TLR7 ligand, induced refractory DCs with suppressive function without causing proinflammatory immune reaction in vitro and in vivo. 1Z1 treated DCs provide suppressive effects on T cell responses without directly altering T cell phenotype or causing long-term T-cell-mediated immunocompromise.

TLR agonists have been used in treatments for allergies, cancer and infectious diseases as well as in vaccines (Sun et al., 2007). The specificity of a TLR7 expression in human leukocytes helps bypass the problems of broad-spectrum immunosuppression and prevent increased susceptibility to opportunistic infection, as in immunosuppressants such as dexamethasone (Bach et al.,2012). The involvement of TLR7 in T1D pathogenesis (Lee 2011; Lien and Zipris, 2009; Zipris 2010), implicates TLR7 ligands for

its treatment; however, the safety concerns associated with these ligands prevent current clinical development (Fidock et al., 2011). PEGylated drugs are considered safe for use in vivo and have been approved for distribution since 1990. The technique involves coupling PEG polymer to a drug or protein and functions to reduce body clearance and to increase the water solubility of very hydrophobic molecules (Pasut and Veronese, 2012). PEGylation has been used to enhance the therapeutic index of small molecule drugs by increasing the solubility of hydrophobic compounds, lowering toxicity, generating a desired pharmacokinetics profile, and enhancing target site accumulation. Thus, PEGylation can create novel drugs with new therapeutic applications (Veronese et al., 2008, Li et al., 2013). Because 1Z1 contains only a short 6 units and maintains a molecular weight less than 1000, pharmacokinetics of 1Z1 in mice was similar to the parent compound (Chan, et al 2011). Despite this, proinflammatory activities were successfully eliminated in the PEGylated drug in comparison to its parent compound, 1V136 (Chan et al. 2011). Moreover, in vitro data showed that 1Z1 is far more potent as an inhibitor than an activator (Figure 4). The flexibility gained by the elongated therapeutic window of 1Z1 can be used to address the possible concerns associated with systemic administration of TLR7 agonists (Fidock et al., 2011) in providing a clinical effect while minimizing the chance of severe adverse side effects.

Despite lowered agonistic potency, 1Z1 was still able to induce a refractory state to subsequent TLR7 stimulation in previous studies (Chan et al., 2011). In this project, we presented two possible mechanisms by which 1Z1 acts as immunomodulatory agent. First, 1Z1 pre-treatment reduced the response of proinflammatory cytokines and downstream signaling, corresponding with the upregulation of a negative regulator of the

MyD88/NFκB signaling pathway, IRAK-M, in a manner similar to the model of endotoxin tolerance (Kobayashi et al., 2002; Sato et al., 2002; Zhou et al., 2013). This indicates that 1Z1 retains the ability to induce a refractory state through TLR7 while having a reduced pro-inflammatory profile, which is advantageous in the safe application of this TLR7 ligand for immunotherapeutics. Second, 1Z1 can alter the DC phenotype to a suppressive, moderately matured phenotype without activating NFκB. These semi-mature DC exhibited functionally suppressor effects on antigen-specific T cell proliferation both in vitro and in vivo. DCs with suppressor phenotypes have been induced by exposure to TNFα or short term exposure to low dose of the TLR4 ligand, LPS (Salazar et al., 2007; Kalantari et al., 2011). Further studies are necessary to identify the regulation of TLR tolerance and induction of suppressor DCs in vivo by PEGylated TLR7 ligands.

PD-L1 ligation to its receptor PD-1 negatively attenuates TCR signaling and T cell activation, which contributes to the inhibition of immune-mediated tissue destruction (Keir et al., 2008). In the pathology of T1D, PD-1-PD-L1 interaction has been implicated in the regulation of induction and progression of autoimmune diabetes in the NOD mouse (Ansari et al., 2003). We demonstrated that PD-L1 expression on $CD11c^+$ DCs by 1Z1 increased in a dose dependent manner in vitro. Additionally, PD-L1 expression was elevated on the surface of local pancreatic DCs and DCs in the peripheral blood in 1Z1-treated NOD mice. We observed that in vivo blocking of PD-L1 by anti-PD-L1 greatly accelerated disease onset regardless of treatment (data not shown), pointing to the importance of PD-L1 in maintaining peripheral tolerance in this model.

Human TLR7 expression is limited to pDCs; however, in rodent, both DC subsets and B cells express TLR7 (Kawai and Akira, 2010). When PD-L1 expression was evaluated in the peripheral blood of 1Z1-treated NOD mice, PD-L1 expression was upregulated in the $CD11c^+$ DC population, but not in $B220^+$ cells (Figure 14). The addition of PEGylation to small molecule 1V136 resulted in reduced protein binding (Chan et al., 2011), which may reduce access to intracellular TLR7 in murine B cells. Meanwhile, endocytic function of DCs supports the access to intracellular receptors, suggesting that 1Z1 targets DCs rather than B cells. In fact, while 1Z1 triggered DC maturation at concentrations as low as 0.4 μM in vitro (Figure 5), 1Z1 concentrations up to 10 μM did not cause B cell proliferation, suggested that this drug is more potent in DCs (Figure 15). In a previous study, it was also demonstrated that PEGylated 1Z1 did not induce B cell proliferation in human PBMC (Chan et al., 2009). As abnormal B cell proliferation is one of serious adverse effects of TLR7 ligand treatment, PEGylation may improve the safety of 1Z1 by targeting professional antigen presenting cells.

In T1D, APCs such as DCs and macrophages contribute to priming and activation of autoreactive T cells and destruction of beta cells (Nicolic et al., 2009; Diana et al., 2011). Our data indicated that long-term administration of 1Z1 prevented diabetes onset in NOD mice and that protection from disease persisted after the withdrawal of treatments. T cell activation in the local lymph nodes was suppressed by 1Z1; however, suppressed function in T cells was transient and was not permanent damaged because the autoreactive T cells could proliferate normally when they were restimulated ex vivo with naïve DCs (Figure 9). Thus, 1Z1 primarily influenced DC phenotypes and function, secondarily influencing T cell function in a reversible manner.

In NOD mice, infiltration of lymphocytes and macrophages into the pancreatic islets begins at 3-4 weeks of age followed by overt diabetes beginning at approximately 12 weeks of age (Bach, 1994; Hanafusa et al., 1994). In our study, 1Z1 treatment was initiated in week 8 to 9 of age, in the pre-diabetic stage. Progression from this stage to overt disease and hyperglycemia requires continued beta cell destruction through the activation of autoreactive T cells. Therapeutic application of 1Z1 reduced diabetic incidents in NOD mice after initiating autoreactive T cell activation, indicating partial remission and glycemic control similar to the honeymoon period of human T1D (Abdul-Rasoul et al., 2006). To determine whether the tolerogenic/suppressor phenotype of DCs by 1Z1 treatment is permanent or transient, we are currently conducting an experiment using mice into which DCs that have been exposed in vitro to 1Z1 are adoptively transferred. NOD mice that have received a transfer of in vitro prepared tolerogenic DCs exhibit a slight delay in the onset of diabetes (data not shown).

In summary, the current study demonstrated that 1Z1 ameliorated T1D through induction of refractory DCs with T cell suppressive function. In vivo, 1Z1 exhibited an acceptable safety profile in that up to 600nmol per animal did not induce any cytokine storm. The advantage of 1Z1 treatment was that this drug induced neither cytokines, nor body weight loss, as described as adverse effects of TLR7 treatment (Hayashi et al., 2008), even in doses up to 1000mg/kg. 1Z1 may be a new class of DC targeting treatment for autoimmune diabetes.

