CHARACTERIZATION AND MODULATION OF AUTOREACTIVE $CD4^+$ T CELLS IN TYPE 1 DIABETES

Li Li

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of PhD in the Department of Microbiology and Immunology

Chapel Hill
2007

Approved by

Advisor: Roland M. Tisch, PhD

Reader: Stephen H. Clarke, PhD

Reader: Edward J. Collins, PhD

Reader: Jeffrey A. Frelinger, PhD

Reader: Glenn K. Matsushima, PhD

ABSTRACT

Characterization and Modulation of Autoreactive $CD4^+$ T cells in Type 1 Diabetes

Type 1 diabetes (T1D) is an autoimmune disease mediated by pathogenic β cell-specific T cells. The soluble (s) IA^{g7}- immunoglobulin (Ig) dimers covalently linked to GAD65 peptides or the mimetic BDC2.5 epitope (mBDC) were utilized in two studies. The first use was to enhance the efficacy of peptide treatment. NOD female mice with established β cell autoimmunity received a short course of sIA^{g7}-Ig dimers intravenously (i.v.). NOD mice treated with sIA^{g7}-mBDC continued to develop diabetes. In marked contrast, the majority of NOD mice treated with sIA^{g7}-Ig complexed with the GAD65-specific peptides p217 or p286 remained diabetes-free. Protection correlated with an increased frequency of IL-10 secreting immunoregulatory $CD4^+$ T cells that delayed diabetes in a co-adoptive transfer model. These results demonstrate that treatment with a short-course of sIA^{g7}-GAD65 peptide dimers is an effective approach to suppress T1D.

Secondly, the relative role for BDC2.5 clonotypic $CD4^+$ T cells in the progression of the diabetogenic response was tracked and temporal analyzed using sIA^{g7}-mBDC multimers. The frequency and/or number of T cells binding sIA^{g7}-mBDC multimers (g7-$mBDC^+$) increase with in peripheral blood lymphocytes (PBL) and the islets at the onset of β cell autoimmunity in NOD female mice. In contrast, a reduced frequency and number of g7-$mBDC^+$ T cells was observed in the PBL and islets of NOD male mice. T cell receptor (TCR) variable β (Vβ)

gene complementary determinant region 3 (CDR3) sequences revealed that BDC2.5 clonotypic $CD4^+$ T cells in the islets but not PBL selectively expressed TRBV15. These data demonstrate that g7-$mBDC^+$ T cells are an early indicator of the development of destructive insulitis, and that both clonotypic expansion and preferential usage of TCR characterize islet infiltrating g7-$mBDC^+$ T cells.

ACKNOWLEDGEMENTS

I wish to give my warmest thanks to Dr. Roland Tisch. He introduced me to this field and generously gave me advice and suggestions during these years. I would like to express my gratitude to him for spending valuable time inspiring me and conducting my dissertation research. Likewise, I am grateful to Dr. Bo Wang for providing me with thought-provoking discussions and advice. I am also grateful to my dissertation committee members, Dr. Jeff Frelinger, Dr. Ed Collins, Dr. Glenn Matsushima, and Dr. Steve Clark. Thank you for generously giving me valuable time and advice during the last few years.

Finally, I would like to specially dedicate this work to my wife, Hong Yu and my son, Evan. Thank you for supporting me both intellectually and emotionally during these years. I could not have done this without your immense love. Thank you Evan for bringing felicity to my life. As well as my parents, Shiyan Li and Yaqin Zhao, your endless love for me and continuous encouragement throughout my life are deeply appreciated. Without the generous help of my sister, Yueying Li and brother, Zongrui Li, this investigation would not have been possible.

TABLE OF CONTENTS

Page

LIST OF TABLES……vii

LIST OF FIGURES……..…..ix

LIST OF ABBREVIATIONS…….....x

CHAPTER

1. INTRODUCTION…….……..…1

1.1 Type 1 Diabetes…….……2

1.2 The NOD Mouse…….…….……..4

1.3 Properties of IA^{g7}…….…….…….5

1.4 T1D is a T cell-mediated Autoimmune Disease……..……..6

1.5 Multiple β cell Autoantigens are Targeted in T1D……9

1.6 Multiple Defects Account for the Breakdown of Self-tolerance to β cells…….11

1.6.1 Thymic Selection……..12

1.6.2 Immunoregulation…….13

1.7 Immunotherapy of T1D……..…….18

1.7.1 Antigen-independent Immunotherapies……...…….18

1.7.2 Antigen-dependent Immunotherapies……...……20

1.8 References……..……..23

2. SUPPRESSION OF AUTOIMMUNE DIABETES BY TREATMENT WITH PEPTIDE-MHC CLASS II DIMERS……36

2.1 Abstract……37

2.2 Introduction……38

2.3 Results……41

The production and characterization of sIA^{g7}-Ig dimers……41

sIA^{g7}-p217 and sIA^{g7}-p286 but not sIA^{g7}-mBDC dimers prevent diabetes in NOD mice……43

Protection mediated by sIA^{g7}-p217 and sIA^{g7}-p286 dimers correlates with the induction of peptide-specific immunoregulatory Tr1 cells……44

2.4 Discussion……46

2.5 Materials and Methods……50

2.6 References……61

3. SELECTIVE EXPANSION OF β CELL-SPECIFIC T CELL RECEPTORS IN AUTOIMMUNE DIABETES……66

3.1 Abstract……67

3.2 Introduction……68

3.3 Results……71

Detection of mBDC-specific $CD4^+$ T cells in PBL and the islets……71

The TCR Vβ repertoire of mBDC2.5-specific $CD4^+$ T cells is skewed in the Islets……72

3.4 Discussion……74

3.5 Materials and Methods……77

3.6 References……93

4. FUTURE PERSPECTIVES……95

4.1 sIA^{g7}-Ig dimer administration: A potent strategy of peptide vaccination……..……....96

4.2 Characterization of autoreactive T cells in islet infiltrates……..……97

4.3 References……..100

5. APPENDICES……..101

Appendix I……101

Appendix II……...132

LIST OF TABLES

Page

Table 3.1 CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 1……83

Table 3.2 CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 2……84

Table 3.3 CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 3……85

Table 3.4 CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 4……86

Table 3.5 CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 5……87

Table 3.6 CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 6……88

Table 3.7 CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 7……89

Table 3.8 CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 8……90

Table 3.9 CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 9……91

Table 3.10 CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 10……..…92

LIST OF FIGURES

Page

Figure 2.1 Characterization of sIA^{g7}-Ig dimers……..…53

Figure 2.2 T cell binding by sIA^{g7}-Ig multimers is peptide specific…….54

Figure 2.3 sIA^{g7}-Ig dimers induce T cell proliferation in a peptide-specific manner…....55

Figure 2.4 *In vivo* detection of sIA^{g7}-Ig dimers……...……..56

Figure 2.5 sIA^{g7}-p217 and sIA^{g7}-p286 but not sIA^{g7}-mBDC dimers prevent diabetes in NOD female mice…….……57

Figure 2.6 Treatment of sIA^{g7}-p217 or sIA^{g7}-p286 dimers blocks the progression of insulitis…….…..……58

Figure 2.7 Protection mediated by sIA^{g7}-p217 and sIA^{g7}-p286 dimers correlates with the induction of peptide-specific immunoregulatory Tr1 cells……59

Figure 2.8 sIA^{g7}-p217 dimer treatment induces immuoregulatory T cells……60

Figure 3.1 g7-mBDC^{+} CD4^{+} T cells are increased in PBL and the islets of NOD female mice……79

Figure 3.2 The Vβ repertoire of most islet infiltrating g7-mBDC^{+} CD4^{+} T cells is skewed……..……80

LIST OF ABBREVIATIONS AND SYMBOLS

AC	apoptotic cell
Ag	antigen
APC	antigen presenting cell
CCR	chemokine receptor
CDR3	complementarity determining region 3
ConA	concavilin A
CTE	cortical thymic epithelial cell
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen
DC	dendritic cell
DN	double-negative
DP	double-positive
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immuospot asay
FACS	fluorescent activated cell sorting
FITC	fluorescein isothiocyanate
FoxP3	Forkhead box P3
GAD65	glutamic acid decarboxylase 65
GITR	glucocorticoid-induced tumor necrosis factor receptor family-related gene
HA	hemagglutinin
HEL	hen egg lysozyme
HLA	human leukocyte antigen

HRP	horseradish peroxidase
IA-2	insulinoma-associated protein 2
Idd	insulin-dependent genes
IDDM	Insulin dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IGRP	islet-specific glucose-6-phosphatase catalytic subunit–related protein
IL	interleukine
InsB	insulin B
i.v.	intravenous
MAb	monoclonal antibody
MHC	major histocompatibility complex
MTE	medullary thymic epithelial cell
MΦ	macrophage
NK	natural killer
NOD	non-obese diabetic
Ova	ovalbumin
PBL	peripheral blood lymphocyte
PE	phyco-erythrin
PerCP	peridinin-chlorophyll protein
PLN	pancreatic lymph node
RIP	rat insulin promoter
RT-PCR	reverse transcription-polymerase chain reaction

SAV	streptavidin
mBDC	mimetic BDC
scid	severe-combined immunodeficient
SP	single-positive
T1D	type 1 diabetes
TCR	t cell receptor
TGF	transforming growth factor
Tg	tansgenic
TH1	type 1 T helper
TH2	type 2 T helper
TNF	tumor necrosis factor
TRBV	T cell receptor beta chain variable gene
Treg	regulatory T cell
α	alpha
β	beta
γ	gamma
μ	micro

CHAPTER 1

INTRODUCTION

1.1 Type 1 Diabetes

Type 1 diabetes (T1D) or insulin dependent diabetes mellitus (IDDM) is a T cell-mediated autoimmune disease characterized by the destruction of the insulin producing β cells of the islets of Langerhans (Bach 1994; Tisch and McDevitt 1996). T1D affects mostly children and young adults, but can occur at any age. Currently, 0.3% of the population in the United States is affected by T1D with 30,000 new clinical cases diagnosed each year and importantly, the incidence of T1D is increasing in developed countries. Diabetic individuals suffer from long-term complications including blindness, kidney failure and premature vascular disease leading to a reduced life expectancy by an average of 15 years. Although diabetes can be controlled by daily insulin injections, there is no effective therapy to prevent or "cure" the disease in humans. Accordingly, there is a pressing need to understand the pathogenic mechanisms of β cell autoimmunity in order to design effective and rational immunotherapies for the prevention and treatment of T1D.

T1D is a multi-factorial disease with both environmental and genetic factors contributing to the development and progression of β cell autoimmunity. Epidemiological studies have found that environmental factors such as diet, toxins, and viral and bacterial infections are associated with T1D (Hyoty and Taylor 2002). The most important evidence for environmental factors having a role in T1D comes from observations that 1) migrants from countries with low incidence rates are more susceptible to T1D in countries with high incidence rates, and 2) genetically identical twins are only 36% concordant in disease development. However, whether environmental insults play a role in the initiation and/or exacerbation of β cell autoimmunity is unknown. Transgenic (Tg) mouse models of T1D

provide evidence that viral infections may trigger β cell autoimmunity through for instance, molecular mimicry between viral and β cell proteins (Ohashi, Oehen et al. 1991; Faideau, Larger et al. 2005) or by direct β cell injury (Oldstone, Southern et al. 1984).

T1D is polygenic and more than 20 chromosal loci have been found to be associated with disease susceptibility in humans and the nonobese diabetic (NOD) mouse, a spontaneous model of T1D (see below) (Davies, Kawaguchi et al. 1994; Todd 1995; Concannon, Gogolin-Ewens et al. 1998). The majority of the susceptibility genes within these loci have yet to be identified with the exception of the major histocompatibility complex (MHC) class II genes, the 5' flanking region of the insulin gene, and the *CTLA4* gene (Rotwein, Chyn et al. 1981; Winter, Beppu et al. 1987; Owerbach and Gabbay 1993; Kristiansen, Larsen et al. 2000). The strongest genetic association with T1D susceptibility and resistance maps to the MHC class II region in both humans (*IDDM1)* and NOD mice (*idd1)*. The HLA-DRB1*0301, HLADRB1*0401, HLA-DQB1*0302, and HLADQA1*0301 alleles confer high-risk susceptibility in humans, whereas other alleles such as HLA-DRB1*0403, HLA-DQB1*0602, and HLADQA1*0102 confer resistance to T1D (Wicker, Todd et al. 1995; Undlien, Lie et al. 2001). Currently, it is believed that MHC class II susceptibility alleles are necessary but not sufficient for the development of diabetes, and that the combined effects of other susceptibility genes influence the progression of β cell autoimmunity in the context of the appropriate environmental insult (Redondo, Rewers et al. 1999).

1.2 The NOD Mouse

NOD mice are considered to be the leading animal model for T1D. β cell autoimmunity spontaneously develops in NOD mice and several aspects of the diabetogenic response closely reflect the human disease. For instance, both NOD mice and humans share a number of T1D genetic susceptible loci, and both autoimmune responses are affected by environmental factors. In addition, multiple immune effector cells, such as $CD4^+$ and $CD8^+$ T cells, B cells, macrophages (MΦ), dendritic cells (DCs), and natural killer (NK) cells are involved in the respective disease processes (Miller, Appel et al. 1988; O'Reilly, Hutchings et al. 1991; Cooke, Phillips et al. 2001). Finally, several β cell autoantigens, such GAD and insulin, are targeted in both diabetic patients and NOD mice (Kent, Chen et al. 2005).

Islet infiltration or insulitis is initially detected in NOD mice at 3-4 weeks of age. The first step in insulitis referred to peri-insulitis, is characterized by pancreatic infiltrates surrounding an islet. Peri-insulitis then progresses to intra-insulitis in which cells invade the islets. MΦs and DCs are the first cells to traffick to the islets (Jansen, Homo-Delarche et al. 1994) and appear to play essential albeit ill-defined roles in the disease process (Jun, Santamaria et al. 1999; Yoon, Yoon et al. 1999). Islet infiltrating antigen-presenting cells (APC) may mediate initial β cell injury through the secretion of proinflammatory cytokines such as interferon-γ (IFNγ), tumor necrosis factor-α (TNFα) and interleukin (IL)-1 and reactive oxygen species, in addition to processing and presenting β cell autoantigens to promote the subsequent recruitment of T cells (Chervonsky, Wang et al. 1997; Kagi, Odermatt et al. 1997; Suarez-Pinzon, Mabley et al. 2001; Ogasawara, Hamerman et al. 2003). Insulitis continues over a period of weeks without significant β cell loss. However, at 12 weeks of age the insulitic

lesion undergoes a qualitative change and β cells are efficiently destroyed (Mathis, Vence et al. 2001). The events that promote this destructive phase of insulitis are poorly understood and may involve changes in the composition of DC subsets residing in the islets (Ludewig, Odermatt et al. 1998; Summers, Behme et al. 2003), affinity/avidity maturation of pathogenic T effectors (Amrani, Verdaguer et al. 2000), and a reduced frequency of immunoregulatory T cells (see below) (Gregori, Giarratana et al. 2003; Pop, Wong et al. 2005). Once 90% of β cell mass is lost, hyperglycemic blood levels are achieved and overt diabetes are established. Typically, diabetes is initially detected at 13-15 weeks of age in NOD mice. The frequency of diabetes differs markedly between NOD male and female mice. By 30 weeks of age 30% and 80% of NOD males and females develop diabetes, respectively. This sex bias in diabetes development appears due to the ill-defined effects of testosterone and estrogen on the immune system (Fox 1992; Bao, Yang et al. 2002).

1.3 Properties of IA^{g7}

The IA^{g7} MHC class II molecule expressed by NOD mice plays a key role in the initiation and progression of T1D . The association of IA^{g7} with T1D is believed to be due to the molecule's unique structure. The α chain of IA^{g7} is identical to that of the IA^{d} α chain. However, the sequence of the IA^{g7} β chain is unique compared to all other mouse IAβ alleles (Acha-Orbea and McDevitt 1987). Specifically, histidine and serine residues are found at positions 56 and 57 in the IA^{g7}β chain, respectively, whereas all other IAβ chain alleles contain proline and aspartic acid residues, respectively. Noteworthy is that the human T1D susceptible DQ8β chain is also characterized by a non-aspartic residue at position 57 (Tisch and McDevitt 1996).

A direct role for IA^{g7} in T1D has been confirmed by introduction of transgenes encoding various IA alleles, such as IA^{k} ($A^{k}\alpha/A^{k}\beta$) or IA^{d} ($A^{d}\beta$) that prevent or reduce the frequency of diabetes in Tg NOD mice (Nishimoto, Kikutani et al. 1987; Lund, O'Reilly et al. 1990). Furthermore, substitution of the histidine and/or serine residues at β56 and β57 with a proline and aspartic acid, respectively, significantly reduces the frequency of insulitis and prevents diabetes in the corresponding lines of Tg NOD mice (Lund, O'Reilly et al. 1990; Quartey-Papafio, Lund et al. 1995). Consequently, it has been proposed that the amino acid residues at β56 and β57 influence the peptide binding properties of IA^{g7} (Carrasco-Marin, Shimizu et al. 1996; Latek, Suri et al. 2000). Nevertheless, the precise mechanism(s) by which the β56 and β57 amino acids govern peptide binding, and in turn the diabetogenic capacity of IA^{g7} remains ill defined. Studies have suggested that IA^{g7} binds to peptides weakly, and/or the surface half-life of IA^{g7}-peptide complexes are relatively short-lived (Carrasco-Marin, Shimizu et al. 1996). Either of these properties would be predicted to impact the repertoire and reactivity of β cell-specific T cells.

1.4 T1D is a T cell-mediated Autoimmune Disease.

As alluded to above, T cells are the primary mediators of β cell destruction (Wicker, Miller et al. 1986; Bach 1994). B cells have also been implicated in T1D, and appear to primarily serve as APC (Baekkeskov, Nielsen et al. 1982; Baekkeskov, Aanstoot et al. 1990; Kaufman, Clare-Salzler et al. 1993). The first evidence indicating that T1D is a T cell-mediated autoimmune disease came from histological examination of pancreases from diabetic patients which demonstrated significant lymphocytic infiltration concomitant with the loss of β cell

mass (Gepts 1965). Subsequent work in prediabetic and diabetic NOD mice has also demonstrated T cell infiltrates in the islets. A direct role for T cells in β cell destruction was initially shown by depleting T cells via administration of anti-CD3 antibody, and preventing diabetes in NOD mice (Hayward and Shreiber 1989; Chatenoud, Thervet et al. 1992; Hayward and Shriber 1992; Chatenoud, Thervet et al. 1994). Furthermore, NOD mice deficient of T cells develop neither insulitis nor diabetes, and diabetes can be adoptively transferred in immunodeficient NOD.*scid* mice by T cells from diabetic NOD donors. A number of Tg mouse lines have been established expressing T cell receptors (TCR) specific for native or neo β cell autoantigens that further substantiate the critical role for T cells in the initiation and progression of β cell autoimmunity (Ohashi, Oehen et al. 1991; Oldstone, Nerenberg et al. 1991; Pankewycz, Strom et al. 1991; Katz, Wang et al. 1993)

Both $CD4^+$ and $CD8^+$ T cells are required to mediate efficient β cell destruction based on adoptive transfer studies using NOD.*scid* or irradiated NOD mice as recipients (Bendelac, Carnaud et al. 1987; Miller, Appel et al. 1988). For example, diabetes is transferred to NOD.*scid* recipients by a mixture of naïve $CD4^+$ and $CD8^+$ T cells prepared from the spleens of diabetic NOD mice, but not by either T cell subset alone (Bendelac, Carnaud et al. 1987; Miller, Appel et al. 1988). These pathogenic β cell-specific $CD4^+$ and $CD8^+$ T cells typically exhibit a type 1 phenotype characterized by the production of IFNγ and TNFα (Healey, Ozegbe et al. 1995; Katz, Benoist et al. 1995; Liblau, Singer et al. 1995).