This work in part includes material currently being prepared for publication: Hayashi, Tomoko; Yao, Shiyin; Crain, Brian; Promessi, Victor J.; Shyu, Luke; Sheng, Caroline; Kang, McNancy; Corr, Maripat; Cottam, Howard; Carson, Dennis.

FIGURES

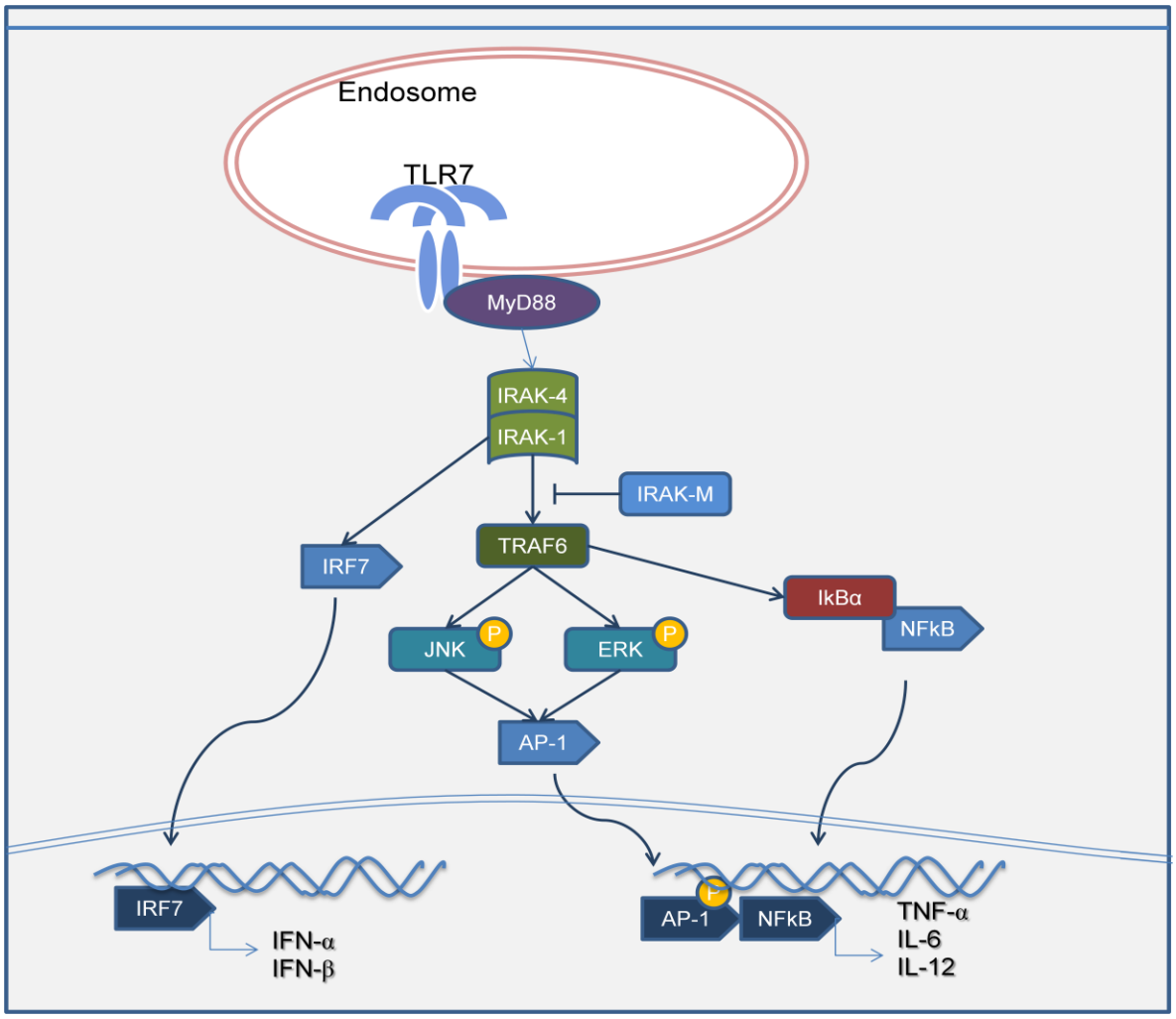

Figure 1: Toll-like receptor 7 signaling. Binding of TLR7 to its ligand causes a signal cascade through its signal adaptors, resulting in the nuclear translocation of the transcription factors NF-κB, AP-1, and IRF7. In the nucleus, NFκB and AP-1 coordinate inflammation by upregulating expression of pro-inflammatory cytokines and chemokines, whereas IRF7 coordinates the viral immune response through the production of Type 1 interferon (Kawai and Akira, 2010).

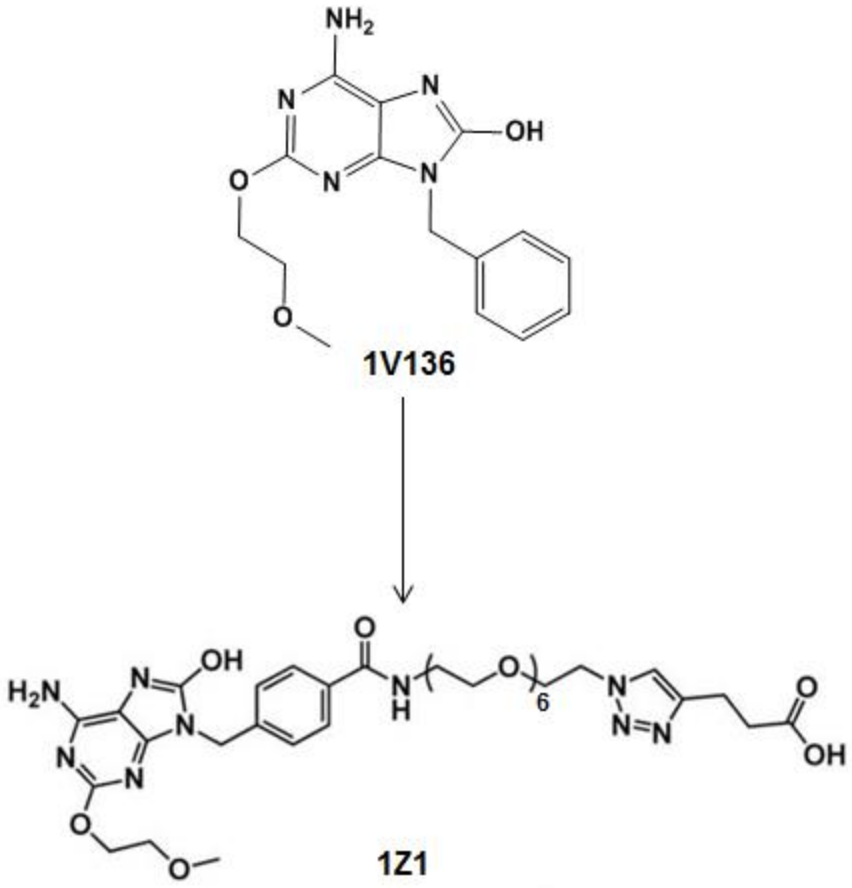

Figure 2: Synthesis and Structure of 1Z1. A known TLR7 ligand, 1V136 (MW= 315.33 g/mol) was conjugated to a 6-unit polyethylene glycol chain (Chan et al., 2011) and designated 1Z1 (MW= 790.59 g/mol). Characterization of 1Z1 showed that the compound exhibited reduced potency in induction of inflammatory cytokines *in vivo* and *in vitro* in comparison to the parent TLR7 ligand (Chan et al., 2011).