The relative contribution of $CD4^+$ and $CD8^+$ T cells in the diabetogenic response continues to be a controversial issue. A number of studies suggest that $CD4^+$ T cells are necessary for

the early and late stages of β cell autoimmunity. Various groups have shown that activated $CD4^+$ T cells alone transfer diabetes to irradiated NOD or NOD.*scid* recipients (Katz, Wang et al. 1993). Furthermore, depletion of $CD4^+$ T cells in NOD mice via anti-CD4 antibody treatment at early and late preclinical stages of T1D prevents diabetes (Shizuru, Taylor-Edwards et al. 1988). Moreover, NOD mice lacking expression of the MHC class II transcriptional regulator CIITA and which have few $CD4^+$ T cells, remain diabetes-free although insulitis is detected (Wong, Visintin et al. 1998). The latter observation is important since NOD mice depleted of $CD8^+$ T cells by anti-CD8 antibody treatment prior to the initiation of β cell autoimmunity, remain diabetes-free but also fail to develop insulitis (Wang, Gonzalez et al. 1996). Prevention of diabetes, however, is only detected when young but not older NOD mice (>5 weeks of age) are treated with anti-CD8 antibody (Wang, Gonzalez et al. 1996). Together, these findings suggest that $CD8^+$ T cells play an important role in the initiation of β cell autoimmunity. Further support for this notion comes from work with NOD mice lacking expression of the β2 microglobulin (β2m) (NOD. $\beta2m^{null}$) gene and which have significantly reduced numbers of $CD8^+$ T cells. NOD. $\beta2m^{null}$ mice exhibit no insulitis and consequently fail to develop diabetes (Serreze, Leiter et al. 1994; Wicker, Leiter et al. 1994). Based on these observations a model has been proposed in which $CD8^+$ T cells are required to promote early β cell injury, and $CD4^+$ T cells amplify the response and drive autoimmunity through the destructive phase of the disease process (Wang, Gonzalez et al. 1996). However, this model is too simplistic. For instance, reports by the Santamaria group demonstrate that detection of increasing numbers of $CD8^+$ T cells specific for islet-specific glucose-6-phosphatase catalytic subunit–related protein $(IGRP)_{206-214}$ in the peripheral blood of euglycemic adult NOD mice coincides with the progression to diabetes (Trudeau, Kelly-

Smith et al. 2003). This result would argue that (at least) IGRP-specific $CD8^+$ T cells are also involved in the late stages of β cell destruction. Therefore, β cell antigen specificity and affinity/avidity of the clonotypic TCR may be additional parameters determining the relative role of $CD4^+$ and $CD8^+$ T cells in the disease process.

1.5 Multiple β cell Autoantigens are Targeted in T1D

Given the overall importance of T cells in the pathogenesis of T1D, a large body of work has been devoted to identifying the β cell antigens that drive the autoimmune T cell responses in NOD mice and diabetic patients. A number of β cell autoantigens are targeted throughout the diabetogenic response, but only a few have been identified. The autoantigens targeted in T1D can be distinguished by tissue distribution, namely: 1) β-cell-specific antigens such as insulin, insulin derivatives, and IGRP; 2) neuroendocrine antigens such as carboxypeptidase H, insulinoma-associated antigen (IA-2), GAD, and carboxypeptidase E; and 3) those that are expressed ubiquitously such as heat shock protein 60 (HSP60) (Anderson and Bluestone 2005). Among these autoantigens, insulin, GAD and IGRP appear to have significant roles in disease initiation and progression. BDC2.5 clonotypic $CD4^+$ T cells also appear to have an important role in the diabetogenic response of NOD mice, which will be discussed in Chapter 3 (Candeias, Katz et al. 1991; Katz, Wang et al. 1993).

Insulin is abundantly expressed by β cells and processed from a precursor molecule, preproinsulin. Mice, unlike humans, express two isoforms of insulin, insulin 1 and 2. In mice, proinsulin 1 is expressed mainly in the pancreas, whereas proinsulin 2 is expressed in both the pancreas and thymus. Thymic expression of proinsulin 2 is believed to be crucial for

inducing self-tolerance to insulin. Compelling evidence suggests that insulin is a major target of pathogenic T cells in NOD mice (French, Allison et al. 1997; Eisenbarth, Moriyama et al. 2002). For instance, $CD4^+$ T cells isolated from islet infiltrates of young NOD mice display high reactivity to insulin, with more than 90% recognizing the insulin B chain derived peptide 9 to 23 (InsB9−23) (Wegmann, Norbury-Glaser et al. 1994). Furthermore, InsB9-23-specific $CD4^+$ T cell lines or clones induce diabetes upon transfer into young NOD or NOD.*scid* mice (Wegmann, Norbury-Glaser et al. 1994; Daniel, Gill et al. 1995). Insulin-specific $CD8^+$ T cells that are $H2K^d$-restricted and recognize an InsB15-23 epitope have also been identified in islet infiltrates. Recently, Nakayama *et al.* demonstrated that insulitis and diabetes are prevented in Tg NOD mice expressing a transgene encoding proinsulin 2 in which the IA^{g7} and $H2K^d$ epitopes had been mutated (Nakayama, Abiru et al. 2005). These results argue that insulin is a key causative self-antigen of T1D in NOD mice.

Two isoforms of GAD exist, namely GAD65 and GAD67, which catalyze the production of the neurotransmitter γ amino butyric acid in the central nerve system (Erlander, Tillakaratne et al. 1991; Martin and Rimvall 1993). Both isoforms of GAD are expressed in β cells, the thymus and brain (Faulkner-Jones, Cram et al. 1993; Kim, Richter et al. 1993). The role of GAD in the pancreas is unclear, and may be involved in regulating the response of β cells to glucose. GAD65 is an important autoantigen implicated in the pathogenesis of T1D. The earliest autoantibodies found in pre-diabetic patients are GAD65-specific (Verge, Gianani et al. 1996) and presentation of these autoantibodies indicate a strong likelihood for the development of diabetes (Baekkeskov, Aanstoot et al. 1990). In NOD mice, the earliest detectable response to islet extracts coincides with detection of GAD65- and GAD67-specific

T cell reactivity (Kaufman, Clare-Salzler et al. 1993; Tisch, Yang et al. 1993). Furthermore, immunization with plasmid DNA (pDNA) encoding GAD65 exacerbates the onset of diabetes in NOD mice, further suggesting a pathogenic role in T1D (Tisch, Wang et al. 2001).

IGRP-specific $CD8^+$ T cells are detected in the earliest insulitic lesions, and up to 30% of islet infiltrating $CD8^+$ T cells in adult NOD female mice are specific for $IGRP_{206-214}$ or the corresponding mimetic peptide NRP-V7 (Anderson, Park et al. 1999). Interestingly, the majority of IGRP-specific $CD8^+$ T cells are characterized by a recurrent amino acid sequence motif in the complementarity determining region 3 (CDR3) of the TCR α chain, with a prevalence of Vα17 joined to the Jα42 segment (Santamaria, Utsugi et al. 1995). Several lines of evidence suggest that IGRP-specific $CD8^+$ T cells play a critical role in the diabetogenic response of NOD mice. First, the frequency and TCR avidity of IGRP-specific $CD8^+$ T cells increase in the islet infiltrates during disease progression (Amrani, Verdaguer et al. 2000; Lieberman, Evans et al. 2003). Second, diabetes onset is accelerated in NOD mice that express a transgenic IGRP-specific TCR (Verdaguer, Yoon et al. 1996). Finally, depletion of IGRP-specific $CD8^+$ T cells via treatment with the mimetic peptide NRP-A7 protects NOD mice from diabetes (Han, Serra et al. 2005).

1.6 Multiple Defects Account for the Breakdown of Self-Tolerance to β cells.

Induction of self–tolerance is essential for regulating the development, activation and expansion of autoreactive lymphocytes. A number of mechanisms exist to establish tolerance to self antigens, including central deletion, peripheral clonal anergy and/or deletion and active immunoregulation (Wraith, Nicolson et al. 2004; Gonzalez-Rey, Chorny et al. 2007).

Studies in NOD mice suggest that the development and expansion of pathogenic β cell-specific T cells are largely due to defective thymic selection, and aberrant peripheral immunoregulation (Thomas-Vaslin, Damotte et al. 1997; Kishimoto and Sprent 2001; Anderson and Bluestone 2005).

1.6.1 Thymic Selection.

Positive selection of thymocytes occurs in the cortex of the thymus. Double-positive (DP) $CD4^+CD8^+$ thymocytes bearing newly rearranged TCR interact with self-peptide/MHC complexes presented by cortical thymic epithelial cells (CTE). This interaction can lead to transduction of a survival signal, thereby ensuring that the TCR expressed by thymocytes is "restricted" to self-MHC (Liu 2006). On the other hand, DP thymocytes expressing TCR that fail to recognize a self-peptide/MHC complex die by neglect (Nossal 1994). DP thymocytes that are positively selected traffick to the corticomedullary junction and medulla to undergo negative selection. The majority of DP thymocytes with autoreactive potential are eliminated by negative selection (Anderson, Partington et al. 1998). DP thymocytes are clonally deleted which express TCR that bind with high affinity/avidity to peptide/MHC complexes on the surface of medullary thymic epithelial (MTE) cells and/or thymic DC (Shortman, Vremec et al. 1998). The efficiency of MTE to mediate negative selection is partly regulated by the transcription factor Aire, which drives expression of a large number of self-antigens normally found in peripheral tissues (Kyewski, Derbinski et al. 2002; Anderson, Venanzi et al. 2005).

Properties of the IA^{g7} molecule and characteristics intrinsic to NOD thymocytes are believed to influence thymic selection and the development of autoreactive β cell-specific T

cells in NOD mice. For instance, Ridgway *et al.* have proposed that the relatively short half-life of peptide/IA^{g7} complexes reduces the "stimulatory" capacity of CTE, therefore resulting in positive selection skewed towards thymocytes expressing TCR with increased affinity/avidity (Ridgway, Fasso et al. 1999). In the periphery, these T cells would be expected to have an increased autoreactive potential. Poor peptide binding by and/or a decreased half-life of peptide/IA^{g7} complexes would also be expected to diminish the efficacy of thymic negative selection, and result in increased development of autoreactive T cells (Carrasco-Marin, Shimizu et al. 1996). Recent work by the Mathis and Benoist group has demonstrated that DP thymocytes from NOD mice exhibit a decreased sensitivity to the apoptotic-inducing events promoted by negative selection (Anderson, Venanzi et al. 2005), which would further promote the development of T cells with a pathogenic potential. Interestingly, CTE of NOD mice have been reported to be inefficient in inducing the development of natural $CD4^+CD25^+$ regulatory T cells (Treg) (Thomas-Vaslin et al., 1997). $CD4^+CD25^+$ Treg play an important role in preventing the expansion and/or differentiation of autoreactive T cells in the periphery (Sakaguchi 2000) (see below). Therefore, defective thymic selection in NOD mice may both enhance the development of autoreactive T cells, and limit the production of natural $CD4^+CD25^+$ Treg.

1.6.2 Immunoregulation

Negative selection of thymocytes is not absolute and mature T cells with autoreactive potential reside in the periphery. Nevertheless, activation, expansion and/or effector cell differentiation of these autoreactive T cells are normally held "in check" through several mechanisms that include induction of clonal anergy and/or deletion (Wraith, Nicolson et al.

2004; Gonzalez-Rey, Chorny et al. 2007). The most dominant mechanism by which self-tolerance within the T cell compartment is maintained, however, is through the function of immunoregulatory T cells. Until recently immunoregulation of T cells was considered in terms of a functional balance between $CD4^+$ T helper (Th)1 and Th2 cells (Abbas, Murphy et al. 1996; Coffman and Reiner 1999). Th1 cells are generally associated with pro-inflammatory/cell-mediated responses, and are characterized by the secretion of IFNγ (Scott 1993). As mentioned above, pathogenic β cell-specific $CD4^+$ T effectors exhibit a Th1 phenotype (Katz, Benoist et al. 1995; Haskins and Wegmann 1996). Th2 cells typically mediate humoral immunity, are characterized by the production of IL-4, and in T1D exhibit an immunoregulatory function (Stevens, Bossie et al. 1988; Kopf, Le Gros et al. 1993). IFNγ and IL-4 have reciprocal down-regulatory effects on the differentiation of naïve Th cells into Th1 or Th2 cells. For example, IFNγ aids in the differentiation of Th1 cells, and blocks Th2 cell development (Mosmann, Cherwinski et al. 1986). In contrast, IL-4 promotes and inhibits the differentiation of Th2 and Th1 cells, respectively (Abbas, Murphy et al. 1996).

The current view of immunoregulation and self-tolerance is considerably more complex. Several subsets of immunoregulatory T cells with distinct phenotypes and mechanisms of action have been identified (Ramsdell 2003). These subsets include: i) Th3 cells, which primarily secrete IL-4 and transforming growth factor-β (TGFβ) and are induced via mechanisms of oral tolerance (Chen, Kuchroo et al. 1994), ii) Tr1 cells, which secrete high levels of IL-10 (Groux, O'Garra et al. 1997), and iii) natural and adaptive $CD4^+CD25^+$ Treg which are defined by the expression of the transcription factor Forkhead box P3 (FoxP3) and exhibit suppressor function mediated by cell-cell contact and secretion of TGFβ (Hori,

Nomura et al. 2003). More recently, $CD8^+$ T cells exhibiting immunoregulatory function have also been identified (Chang, Ciubotariu et al. 2002; Hu, Ikizawa et al. 2004). For the purpose of this thesis and in view of their potent immunoregulatory function, the following discussion will focus primarily on Tr1 cells and $CD4^+CD25^+$ Treg. Discussion of other subsets of immunoregulatory T cells can be obtained in the following reviews (Bluestone and Boehmer 2006; Weaver, Harrington et al. 2006).

Similar to Th1 and Th2 cells, Tr1 cells differentiate from naïve $CD4^+$ T precursors. IL-10 is critical for Tr1 cell differentiation. Tr1 cells are characterized by the secretion of high levels of IL-10, low amounts of IL-5, IFNγ, and no IL-2 and IL-4 production (Groux, O'Garra et al. 1997). The surface phenotype of Tr1 cells is similar to that of naïve T cells with regard to expression levels of CD40L, CD69, CD28, cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Bacchetta, Sartirana et al. 2002). The observation that Tr1 cells express CCR5 and T1-ST2, which are surface markers expressed preferentially by Th1 and Th2 cells, respectively, suggests that Tr1 cells are a phenotypically distinct subset of $CD4^+$ T cells (McGuirk, McCann et al. 2002). Functional studies have shown that upon antigen stimulation, Tr1 cells promote bystander suppression mediated by the local release of IL-10. IL-10 has effects on both APC and T cells (Groux, O'Garra et al. 1997). IL-10 blocks the effector function of APC by inhibiting upregulation of costimulatory molecules and pro-inflammatory cytokine secretion, and directly inhibits IL-2 and TNFα production by $CD4^+$ T cells (Conti, Kempuraj et al. 2003). Numerous studies have demonstrated that Tr1 cells prevent the development of type 1-mediated autoimmune and inflammatory bowel diseases (Roncarolo, Bacchetta et al. 2001; O'Garra, Vieira et al. 2004).

Natural FoxP3-expressing $CD4^+CD25^+$ Treg are considered to be the most potent subset of immunoregulatory T cells, and as such play a pivotal role in establishing and maintaining self-tolerance (Hori, Nomura et al. 2003). Indeed, mice lacking natural $CD4^+CD25^+$ Treg develop a highly aggressive, systemic form of autoimmunity (Chatila, Blaeser et al. 2000; Bennett, Christie et al. 2001; Brunkow, Jeffery et al. 2001). Unlike Th2 or Tr1 cells which differentiate into immunoregulatory T effector cells upon antigen stimulation in the periphery, the suppressor function of natural $CD4^+CD25^+$ Treg is established in the thymus upon recognition of self-peptide/MHC complexes. Expression of FoxP3 is essential for the differentiation of $CD4^+CD25^+$ Treg. For instance, retroviral transduction of a FoxP3 transgene into naïve $CD4^+CD25^-$ T cells is sufficient to induce differentiation of *bona fide* $CD4^+CD25^+$ Treg (Hori, Nomura et al. 2003). Furthermore, humans and mice lacking FoxP3-expression fail to develop natural $CD4^+CD25^+$ Treg (Khattri, Cox et al. 2003; Fontenot, Rasmussen et al. 2005; Sakaguchi 2005). Natural $CD4^+CD25^+$ Treg exhibit an anergic-like phenotype *in vitro* but proliferate extensively *in vivo* (Bluestone and Boehmer 2006), and constitutively express CTLA-4 and the glucocorticoid-induced TNF receptor (GITR) among other surface molecules. Natural $CD4^+CD25^+$ Treg have been shown to mediate suppression *in vitro* by a cell-cell contact mechanism. However, the mechanism by which natural $CD4^+CD25^+$ Treg elicit suppression *in vivo* is a matter of debate, and may also involve the production of TGFβ. This controversy may partly be explained by the presence of "adaptive" FoxP3-expressing $CD4^+CD25^+$ Treg, which differentiate in the periphery from naïve $CD4^+$ T precursors upon antigen stimulation and in the presence of TGFβ (Chen, Jin et al. 2003; Fantini, Becker et al. 2004). Although phenotypically identical, the effector

function of adaptive versus natural $CD4^+CD25^+$ Treg may nevertheless differ. Natural $CD4^+CD25^+$ Treg can directly inhibit $CD4^+$ and $CD8^+$ T cells regardless of the activation, proliferative and effector status of the T cells (Sakaguchi 2000; Shevach 2002). Immunoregulation of T cells by natural $CD4^+CD25^+$ Treg can also be achieved indirectly by the latter's modulatory effects on DC. For example, DC co-cultured with natural $CD4^+CD25^+$ Treg produce indoleamine 2,3-dioxygenase (IDO) which catalyzes the degradation of tryptophans and in turn promotes T cell apoptosis (Fallarino, Grohmann et al. 2003).

The breakdown of T cell immunoregulation is believed to be a key factor in driving β cell autoimmunity in NOD mice and diabetic individuals. In NOD female mice, the frequency of β cell-specific Th2 and Tr1 cells is reduced relative to NOD males, which develop diabetes less frequently (Haskins and Wegmann 1996). Notably, work by Peakman and colleagues has shown that in individuals that are at high risk of developing diabetes, the frequency of proinsulin- and IA-2-specific type 1 T effectors is increased, and the percentage of Tr1 cells markedly decreased compared to HLA-matched healthy control subjects (Peakman, Stevens et al. 1999). Furthermore, the frequency and function of natural FoxP3-expressing $CD4^+CD25^+$ Treg progressively decline with age in NOD female but not male mice (Pop, Wong et al. 2005). In diabetic subjects, FoxP3-expressing $CD4^+CD25^+$ Treg exhibit a reduced suppressor activity *in vitro* (Gregori, Giarratana et al. 2003). Finally, adoptive transfer of Th2 cells, Tr1 cells or $CD4^+CD25^+$ Treg into NOD female mice can effectively prevent the development of diabetes, providing additional evidence that β cell autoimmunity progresses due to insufficient T cell immunoregulation (Healey, Ozegbe et al. 1995; Chen, Lee et al. 2003; Green, Gorelik et al. 2003).

1.7 Immunotherapy of T1D

A number of different strategies of immunotherapy have been tested experimentally and in the clinic to prevent and/or suppress β cell autoimmunity. These immunotherapies can be generally categorized as antigen-independent versus antigen-dependent strategies. Antigen-independent strategies typically have the benefit of targeting large numbers of autoreactive T cells that are found at late preclinical T1D or once diabetes has been established. However, a major drawback of this approach is that both autoreactive and nonautoreactive T cells are affected, therefore raising the possibility that a treated subject may be immunocompromised. "Vaccinating" with a β cell autoantigen provides the means to selectively target disease relevant T cells, leaving the nonautoimmune component of the immune system unaffected. However, the efficacy of antigen-specific immunotherapies tends wane at late stages of disease progression due to the high numbers of established pathogenic T effectors, and the relatively low frequency of immunoregulatory T cells. In general, the most effective antigen-independent or –dependent immunotherapies at late stages of β cell autoimmunity promote the development of immunoregulatory T cells. As alluded to above, immunoregulatory T cells when found at a sufficient frequency are highly effective at inhibiting the differentiation of type 1 T effectors, in addition to suppressing the activity of established pathogenic T cells depending on the subset of immunoregulatory T cell.

1.7.1 Antigen-independent Immunotherapies.

One of the first approaches tested in the clinic to target T cells and treat T1D was the administration of cyclosporine A to recent onset diabetic children (Stiller, Laupacis et al.

1983; Stiller, Dupre et al. 1984). Remission of diabetes was observed but the treatment had to be discontinued due to adverse effects, and unfortunately both T cells and diabetes reappeared. This study provided proof of principle that targeting T cells in the clinic can be effective for the treatment of T1D, and that establishing self-tolerance (e.g. immunoregulation) is essential for suppressing β cell autoimmunity long-term. A variety of approaches have since been investigated attempting to modulate the activity of β cell-specific T cells. Continuous administration of anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and TGFβ to young NOD mice prevents diabetes (Cameron, Arreaza et al. 1997; Nitta, Tashiro et al. 1998; Piccirillo, Chang et al. 1998; Zaccone, Phillips et al. 1999). However, the efficacy of cytokine immunotherapy is significantly diminished when initiated after β cell autoimmunity is well established. Noteworthy, is that treatment with a short course of IL-10 and rapamycin protects islet grafts in diabetic NOD recipients via induction of Tr1 cells (Battaglia, Stabilini et al. 2006). T cell-depleting antibodies specific for CD4, CD8, and CD25 have been used in NOD mice and Tg models of T1D to successfully prevent or induce remission of diabetes (Wang, Hao et al. 1987; Shizuru, Taylor-Edwards et al. 1988; Lenschow, Ho et al. 1995; Wang, Gonzalez et al. 1996; Balasa, Krahl et al. 1997; Kuttler, Rosing et al. 1999). Despite their efficacy, the depleting action of these antibodies establishes a state of immunosuppression, and protection is only maintained by continuous treatment with the antibodies. Nevertheless, an approach based on low dose administration of an anti-CD3 $(Fab')_2$ antibody has proven to be highly effective at inducing remission in recent onset diabetic NOD mice. At effective doses, T cell depletion is minimal and long-term protection is mediated by adaptive FoxP3-expressing $CD4^+CD25^+$ Treg that express TGFβ (Chatenoud, 2005) . A clinical Phase II trial has shown that a short course of anti-CD3 antibody treatment

protects β cell mass in recent onset diabetics (Herold, Bluestone et al. 1992; Chatenoud 2005; Herold, Gitelman et al. 2005; Schwartz 2005; Li, Davis et al. 2006). The protective effect, however, is only transient and there are concerns regarding adverse cytokine release due to T cell activation, and recurrent viral infections due to transient depletion of T cells (Xu, Wang et al. 2005; Chatenoud 2006).