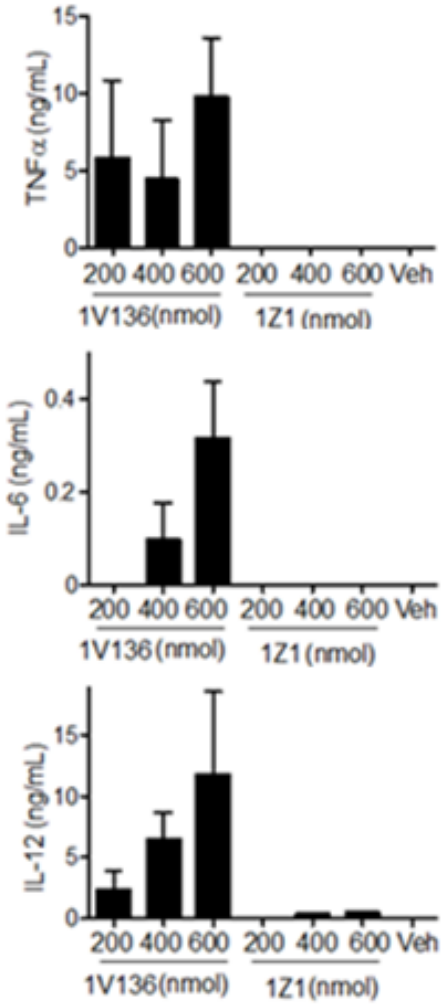

Figure 3: 1Z1 treatment does not induce cytokine release. C57BL/6 mice were given s.c. injections of 200, 400 or 600 nmol 1V136 or 1Z1. Two hours after the injection, the levels of TNFα, IL-6, and IL-12 in the sera were determined by Luminex bead-based assay.

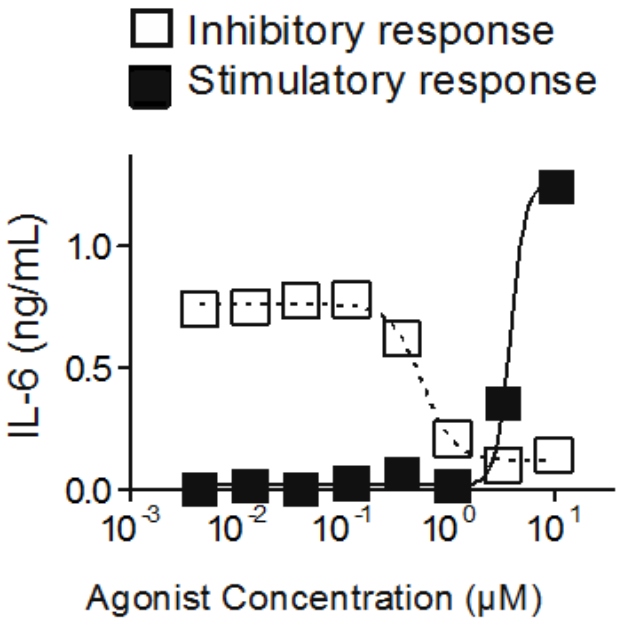

Figure 4: Pretreatment with 1Z1 induces hyporesponsiveness to subsequent TLR7 stimulation. BMDMs were stimulated overnight with graded concentrations of 1Z1 (indicated) and the culture supernatants were collected. The cells were washed twice with fresh media and cultured with 1 µM reference TLR7 ligand, 1V136 for 18 h. IL-6 levels in the culture supernatants of pretreatment (closed square) and after restimulation (open square) were compared. Data shown are representative of two independent experiments.

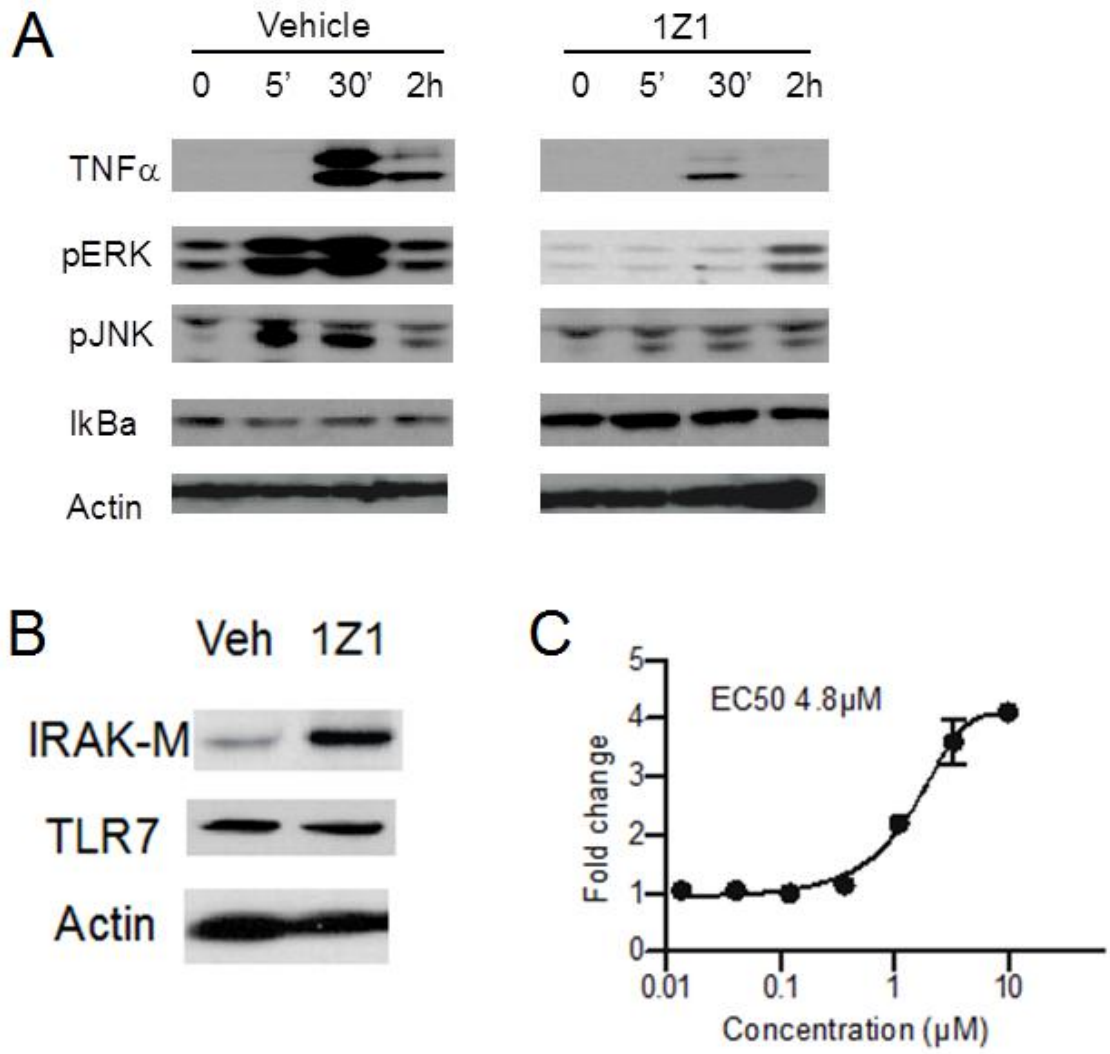

Figure 5: 1Z1 treatment results in downregulation of MyD88/NFκB signaling in response to subsequent TLR7 stimulation. *A) Less phosphorylation of ERK and JNK and less degradation of IκBα.* BMDM were treated with vehicle or 1Z1 overnight (1st treatment) and restimulated with reference TLR7 ligands (2nd treatment) for indicated period (min). The cell lysate were separated in SDS-PAGE and proteins were detected by immunoblotting. *B, C) Upregulation of negative regulatory molecule of MyD88 pathway, IRAK-M. B)* IRAK-M and TLR7 expression were detected by immunoblotting. *C)* IRAK-M expression was evaluated by quantitative RT-PCR 1h after 2nd treatment. Fold increase of IRAK-M expression by graded concentrations of 1Z1 were plotted. EC50 was calculated using Prism 6.0.

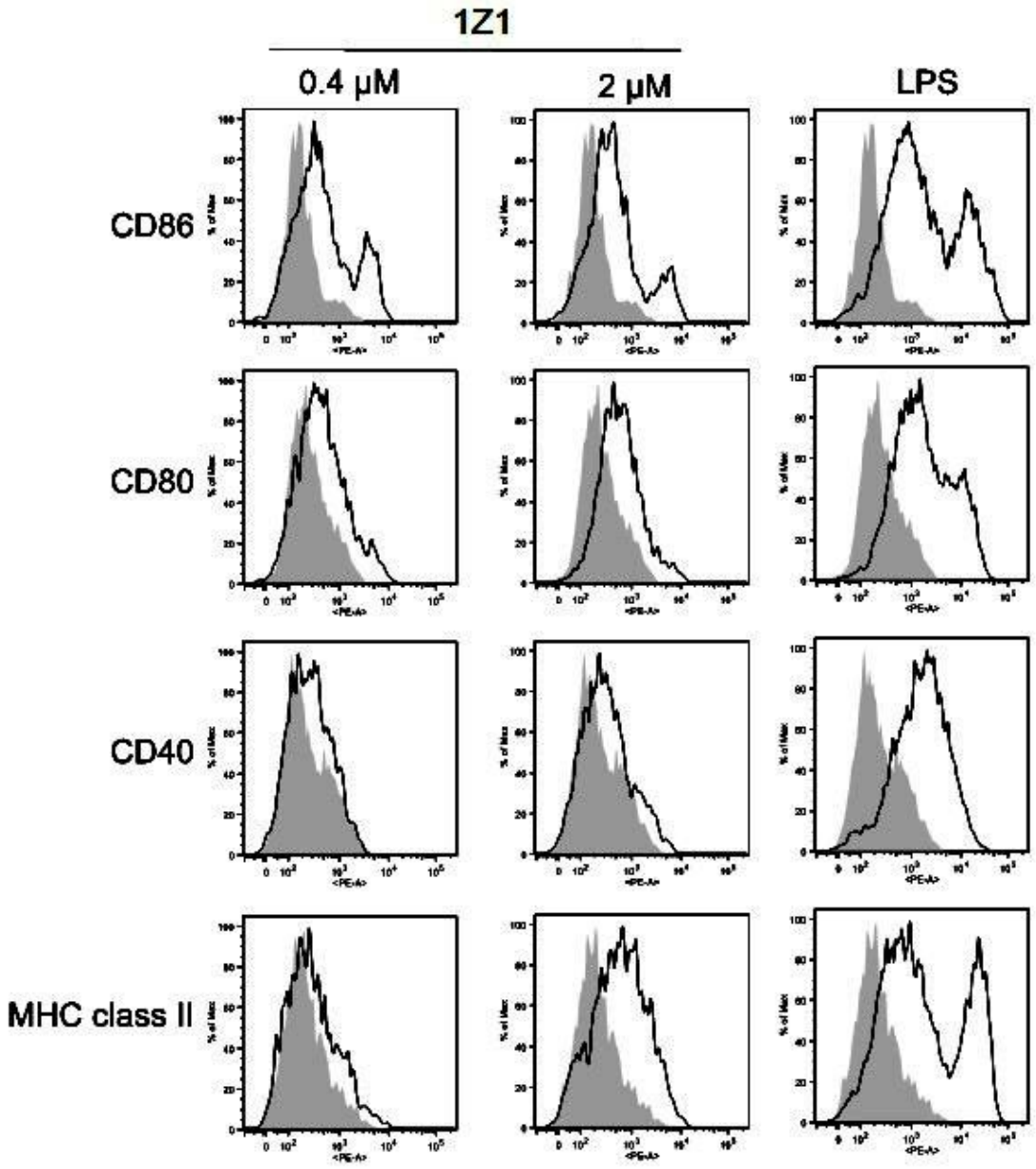

Figure 6: 1Z1 treatment induces modest increase in expression of costimulatory molecules on DCs. C57BL/6 BMDCs were incubated overnight with indicated concentration of 1Z1 or LPS (100ng/mL). The expression of CD86, CD80, CD40 or MHC class II was evaluated in $CD11c^{+}$-gated population (solid black line). Shaded grey indicates the expression pattern of vehicle-treated cells.

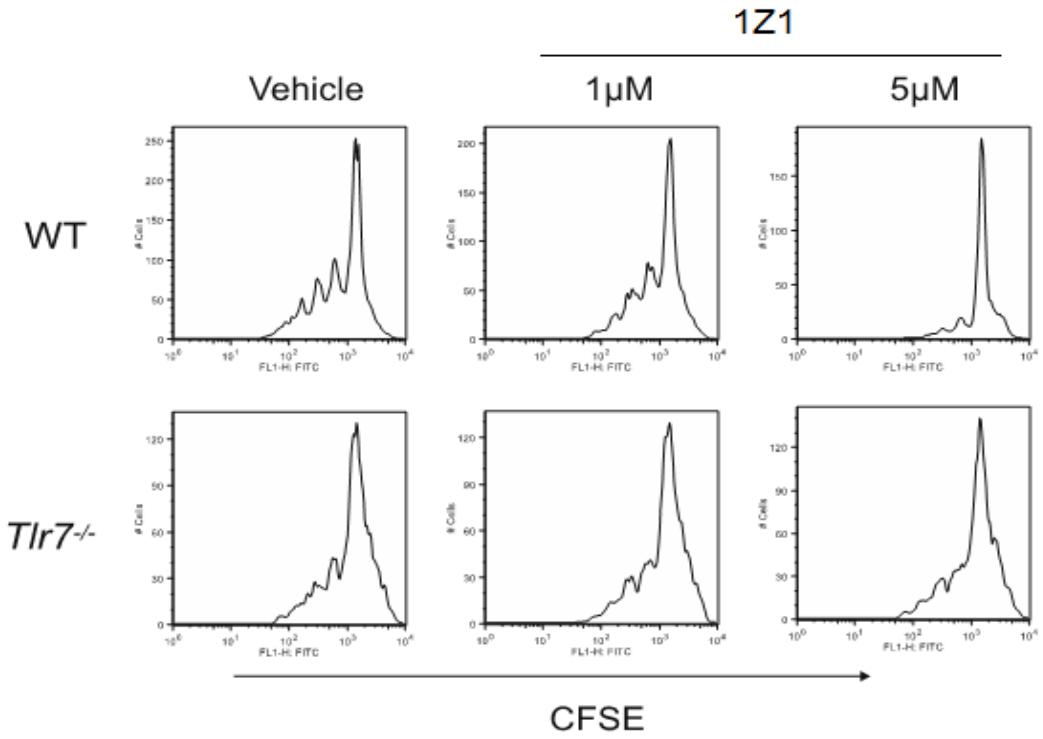

Figure 7: Antigen-specific T cell proliferation inhibited by in vitro 1Z1 treatment. OVA primed $CD4^+$ T cells were labeled with CFSE and incubated with WT or $TLR7^{-/-}$ BMDC, with OVA in the presence or absence of 1Z1 (1 or 5 μM) or vehicle for 5 days. T cell proliferation was monitored by CFSE dilution.