1.7.2 Antigen-dependent Immunotherapies.

Treatment with self-antigen can affect autoreactive T cells in two mutually nonexclusive ways. First, T cells may undergo clonal anergy and/or deletion, which is typically seen when soluble antigen is administered at high doses (Liblau, Pearson et al. 1994; Liblau, Tisch et al. 1996). This approach is effective if there is a single or dominant autoantigen as in the case of myasthenia gravis (Drachman, Okumura et al. 1996; Barchan, Souroujon et al. 1999). However, inducing anergy/deletion in a select set of clonotypes is only marginally effective when multiple autoantigens are targeted, as seen in the late stages of T1D (Sohnlein, Muller et al. 2000; Tian, Gregori et al. 2001). The second possible outcome of self-antigen vaccination is the induction/expansion of immunoregulatory T cells. This outcome is appealing since once established, immunoregulatory T cells traffick to the site of inflammation and suppress the differentiation and/or activity of pathogenic T effectors independent of antigen-specificity. Indeed, administration of whole GAD65 or a pool of GAD65-specific peptides (e.g. p217-236, p290-309) induces GAD65-specific immunoregulatory $CD4^+$ T cells in 12 week-old NOD female mice, which traffick to the islets and draining pancreatic lymph nodes (PLN) to suppress β cell autoimmunity and prevent diabetes (Tisch, Liblau et al. 1998; Tisch, Wang et al. 1999). Importantly, the

extracellular milieu established by GAD65-specific immunoregulatory T cells also promotes the development of additional immunoregulatory T cells with distinct β cell-specificities to amplify the protective effect (Kaufman, Clare-Salzler et al. 1993; Tisch, Yang et al. 1993; Elliott, Qin et al. 1994).

There are at least two key issues that need to be addressed when developing an antigen-based approach to induce β cell-specific immunoregulatory T cells. First, the identity of the β cell antigen used for vaccination is critical. Preventing the initiation of the diabetogenic response in young NOD mice has been readily achieved by treatment with several β cell autoantigens such as insulin, GAD65, and HSP60 (Kaufman, Clare-Salzler et al. 1993; Tisch, Yang et al. 1993; Daniel and Wegmann 1996; Cohen 1997; Elias, Meilin et al. 1997). However, in older NOD mice in which β cell autoimmunity is ongoing, only administration of intact GAD65 or derived peptides has consistently induced a sufficient frequency of immunoregulatory T cells to prevent diabetes (Kaufman, Clare-Salzler et al. 1993; Tian, Clare-Salzler et al. 1996; Tisch, Liblau et al. 1998; Tisch, Wang et al. 1999). The efficacy of GAD65 treatment may partly be explained by a significant number of naïve GAD65-specific T precursors present at the late stages of T1D, which in turn can differentiate into immunoregulatory T effectors. The second key issue is how immunoregulatory versus pathogenic T effectors can be selectively induced/expanded by self-antigen vaccination. The use of adjuvants such as complete Freund's adjuvant or alum have proven to be relatively effective in inducing antigen-specific Th2 and Tr1-like cells (Sadelain, Qin et al. 1990; Qin, Sadelain et al. 1993). Furthermore, co-administration of antigen and anti-inflammatory cytokines such as IL-4 and/or IL-10 provides the means to preferentially induce specific

subsets of immunoregulatory T cells (Tisch, Wang et al. 2001; Weaver, Liu et al. 2001; Pop, Wong et al. 2007). Properties intrinsic to a given approach of self-antigen vaccination may also skew towards the induction/expansion of immunoregulatory T cells. One such example is the use of soluble MHC class II molecules containing a covalently linked peptide (Casares, Bona et al. 1997; Casares, Zong et al. 1999; Appel, Seth et al. 2001; Zuo, Cullen et al. 2002). These fusion molecules consist of the extracellular domains of an MHC class II molecule that is supported by an immunoglobulin (Ig) scaffold. Remission of diabetes has been reported in a TCR Tg mouse model following treatment with a peptide-MHC class II-Ig fusion molecule (Casares, Hurtado et al. 2002; Masteller, Warner et al. 2003). Notably, protection corresponds with clonal anergy/deletion of pathogenic T effectors, and induction of IL-10 producing Tr1 cells (Casares, Hurtado et al. 2002; Masteller, Warner et al. 2003).

1.8 References

Abbas, A. K., K. M. Murphy, et al. (1996). "Functional diversity of helper T lymphocytes." Nature **383**(6603): 787-93.

Acha-Orbea, H. and H. O. McDevitt (1987). "The first external domain of the nonobese diabetic mouse class II I-A beta chain is unique." Proc Natl Acad Sci U S A **84**(8): 2435-9.

Amrani, A., J. Verdaguer, et al. (2000). "Progression of autoimmune diabetes driven by avidity maturation of a T-cell population." Nature **406**(6797): 739-42.

Anderson, B., B. J. Park, et al. (1999). "Prevalent CD8(+) T cell response against one peptide/MHC complex in autoimmune diabetes." Proc Natl Acad Sci U S A **96**(16): 9311-6.

Anderson, G., K. M. Partington, et al. (1998). "Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus." J Immunol **161**(12): 6599-603.

Anderson, M. S. and J. A. Bluestone (2005). "The NOD mouse: a model of immune dysregulation." Annu Rev Immunol **23**: 447-85.

Anderson, M. S., E. S. Venanzi, et al. (2005). "The cellular mechanism of Aire control of T cell tolerance." Immunity **23**(2): 227-39.

Appel, H., N. P. Seth, et al. (2001). "Anergy induction by dimeric TCR ligands." J Immunol **166**(8): 5279-85.

Bacchetta, R., C. Sartirana, et al. (2002). "Growth and expansion of human T regulatory type 1 cells are independent from TCR activation but require exogenous cytokines." Eur J Immunol **32**(8): 2237-45.

Bach, J. F. (1994). "Insulin-dependent diabetes mellitus as an autoimmune disease." Endocr Rev **15**(4): 516-42.

Baekkeskov, S., H. J. Aanstoot, et al. (1990). "Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase." Nature **347**(6289): 151-6.

Baekkeskov, S., J. H. Nielsen, et al. (1982). "Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins." Nature **298**(5870): 167-9.

Balasa, B., T. Krahl, et al. (1997). "CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice." J Immunol **159**(9): 4620-7.

Bao, M., Y. Yang, et al. (2002). "Molecular mechanisms for gender differences in susceptibility to T cell-mediated autoimmune diabetes in nonobese diabetic mice." J Immunol **168**(10): 5369-75.

Barchan, D., M. C. Souroujon, et al. (1999). "Antigen-specific modulation of experimental myasthenia gravis: nasal tolerization with recombinant fragments of the human acetylcholine receptor alpha-subunit." Proc Natl Acad Sci U S A **96**(14): 8086-91.

Battaglia, M., A. Stabilini, et al. (2006). "Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance." Diabetes **55**(1): 40-9.

Bendelac, A., C. Carnaud, et al. (1987). "Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells." J Exp Med **166**(4): 823-32.

Bennett, C. L., J. Christie, et al. (2001). "The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3." Nat Genet **27**(1): 20-1.

Bluestone, J. A. and H. Boehmer (2006). "Regulatory T cells." Semin Immunol **18**(2): 77.

Brunkow, M. E., E. W. Jeffery, et al. (2001). "Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse." Nat Genet **27**(1): 68-73.

Cameron, M. J., G. A. Arreaza, et al. (1997). "IL-4 prevents insulitis and insulin-dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function." J Immunol **159**(10): 4686-92.

Candeias, S., J. Katz, et al. (1991). "Islet-specific T-cell clones from nonobese diabetic mice express heterogeneous T-cell receptors." Proc Natl Acad Sci U S A **88**(14): 6167-70.

Carrasco-Marin, E., J. Shimizu, et al. (1996). "The class II MHC I-Ag7 molecules from non-obese diabetic mice are poor peptide binders." J Immunol **156**(2): 450-8.

Casares, S., C. A. Bona, et al. (1997). "Engineering and characterization of a murine MHC class II-immunoglobulin chimera expressing an immunodominant CD4 T viral epitope." Protein Eng **10**(11): 1295-301.

Casares, S., A. Hurtado, et al. (2002). "Down-regulation of diabetogenic CD4+ T cells by a soluble dimeric peptide-MHC class II chimera." Nat Immunol **3**(4): 383-91.

Casares, S., C. S. Zong, et al. (1999). "Antigen-specific signaling by a soluble, dimeric peptide/major histocompatibility complex class II/Fc chimera leading to T helper cell type 2 differentiation." J Exp Med **190**(4): 543-53.

Chang, C. C., R. Ciubotariu, et al. (2002). "Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4." Nat Immunol **3**(3): 237-43.

Chatenoud, L. (2005). "CD3-specific antibodies restore self-tolerance: mechanisms and clinical applications." Curr Opin Immunol **17**(6): 632-7.

Chatenoud, L. (2006). "[Anti-CD3 monoclonal antibodies: a new step towards therapy in new-onset type 1 diabetes]." Med Sci (Paris) **22**(1): 5-6.

Chatenoud, L., E. Thervet, et al. (1992). "[Remission of established disease in diabetic NOD mice induced by anti-CD3 monoclonal antibody]." C R Acad Sci III **315**(6): 225-8.

Chatenoud, L., E. Thervet, et al. (1994). "Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice." Proc Natl Acad Sci U S A **91**(1): 123-7.

Chatila, T. A., F. Blaeser, et al. (2000). "JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome." J Clin Invest **106**(12): R75-81.

Chen, C., W. H. Lee, et al. (2003). "Induction of autoantigen-specific Th2 and Tr1 regulatory T cells and modulation of autoimmune diabetes." J Immunol **171**(2): 733-44.

Chen, W., W. Jin, et al. (2003). "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3." J Exp Med **198**(12): 1875-86.

Chen, Y., V. K. Kuchroo, et al. (1994). "Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis." Science **265**(5176): 1237-40.