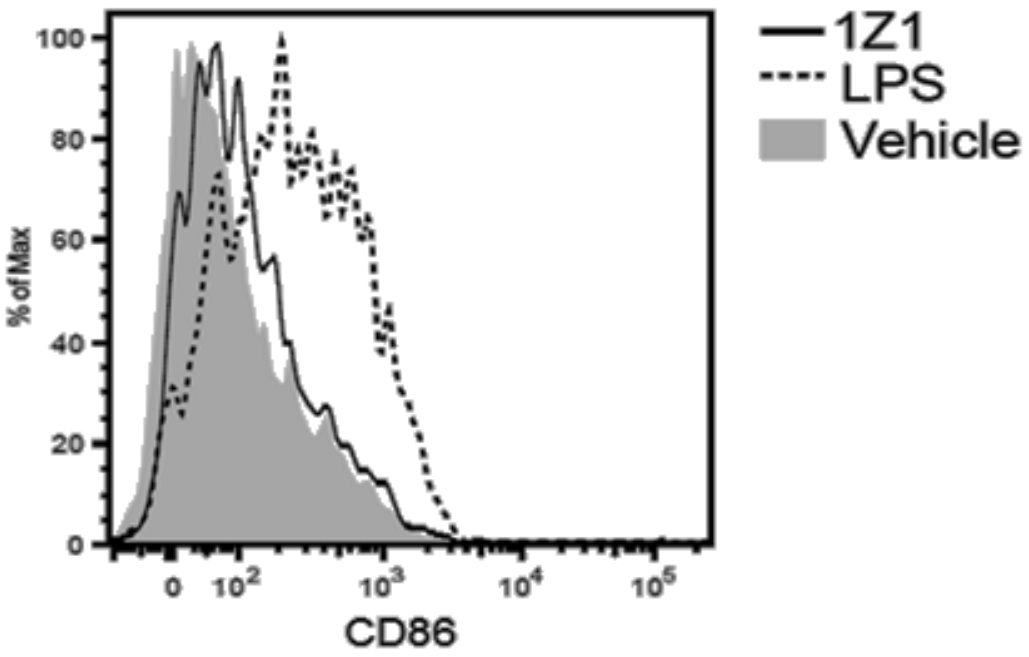

Figure 8: DCs treated with 1Z1 in vivo exhibit semi-mature phenotype. NOD mice (n=3-5/group) were treated daily with vehicle or 1Z1 for 7 days. LPS group received LPS (100µg/animal) 5h prior to sacrifice. CD86 expression was evaluated in gated $CD11c^+$ population in PLNs.

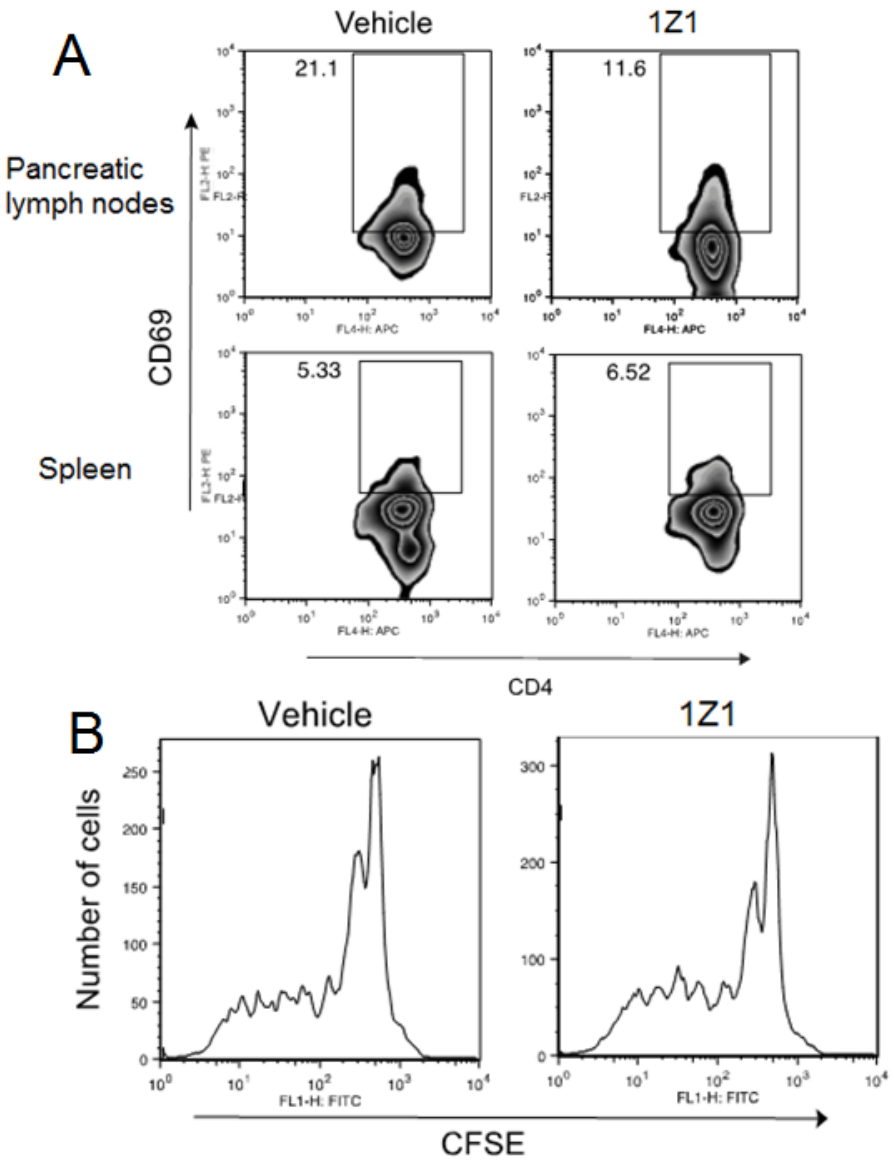

Figure 9: 1Z1-treated DCs suppress T cell proliferation reversibly. *A) Decreased T cell activation in PLN of 1Z1 treated mice***.** NOD mice were treated s.c. with 400 nmol 1Z1 from 8 to 12 weeks of age. PLNs and splenocytes from 1Z1 treated NOD mice were harvested, fixed, and stained for CD4 and CD69. CD69 expression on $CD4^+$ gated population was analyzed by flow cytometry. *B) T cell function is restored when cultured with naïve untreated BMDC.* $CD4^+$ T cells isolated from NOD mice treated with 1Z1 (daily treatment from 8 to 12 weeks of age) were labeled with CFSE and cultured with BMDC collected from naive non-diabetic NOD mice, pulsed with insulin peptide B9-23. Proliferation of T cells was monitored by CFSE dilution.