Chervonsky, A. V., Y. Wang, et al. (1997). "The role of Fas in autoimmune diabetes." Cell **89**(1): 17-24.

Coffman, R. L. and S. L. Reiner (1999). "Instruction, selection, or tampering with the odds?" Science **284**(5418): 1283, 1285.

Cohen, I. R. (1997). "The Th1/Th2 dichotomy, hsp60 autoimmunity, and type I diabetes." Clin Immunol Immunopathol **84**(2): 103-6.

Concannon, P., K. J. Gogolin-Ewens, et al. (1998). "A second-generation screen of the human genome for susceptibility to insulin-dependent diabetes mellitus." Nat Genet **19**(3): 292-6.

Conti, P., D. Kempuraj, et al. (2003). "IL-10 subfamily members: IL-19, IL-20, IL-22, IL-24 and IL-26." Immunol Lett **88**(3): 171-4.

Cooke, A., J. M. Phillips, et al. (2001). "Tolerogenic strategies to halt or prevent type 1 diabetes." Nat Immunol **2**(9): 810-5.

Daniel, D., R. G. Gill, et al. (1995). "Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice." Eur J Immunol **25**(4): 1056-62.

Daniel, D. and D. R. Wegmann (1996). "Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23)." Proc Natl Acad Sci U S A **93**(2): 956-60.

Davies, J. L., Y. Kawaguchi, et al. (1994). "A genome-wide search for human type 1 diabetes susceptibility genes." Nature **371**(6493): 130-6.

Drachman, D. B., S. Okumura, et al. (1996). "Oral tolerance in myasthenia gravis." Ann N Y Acad Sci **778**: 258-72.

Eisenbarth, G. S., H. Moriyama, et al. (2002). "Insulin autoimmunity: prediction/precipitation/prevention type 1A diabetes." Autoimmun Rev **1**(3): 139-45.

Elias, D., A. Meilin, et al. (1997). "Hsp60 peptide therapy of NOD mouse diabetes induces a Th2 cytokine burst and downregulates autoimmunity to various beta-cell antigens." Diabetes **46**(5): 758-64.

Elliott, J. F., H. Y. Qin, et al. (1994). "Immunization with the larger isoform of mouse glutamic acid decarboxylase (GAD67) prevents autoimmune diabetes in NOD mice." Diabetes **43**(12): 1494-9.

Erlander, M. G., N. J. Tillakaratne, et al. (1991). "Two genes encode distinct glutamate decarboxylases." Neuron **7**(1): 91-100.

Faideau, B., E. Larger, et al. (2005). "Role of beta-cells in type 1 diabetes pathogenesis." Diabetes **54 Suppl 2**: S87-96.

Fallarino, F., U. Grohmann, et al. (2003). "Modulation of tryptophan catabolism by regulatory T cells." Nat Immunol **4**(12): 1206-12.

Fantini, M. C., C. Becker, et al. (2004). "Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7." J Immunol **172**(9): 5149-53.

Faulkner-Jones, B. E., D. S. Cram, et al. (1993). "Localization and quantitation of expression of two glutamate decarboxylase genes in pancreatic beta-cells and other peripheral tissues of mouse and rat." Endocrinology **133**(6): 2962-72.

Fontenot, J. D., J. P. Rasmussen, et al. (2005). "Regulatory T cell lineage specification by the forkhead transcription factor foxp3." Immunity **22**(3): 329-41.

Fox, H. S. (1992). "Androgen treatment prevents diabetes in nonobese diabetic mice." J Exp Med **175**(5): 1409-12.

French, M. B., J. Allison, et al. (1997). "Transgenic expression of mouse proinsulin II prevents diabetes in nonobese diabetic mice." Diabetes **46**(1): 34-9.

Gepts, W. (1965). "Pathologic anatomy of the pancreas in juvenile diabetes mellitus." Diabetes **14**(10): 619-33.

Gonzalez-Rey, E., A. Chorny, et al. (2007). "Regulation of immune tolerance by anti-inflammatory neuropeptides." Nat Rev Immunol **7**(1): 52-63.

Gorelik, L. and R. A. Flavell (2000). "Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease." Immunity **12**(2): 171-81.

Green, E. A., L. Gorelik, et al. (2003). "CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes." Proc Natl Acad Sci U S A **100**(19): 10878-83.

Gregori, S., N. Giarratana, et al. (2003). "Dynamics of pathogenic and suppressor T cells in autoimmune diabetes development." J Immunol **171**(8): 4040-7.

Groux, H., A. O'Garra, et al. (1997). "A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis." Nature **389**(6652): 737-42.

Han, B., P. Serra, et al. (2005). "Prevention of diabetes by manipulation of anti-IGRP autoimmunity: high efficiency of a low-affinity peptide." Nat Med **11**(6): 645-52.

Haskins, K. and D. Wegmann (1996). "Diabetogenic T-cell clones." Diabetes **45**(10): 1299-305.

Hayward, A. R. and M. Shreiber (1989). "Neonatal injection of CD3 antibody into nonobese diabetic mice reduces the incidence of insulitis and diabetes." J Immunol **143**(5): 1555-9.

Hayward, A. R. and M. Shriber (1992). "Reduced incidence of insulitis in NOD mice following anti-CD3 injection: requirement for neonatal injection." J Autoimmun **5**(1): 59-67.

Healey, D., P. Ozegbe, et al. (1995). "In vivo activity and in vitro specificity of CD4+ Th1 and Th2 cells derived from the spleens of diabetic NOD mice." J Clin Invest **95**(6): 2979-85.

Herold, K. C., J. A. Bluestone, et al. (1992). "Prevention of autoimmune diabetes with nonactivating anti-CD3 monoclonal antibody." Diabetes **41**(3): 385-91.

Herold, K. C., S. E. Gitelman, et al. (2005). "A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes." Diabetes **54**(6): 1763-9.

Hori, S., T. Nomura, et al. (2003). "Control of regulatory T cell development by the transcription factor Foxp3." Science **299**(5609): 1057-61.

Hu, D., K. Ikizawa, et al. (2004). "Analysis of regulatory CD8 T cells in Qa-1-deficient mice." Nat Immunol **5**(5): 516-23.

Hyoty, H. and K. W. Taylor (2002). "The role of viruses in human diabetes." Diabetologia **45**(10): 1353-61.

Jansen, A., F. Homo-Delarche, et al. (1994). "Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and beta-cell destruction in NOD mice." Diabetes **43**(5): 667-75.

Jun, H. S., P. Santamaria, et al. (1999). "Absolute requirement of macrophages for the development and activation of beta-cell cytotoxic CD8+ T-cells in T-cell receptor transgenic NOD mice." Diabetes **48**(1): 34-42.

Jun, H. S. and J. W. Yoon (2001). "The role of viruses in type I diabetes: two distinct cellular and molecular pathogenic mechanisms of virus-induced diabetes in animals." Diabetologia **44**(3): 271-85.

Kagi, D., B. Odermatt, et al. (1997). "Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice." J Exp Med **186**(7): 989-97.

Katz, J. D., C. Benoist, et al. (1995). "T helper cell subsets in insulin-dependent diabetes." Science **268**(5214): 1185-8.

Katz, J. D., B. Wang, et al. (1993). "Following a diabetogenic T cell from genesis through pathogenesis." Cell **74**(6): 1089-100.

Kaufman, D. L., M. Clare-Salzler, et al. (1993). "Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes." Nature **366**(6450): 69-72.

Kent, S. C., Y. Chen, et al. (2005). "Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope." Nature **435**(7039): 224-8.

Khattri, R., T. Cox, et al. (2003). "An essential role for Scurfin in CD4+CD25+ T regulatory cells." Nat Immunol **4**(4): 337-42.

Kim, J., W. Richter, et al. (1993). "Differential expression of GAD65 and GAD67 in human, rat, and mouse pancreatic islets." Diabetes **42**(12): 1799-808.

Kishimoto, H. and J. Sprent (2001). "A defect in central tolerance in NOD mice." Nat Immunol **2**(11): 1025-31.

Kopf, M., G. Le Gros, et al. (1993). "Disruption of the murine IL-4 gene blocks Th2 cytokine responses." Nature **362**(6417): 245-8.

Krishnamurthy, B., N. L. Dudek, et al. (2006). "Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP." J Clin Invest **116**(12): 3258-65.

Kristiansen, O. P., Z. M. Larsen, et al. (2000). "CTLA-4 in autoimmune diseases--a general susceptibility gene to autoimmunity?" Genes Immun **1**(3): 170-84.

Kuttler, B., K. Rosing, et al. (1999). "Prevention of autoimmune but not allogeneic destruction of grafted islets by different therapeutic strategies." J Mol Med **77**(1): 226-9.

Kyewski, B., J. Derbinski, et al. (2002). "Promiscuous gene expression and central T-cell tolerance: more than meets the eye." Trends Immunol **23**(7): 364-71.

Latek, R. R., A. Suri, et al. (2000). "Structural basis of peptide binding and presentation by the type I diabetes-associated MHC class II molecule of NOD mice." Immunity **12**(6): 699-710.

Lenschow, D. J., S. C. Ho, et al. (1995). "Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse." J Exp Med **181**(3): 1145-55.

Li, J., J. Davis, et al. (2006). "Modulation of antigen-specific T cell response by a non-mitogenic anti-CD3 antibody." Int Immunopharmacol **6**(6): 880-91.

Liblau, R. S., C. I. Pearson, et al. (1994). "High-dose soluble antigen: peripheral T-cell proliferation or apoptosis." Immunol Rev **142**: 193-208.

Liblau, R. S., S. M. Singer, et al. (1995). "Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases." Immunol Today **16**(1): 34-8.

Liblau, R. S., R. Tisch, et al. (1996). "Intravenous injection of soluble antigen induces thymic and peripheral T-cells apoptosis." Proc Natl Acad Sci U S A **93**(7): 3031-6.

Lieberman, S. M., A. M. Evans, et al. (2003). "Identification of the beta cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes." Proc Natl Acad Sci U S A **100**(14): 8384-8.

Liu, Y. J. (2006). "A unified theory of central tolerance in the thymus." Trends Immunol **27**(5): 215-21.

Ludewig, B., B. Odermatt, et al. (1998). "Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue." J Exp Med **188**(8): 1493-501.

Lund, T., L. O'Reilly, et al. (1990). "Prevention of insulin-dependent diabetes mellitus in non-obese diabetic mice by transgenes encoding modified I-A beta-chain or normal I-E alpha-chain." Nature **345**(6277): 727-9.

Martin, D. L. and K. Rimvall (1993). "Regulation of gamma-aminobutyric acid synthesis in the brain." J Neurochem **60**(2): 395-407.

Masteller, E. L., M. R. Warner, et al. (2003). "Peptide-MHC class II dimers as therapeutics to modulate antigen-specific T cell responses in autoimmune diabetes." J Immunol **171**(10): 5587-95.

Mathis, D., L. Vence, et al. (2001). "beta-Cell death during progression to diabetes." Nature **414**(6865): 792-8.

McGuirk, P., C. McCann, et al. (2002). "Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis." J Exp Med **195**(2): 221-31.

Miller, B. J., M. C. Appel, et al. (1988). "Both the Lyt-2+ and L3T4+ T cell subsets are required for the transfer of diabetes in nonobese diabetic mice." J Immunol **140**(1): 52-8.

Mosmann, T. R., H. Cherwinski, et al. (1986). "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." J Immunol **136**(7): 2348-57.

Nakayama, M., N. Abiru, et al. (2005). "Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice." Nature **435**(7039): 220-3.

Nishimoto, H., H. Kikutani, et al. (1987). "Prevention of autoimmune insulitis by expression of I-E molecules in NOD mice." Nature **328**(6129): 432-4.

Nitta, Y., F. Tashiro, et al. (1998). "Systemic delivery of interleukin 10 by intramuscular injection of expression plasmid DNA prevents autoimmune diabetes in nonobese diabetic mice." Hum Gene Ther **9**(12): 1701-7.

Nossal, G. J. (1994). "Negative selection of lymphocytes." Cell **76**(2): 229-39.

O'Garra, A., P. L. Vieira, et al. (2004). "IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage." J Clin Invest **114**(10): 1372-8.

Ogasawara, K., J. A. Hamerman, et al. (2003). "Impairment of NK cell function by NKG2D modulation in NOD mice." Immunity **18**(1): 41-51.

Ohashi, P. S., S. Oehen, et al. (1991). "Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice." Cell **65**(2): 305-17.

Oldstone, M. B., M. Nerenberg, et al. (1991). "Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response." Cell **65**(2): 319-31.

Oldstone, M. B., P. Southern, et al. (1984). "Virus persists in beta cells of islets of Langerhans and is associated with chemical manifestations of diabetes." Science **224**(4656): 1440-3.

O'Reilly, L. A., P. R. Hutchings, et al. (1991). "Characterization of pancreatic islet cell infiltrates in NOD mice: effect of cell transfer and transgene expression." Eur J Immunol **21**(5): 1171-80.

Owerbach, D. and K. H. Gabbay (1993). "Localization of a type I diabetes susceptibility locus to the variable tandem repeat region flanking the insulin gene." Diabetes **42**(12): 1708-14.

Pankewycz, O., T. B. Strom, et al. (1991). "Islet-infiltrating T cell clones from non-obese diabetic mice that promote or prevent accelerated onset diabetes." Eur J Immunol **21**(4): 873-9.

Peakman, M., E. J. Stevens, et al. (1999). "Naturally processed and presented epitopes of the islet cell autoantigen IA-2 eluted from HLA-DR4." J Clin Invest **104**(10): 1449-57.

Piccirillo, C. A., Y. Chang, et al. (1998). "TGF-beta1 somatic gene therapy prevents autoimmune disease in nonobese diabetic mice." J Immunol **161**(8): 3950-6.

Pop, S. M., C. P. Wong, et al. (2005). "Single cell analysis shows decreasing FoxP3 and TGFbeta1 coexpressing CD4+CD25+ regulatory T cells during autoimmune diabetes." J Exp Med **201**(8): 1333-46.

Pop, S. M., C. P. Wong, et al. (2007). "The type and frequency of immunoregulatory CD4+ T-cells govern the efficacy of antigen-specific immunotherapy in nonobese diabetic mice." Diabetes **56**(5): 1395-402.

Qin, H. Y., M. W. Sadelain, et al. (1993). "Complete Freund's adjuvant-induced T cells prevent the development and adoptive transfer of diabetes in nonobese diabetic mice." J Immunol **150**(5): 2072-80.

Quartey-Papafio, R., T. Lund, et al. (1995). "Aspartate at position 57 of nonobese diabetic I-Ag7 beta-chain diminishes the spontaneous incidence of insulin-dependent diabetes mellitus." J Immunol **154**(10): 5567-75.

Ramsdell, F. (2003). "Foxp3 and natural regulatory T cells: key to a cell lineage?" Immunity **19**(2): 165-8.

Redondo, M. J., M. Rewers, et al. (1999). "Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-twin siblings of patients with type 1 diabetes: prospective twin study." Bmj **318**(7185): 698-702.

Ridgway, W. M., M. Fasso, et al. (1999). "A new look at MHC and autoimmune disease." Science **284**(5415): 749, 751.

Roncarolo, M. G., R. Bacchetta, et al. (2001). "Type 1 T regulatory cells." Immunol Rev **182**: 68-79.

Rotwein, P., R. Chyn, et al. (1981). "Polymorphism in the 5'-flanking region of the human insulin gene and its possible relation to type 2 diabetes." Science **213**(4512): 1117-20.

Sadelain, M. W., H. Y. Qin, et al. (1990). "Prevention of type I diabetes in NOD mice by adjuvant immunotherapy." Diabetes **39**(5): 583-9.

Sakaguchi, S. (2000). "Regulatory T cells: key controllers of immunologic self-tolerance." Cell **101**(5): 455-8.

Sakaguchi, S. (2005). "Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self." Nat Immunol **6**(4): 345-52.

Santamaria, P., T. Utsugi, et al. (1995). "Beta-cell-cytotoxic CD8+ T cells from nonobese diabetic mice use highly homologous T cell receptor alpha-chain CDR3 sequences." J Immunol **154**(5): 2494-503.

Schwartz, I. D. (2005). "CD3-antibody therapy in new-onset type 1 diabetes mellitus." N Engl J Med **353**(19): 2086-7; author reply 2086-7.

Scott, P. (1993). "IL-12: initiation cytokine for cell-mediated immunity." Science **260**(5107): 496-7.

Serreze, D. V., E. H. Leiter, et al. (1994). "Major histocompatibility complex class I-deficient NOD-B2mnull mice are diabetes and insulitis resistant." Diabetes **43**(3): 505-9.

Shevach, E. M. (2002). "CD4+ CD25+ suppressor T cells: more questions than answers." Nat Rev Immunol **2**(6): 389-400.

Shizuru, J. A., C. Taylor-Edwards, et al. (1988). "Immunotherapy of the nonobese diabetic mouse: treatment with an antibody to T-helper lymphocytes." Science **240**(4852): 659-62.

Shortman, K., D. Vremec, et al. (1998). "The linkage between T-cell and dendritic cell development in the mouse thymus." Immunol Rev **165**: 39-46.

Sohnlein, P., M. Muller, et al. (2000). "Epitope spreading and a varying but not disease-specific GAD65 antibody response in Type I diabetes. The Childhood Diabetes in Finland Study Group." Diabetologia **43**(2): 210-7.

Stevens, T. L., A. Bossie, et al. (1988). "Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells." Nature **334**(6179): 255-8.

Stiller, C. R., J. Dupre, et al. (1984). "Effects of cyclosporine immunosuppression in insulin-dependent diabetes mellitus of recent onset." Science **223**(4643): 1362-7.

Stiller, C. R., A. Laupacis, et al. (1983). "Cyclosporine for treatment of early type I diabetes: preliminary results." N Engl J Med **308**(20): 1226-7.

Suarez-Pinzon, W. L., J. G. Mabley, et al. (2001). "An inhibitor of inducible nitric oxide synthase and scavenger of peroxynitrite prevents diabetes development in NOD mice." J Autoimmun **16**(4): 449-55.

Summers, K. L., M. T. Behme, et al. (2003). "Characterization of dendritic cells in humans with type 1 diabetes." Ann N Y Acad Sci **1005**: 226-9.

Thomas-Vaslin, V., D. Damotte, et al. (1997). "Abnormal T cell selection on nod thymic epithelium is sufficient to induce autoimmune manifestations in C57BL/6 athymic nude mice." Proc Natl Acad Sci U S A **94**(9): 4598-603.

Tian, J., M. Clare-Salzler, et al. (1996). "Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice." Nat Med **2**(12): 1348-53.

Tian, J., S. Gregori, et al. (2001). "The frequency of high avidity T cells determines the hierarchy of determinant spreading." J Immunol **166**(12): 7144-50.

Tisch, R., R. S. Liblau, et al. (1998). "Induction of GAD65-specific regulatory T-cells inhibits ongoing autoimmune diabetes in nonobese diabetic mice." Diabetes **47**(6): 894-9.

Tisch, R. and H. McDevitt (1996). "Insulin-dependent diabetes mellitus." Cell **85**(3): 291-7.

Tisch, R., B. Wang, et al. (1999). "Induction of glutamic acid decarboxylase 65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope dependent." J Immunol **163**(3): 1178-87.

Tisch, R., B. Wang, et al. (2001). "Antigen-specific mediated suppression of beta cell autoimmunity by plasmid DNA vaccination." J Immunol **166**(3): 2122-32.

Tisch, R., X. D. Yang, et al. (1993). "Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice." Nature **366**(6450): 72-5.

Todd, J. A. (1995). "Genetic analysis of type 1 diabetes using whole genome approaches." Proc Natl Acad Sci U S A **92**(19): 8560-5.

Trudeau, J. D., C. Kelly-Smith, et al. (2003). "Prediction of spontaneous autoimmune diabetes in NOD mice by quantification of autoreactive T cells in peripheral blood." J Clin Invest **111**(2): 217-23.

Undlien, D. E., B. A. Lie, et al. (2001). "HLA complex genes in type 1 diabetes and other autoimmune diseases. Which genes are involved?" Trends Genet **17**(2): 93-100.

Verdaguer, J., J. W. Yoon, et al. (1996). "Acceleration of spontaneous diabetes in TCR-beta-transgenic nonobese diabetic mice by beta-cell cytotoxic CD8+ T cells expressing identical endogenous TCR-alpha chains." J Immunol **157**(10): 4726-35.

Verge, C. F., R. Gianani, et al. (1996). "Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies." Diabetes **45**(7): 926-33.

Wang, B., A. Gonzalez, et al. (1996). "The role of CD8+ T cells in the initiation of insulin-dependent diabetes mellitus." Eur J Immunol **26**(8): 1762-9.

Wang, Y., L. Hao, et al. (1987). "Autoimmune diabetes in NOD mouse is L3T4 T-lymphocyte dependent." Diabetes **36**(4): 535-8.

Weaver, C. T., L. E. Harrington, et al. (2006). "Th17: an effector CD4 T cell lineage with regulatory T cell ties." Immunity **24**(6): 677-88.