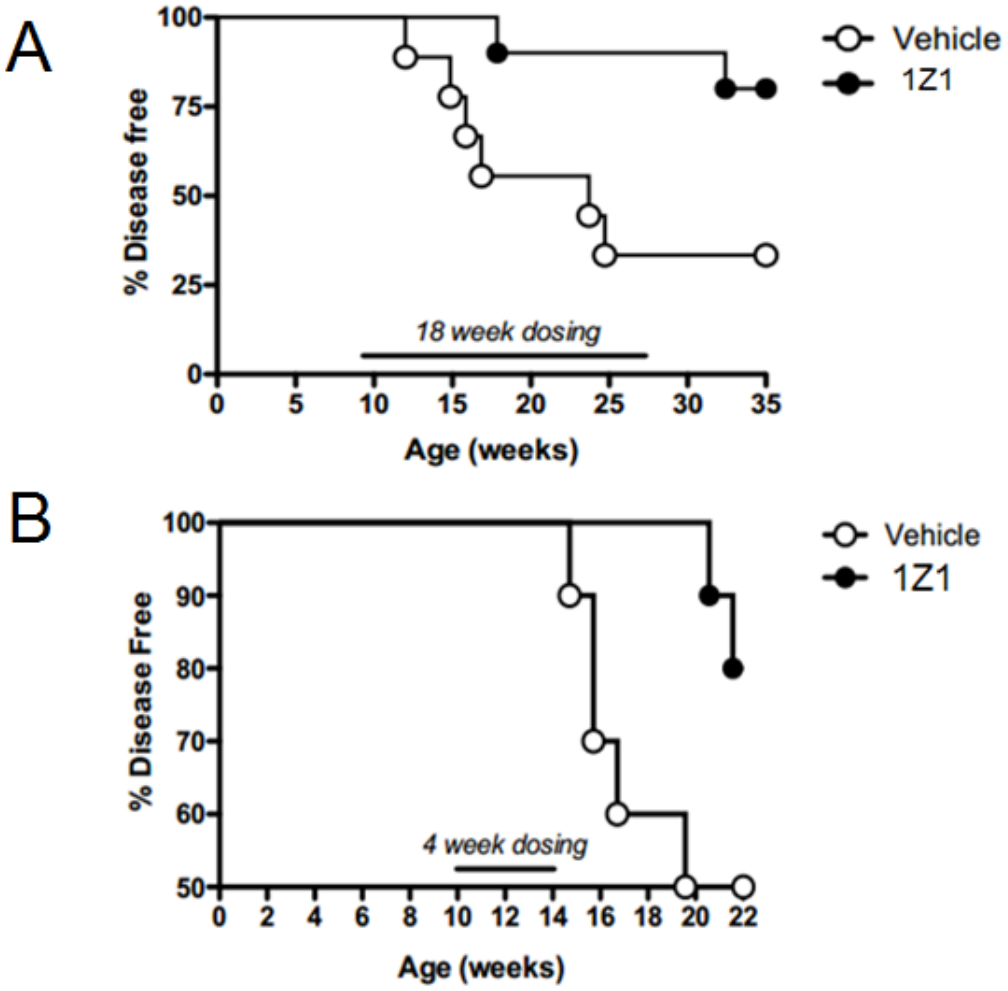

Figure 10: Daily 1Z1 treatment decreases disease incidence in NOD mice. *A) Long term daily treatment prevents disease onset.* Prediabetic NOD mice (n=10) were treated subcutaneously (s.c.) daily with 1Z1 starting at 9 weeks of age. The 18 week treatment period is indicated. Diabetes status was evaluated weekly by urine glucose levels. Upon detection of glucosuria, blood glucose levels were determined. Hyperglycemia (>300 mg/dL) was confirmed by additional evaluations of blood glucose levels 24 h later. Two consecutive positive tests for hyperglycemia led to disease diagnosis. *B) Short term treatment still exerts sustained disease preventative effect.* NOD mice (n=10) were treated s.c. with 1Z1 daily from 8 to 12 weeks of age (treatment period indicated). Disease was monitored as previously described.

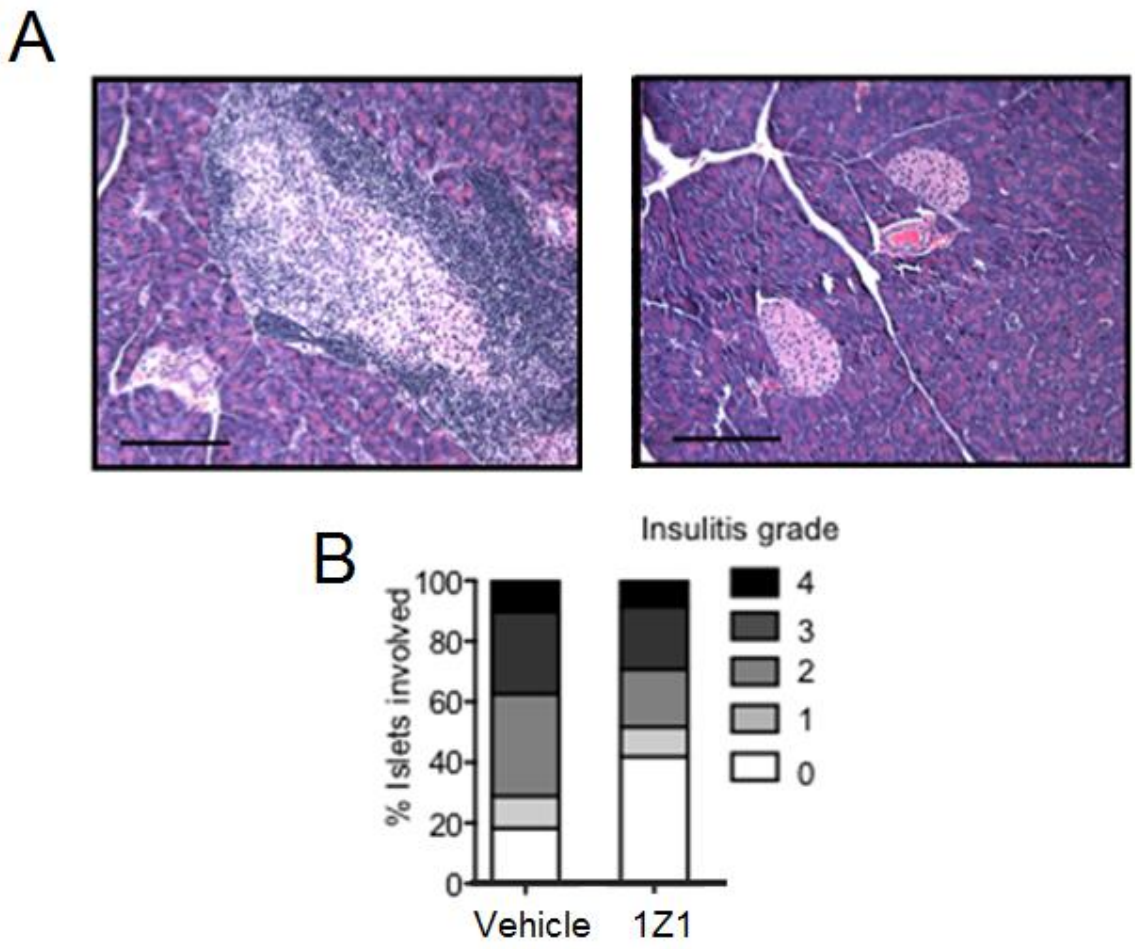

Figure 11: 1Z1-treated NOD mice exhibit lower degree of insulitis. 8 week old NOD mice were treated with 1Z1 or vehicle for 4 weeks. After this, pancreata were harvested and stained with H&E and 10 to 40 islets from each pancreas were scored. The severity of insulitis was determined according to the criteria described in Table 4. Data shown are representative of two independent experiments that had similar results. H&E stained representative pancreas islets. Original magnification x200. Scale bar indicates 200 μm.

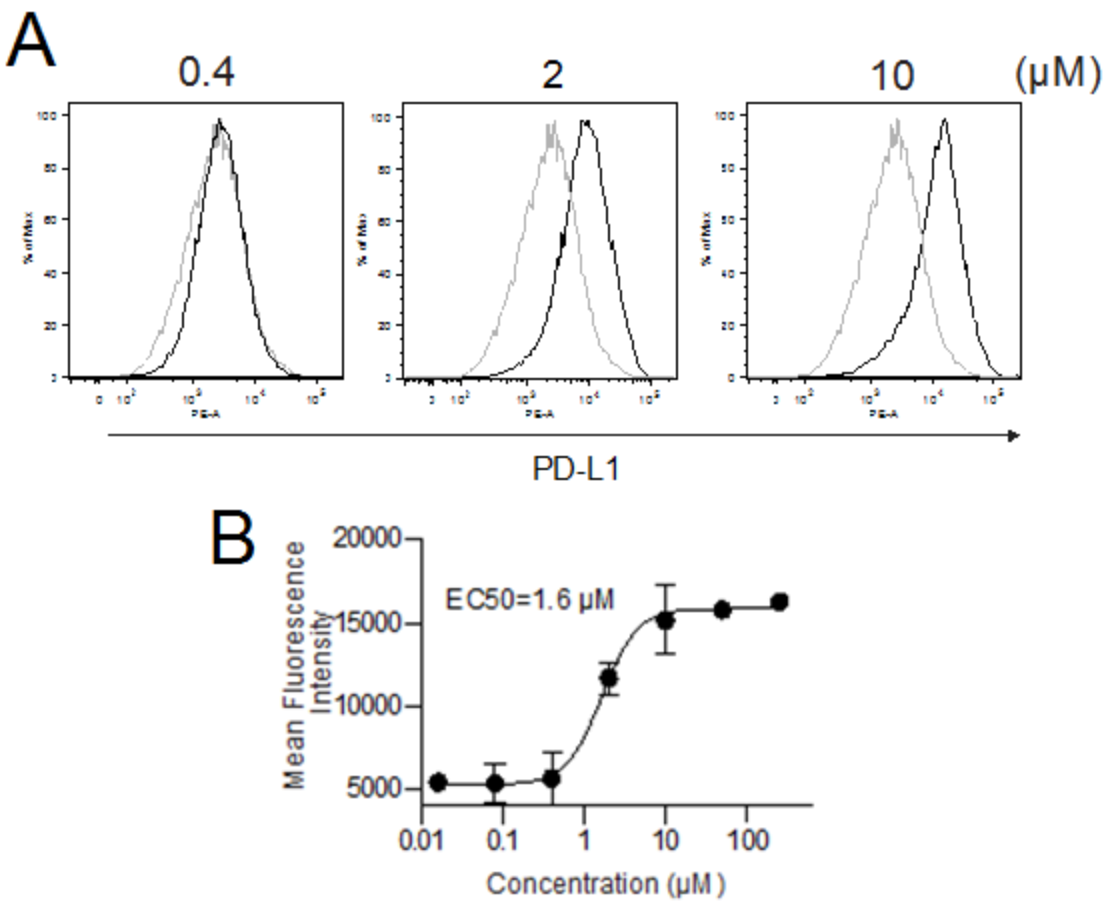

Figure 12: PD-L1 upregulated on DCs by in vitro 1Z1 treatment in a dose-dependent manner. *A)* BMDC prepared from C57BL/6 mice were incubated with vehicle (light grey line) or graded concentrations of 1Z1 (black line) overnight and PD-L1 expression was determined by flow cytometry. *B)* Mean fluorescence intensity (MFI) of PD-L1 expression. EC50=1.6 μM was calculated using Prism 5.

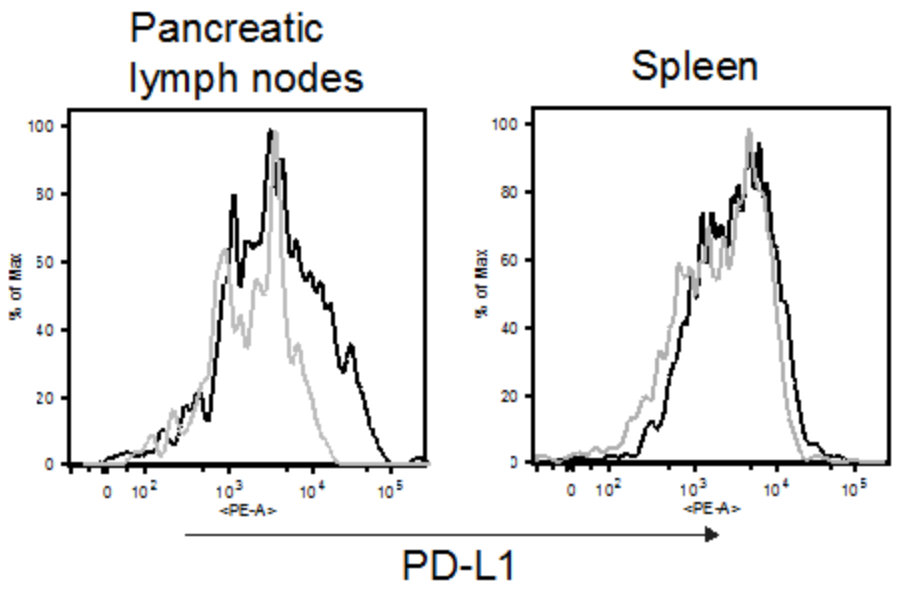

Figure 13: 1Z1 treatment increased PD-L1 expression on DCs in local lymph nodes in vivo. NOD mice were treated with 1Z1 or vehicle for 7 days and pancreatic lymph nodes and spleen were harvested. PD-L1 expression of $CD11c^{+}$-gated populations in pancreatic lymph nodes and spleens isolated from 1Z1-treated (dark line) and vehicle-treated (grey line) mice was compared.

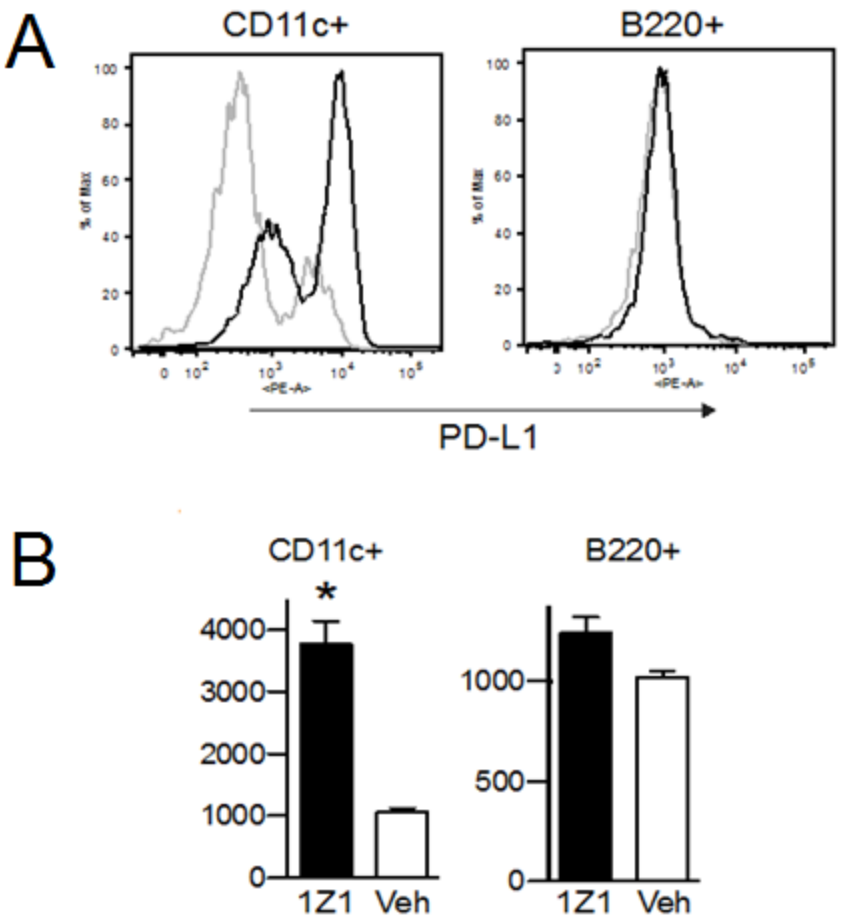

Figure 14: 1Z1 treatment did not increase PD-L1 expression on B cells in vivo. *A)* NOD mice were treated with 1Z1 or vehicle for 7 days and buffy coat was harvested. PD-L1 expression of gated $CD11c^+$ or $B220^+$ cells from mice treated with 1Z1 (dark line) or vehicle (grey line) was compared. *B)* Bar graphs indicate MFI of PD-L1 expression on the gated $CD11c^+$ or $B220^+$ population. Bars indicate the average ± SEM of 5-7 mice per group. *: $p<0.05$ compared to vehicle group.

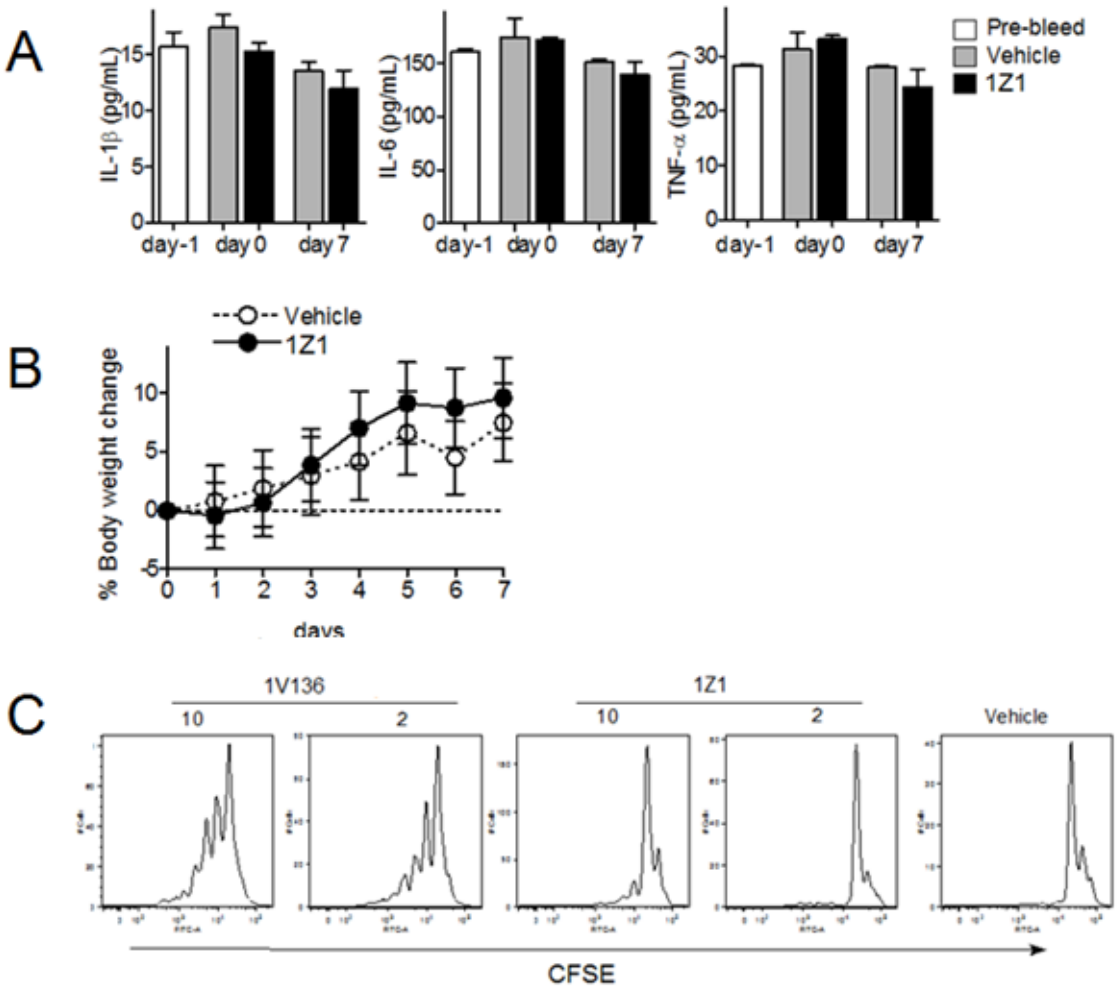

Figure 15: 1Z1 treatment is safe in vivo and in vitro. *A) and B)* 8 week old NOD mice (n = 10) were given daily injections of 400nmol 1Z1 (s.c.). *A) 1Z1 treatment does not cause cytokine storm in vivo.* 2h after the 1st and 7th injections, IL-1β, IL-6, and TNF-α levels were measured by Luminex beads assay. *B*) *1Z1 treatment does not cause body weight loss.* Body weight was measured daily and is here expressed as % change from the initial body weight. The average initial body weights for the vehicle-treated and 1Z1-treated mice were 18.8 ± 0.4g and 18.4 ± 0.4g, respectively. *C) 1Z1 treatment does not induce B cell proliferation in vitro.* Splenocytes from C57BL/6 were incubated with 1Z1 or 1V136 for 3 days. B cell proliferation was evaluated by CFSE dilution of $B220^{+}$ population.

TABLES

Table 1: Toll-Like receptors: ligands and associated proteins

Receptor	**Receptor localization**	**Ligand**	**Adaptor proteins**	**Transcription Factors**
TLR1	Plasma membrane	Triacyl lipopeptides	TIRAP, MyD88	NFκB
TLR2	Plasma membrane	Peptidoglycan	TIRAP, MyD88	NFκB
TLR3	Endosome	ssRNA, dsRNA	TRIF	NFκB, IRF3, IRF 7
TLR4	Plasma membrane	LPS	TIRAP, MyD88, TRAM, TRIF	NFκB, IRF3, IRF7
TLR5	Plasma membrane	Flagellin	MyD88	NFκB
TLR6	Plasma membrane	Diacyl lipopeptides	TIRAP, MyD88	NFκB
TLR7	Endosome	ssRNA	MyD88	NFκB, IRF7
TLR8 (human only)	Endosome	ssRNA	MyD88	NFκB, IRF7
TLR9	Endosome	dsRNA	MyD88	NFκB, IRF7
TLR10	unknown	unknown	unknown	unknown
TLR11 (mouse only)	Plasma membrane	Profilin-like molecule	MyD88	NFκB
TLR12 (mouse only)	Plasma membrane	Profilin-like molecule	MyD88	NFκB
TLR13 (mouse only)	Endosomal	Bacterial rRNA	MyD88	NFκB

Table 2: Expression of TLR7 in human and mouse

(Barchet et al., 2008)

Cell type	Human	Mouse
Plasmacytoid dendritic cells	++	++
Myeloid dendritic cells	-	+
Monocytes	-	++
Macrophages	-	+inducible
B cells	+ inducible	+inducible
T cells	-	-
Mast cells	+	+

Table 3: Mouse models of autoimmune disease in which TLR7 ligands are effective

Mouse Models	**TLR7 Ligand**	**Reference**
Thioglycolate induced peritonitis	1Z1, 1V136	Hayashi T, et al. (2012)
Serum-transferred Arthritis	1V136	Hayashi T, et al. (2009)
DSS-colitis/TNBS colitis prophylactic/therapeutic	1Z1, 1V136	Hayashi T, et al. (2012)
Experimental Allergic Encephalomyelitis	1V136	Hayashi T, et al. (2009)
TMA-induced chronic contact dermatitis	1Z1	Unpublished
NOD model	1Z1	Unpublished

Table 4: Clinical score for severity of insulitis

Clinical Score	**Description**
Grade 0	No infiltration
Grade 1	perivascular/periductular infiltrates with leukocytes touching islet perimeters, but not penetrating
Grade 2	leukocytic penetration of up to 25% of islet mass
Grade 3	leukocytic penetration of up to 75% of islet mass
Grade 4	end-stage insulitis, <20% of islet mass remaining

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