Weaver, D. J., Jr., B. Liu, et al. (2001). "Plasmid DNAs encoding insulin and glutamic acid decarboxylase 65 have distinct effects on the progression of autoimmune diabetes in nonobese diabetic mice." J Immunol **167**(1): 586-92.

Wegmann, D. R., M. Norbury-Glaser, et al. (1994). "Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice." Eur J Immunol **24**(8): 1853-7.

Wicker, L. S., E. H. Leiter, et al. (1994). "Beta 2-microglobulin-deficient NOD mice do not develop insulitis or diabetes." Diabetes **43**(3): 500-4.

Wicker, L. S., B. J. Miller, et al. (1986). "Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice." Diabetes **35**(8): 855-60.

Wicker, L. S., J. A. Todd, et al. (1995). "Genetic control of autoimmune diabetes in the NOD mouse." Annu Rev Immunol **13**: 179-200.

Winter, W. E., H. Beppu, et al. (1987). "Restriction-fragment-length polymorphisms of 5'-flanking region of insulin I gene in BB and other rat strains. Absence of association with IDDM." Diabetes **36**(2): 193-8.

Wong, F. S., J. Karttunen, et al. (1999). "Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library." Nat Med **5**(9): 1026-31.

Wong, F. S., I. Visintin, et al. (1998). "The role of lymphocyte subsets in accelerated diabetes in nonobese diabetic-rat insulin promoter-B7-1 (NOD-RIP-B7-1) mice." J Exp Med **187**(12): 1985-93.

Wraith, D. C., K. S. Nicolson, et al. (2004). "Regulatory CD4+ T cells and the control of autoimmune disease." Curr Opin Immunol **16**(6): 695-701.

Xu, R. N., J. N. Wang, et al. (2005). "The effect of CD3-specific monoclonal antibody on treating experimental autoimmune myasthenia gravis." Cell Mol Immunol **2**(6): 461-5.

Yoon, J. W., C. S. Yoon, et al. (1999). "Control of autoimmune diabetes in NOD mice by GAD expression or suppression in beta cells." Science **284**(5417): 1183-7.

Zaccone, P., J. Phillips, et al. (1999). "Interleukin-13 prevents autoimmune diabetes in NOD mice." Diabetes **48**(8): 1522-8.

Zuo, L., C. M. Cullen, et al. (2002). "A single-chain class II MHC-IgG3 fusion protein inhibits autoimmune arthritis by induction of antigen-specific hyporesponsiveness." J Immunol **168**(5): 2554-9.

CHAPTER 2

SUPPRESSION OF AUTOIMMUNE DIABETES BY TREATMENT WITH PEPTIDE-MHC CLASS II DIMERS

Li Li, Kevin Goudy, Rui Zhang, Bo Wang, Roland Tisch

Department of Microbiology and Immunology

School of Medicine

University of North Carolina, Chapel Hill

Chapel Hill, North Carolina

2.1 Abstract

Type 1 diabetes (T1D) is an autoimmune disease mediated by pathogenic β cell-specific T cells. The use of antigen-specific based immunotherapy is one strategy to selectively target β cell-specific T cells, leaving the remainder of the immune system intact. Administration of peptides derived from β cell autoantigens such as GAD65 prevents T1D in nonobese diabetic (NOD) mice. However, as β cell autoimmunity progresses, the conditions for peptide treatment to suppress T1D become highly stringent. To enhance the efficacy of peptide treatment, soluble (s) IA^{g7}- immunoglobulin (Ig) dimers covalently linked to GAD65 peptides (p217-236, p286-309) or the mimetic BDC2.5 epitope (mBDC) were tested. Twelve week-old NOD female mice with established β cell autoimmunity received a short course of sIA^{g7}-Ig dimers intravenously (i.v.). Treatment with sIA^{g7}-mBDC expanded mBDC-specific $CD4^+$ Th1 cells, and NOD mice continued to develop diabetes. In marked contrast, the majority of NOD mice treated with sIA^{g7}-p217 or sIA^{g7}-p286 remained diabetes-free. Protection correlated with an increased frequency of IL-10 secreting immunoregulatory $CD4^+$ T cells that delayed diabetes in a co-adoptive transfer model. These results demonstrate that treatment with a short-course of sIA^{g7}-GAD65 peptide dimers is an effective approach to suppress T1D.

2.2 Introduction

T1D is an autoimmune disease characterized by the T cell-mediated destruction of insulin-producing β cells (Tisch and McDevitt 1996). Studies in the NOD mouse, a spontaneous model of T1D, suggest that the diabetogenic response progresses in discrete stages culminating in massive β cell destruction. Infiltration of the islets is first detected in NOD mice at 3 weeks of age. Insulitis progresses for a number of weeks with only a minimal effect on β cell mass. However, at 12 weeks of age the destructive phase of insulitis is initiated. During this stage β cells are readily destroyed leading to hyperglycemic blood levels, and the development of overt diabetes. β cell destruction is driven by $CD4^+$ and $CD8^+$ T cells recognizing multiple autoantigens, of which only a few have been identified (Anderson and Bluestone 2005). The latter group includes insulin, glutamic acid decarboxylase 65 (GAD65), and insulinoma-associated antigen (IA-2) (Tisch, Yang et al. 1993; Wegmann, Norbury-Glaser et al. 1994; Verge, Gianani et al. 1996; Hawa, Rowe et al. 1997; Eisenbarth, Moriyama et al. 2002). $CD4^+$ T cells expressing the diabetogenic BDC2.5 clonotypic T cell receptor (TCR) and recognizing a mimetic peptide (mBDC), represent an unidentified β cell specificity that also has a key role in the pathogenesis of T1D (Katz, Wang et al. 1993; Bergman and Haskins 1994; Kurrer, Pakala et al. 1997; Yoshida, Martin et al. 2002).

To date, a considerable effort has been devoted to developing therapeutic approaches to target T cells and prevent and/or treat T1D. Strategies based on administration of immunosuppressant drugs, anti-inflammatory cytokines, and antibodies specific for T cells

have been successfully used in experimental models, and in some instances the clinic (Shizuru, Taylor-Edwards et al. 1988; Yang, Tisch et al. 1994; Lenschow, Ho et al. 1995; Wang, Gonzalez et al. 1996; Balasa, Krahl et al. 1997; Cameron, Arreaza et al. 1997; Nitta, Tashiro et al. 1998; Piccirillo, Chang et al. 1998; Kuttler, Rosing et al. 1999; Nicoletti, Zaccone et al. 1999; Zaccone, Phillips et al. 1999; Chatenoud 2005). However, these approaches fail to discriminate between autoreactive and nonautoreactive T cells, and as a result induce varying degrees of immunosuppression.

Antigen-specific immunotherapies offer an approach to selectively target autoreactive T cells, leaving the remainder of the immune system intact (Bach 2001). Administration of insulin, insulin B chain or GAD65 to young NOD mice effectively prevents insulitis and the development of T1D (Daniel and Wegmann 1996; Tian, Atkinson et al. 1996; Tian, Clare-Salzler et al. 1996; Maron, Melican et al. 1999; Tisch, Wang et al. 1999). However, at late preclinical stages of T1D conditions for antigen-specific immunotherapy become far more stringent due to the high number of pathogenic T effectors. For instance, β cell autoimmunity is readily prevented in young NOD female mice with a single injection of GAD65-specific peptides p217-236 (p217) or p286-309 (p286) prepared in incomplete Freund's adjuvant (IFA) (Tisch, Wang et al. 1999). However, once β cell autoimmunity is established (e.g. 12 week old NOD female mice), diabetes can be prevented only after multiple injections of high doses of a mixture of GAD65 p217 and p286 in IFA (Tisch, Wang et al. 1999). Protection corresponds with clonal deletion of GAD65-specific Th1 effectors, and the induction of IL-4 secreting peptide-specific $CD4^+$ Th2 cells.

Based on our findings and that of others, peptide immunotherapy can be an effective approach to modulate the activity of β cell-specific T cells (Chao and McDevitt 1997; Zechel, Elliott et al. 1998; Tisch, Wang et al. 1999). Nevertheless, several parameters need to be considered for successful clinical application of this approach including peptide dose and the route of administration, the peptide binding affinity to MHC molecules, and *in vivo* peptide stability. For instance, peptides are rapidly cleared from the circulation and inefficiently presented by APC *in vivo*, which limit therapeutic efficacy (Babbitt, Matsueda et al. 1986; Muller, Adorini et al. 1990; Ishioka, Adorini et al. 1994). One strategy to overcome these limitations has been the engineering of soluble peptide-MHC class II-Ig fusion proteins. Such recombinants consist of the extracellular domains of the MHC class II α and β chains supported by an Ig scaffold, which in turn enhances the stability of the fusion molecule *in vivo* (Casares, Bona et al. 1997; Casares, Zong et al. 1999; Appel, Seth et al. 2001; Zuo, Cullen et al. 2002). In addition, a peptide is tethered to the MHC class II β chain ensuring that each bivalent fusion molecule presents a peptide. Soluble bivalent peptide-MHC-Ig molecules stimulate T cells by delivering a signal through TCR (Hamad, O'Herrin et al. 1998; Casares, Zong et al. 1999; Appel, Gauthier et al. 2000; Cochran, Cameron et al. 2000). Recently, peptide-MHC-Ig molecules have been reported to: i) ameliorate collagen-induced arthritis in mice by induction of antigen-specific hyporesponsiveness (Zuo, Cullen et al. 2002), and ii) promote remission of diabetes in a transgenic (Tg) mouse model of T1D via induction of immunoregulatory Tr1 cells (Casares, Hurtado et al. 2002). With this in mind, the current study was initiated to test the hypothesis that administration of sIA^{g7}-Ig dimers complexed with β cell peptides effectively suppresses ongoing β cell autoimmunity, and blocks the development of overt diabetes in NOD female mice.

2.3 Results

The production and characterization of sIA^{g7}-Ig dimers

sIA^{g7}-Ig dimers were engineered using recombinant DNA techniques. The transmembrane and cytoplasmic domains of the IA$^{d}\alpha$- and the IA$^{g7}\beta$-chains were replaced with leucine zipper dimerization domains to promote assembly of the two chains. Peptides were covalently linked to the NH_2-terminus of the IA$^{g7}\beta$-chain. For this study sIA^{g7}-Ig dimers were established tethered to: i) GAD65-specific peptides p217 and p286; ii) the mimetic peptide recognized by BDC2.5 CD4$^+$ T cells (mBDC); and iii) the hen egg lysozyme (HEL) epitope p12-26. The IA$^{d}\alpha$-chain was modified by the addition of the murine IgG2a Fc domain to establish a divalent structure (Malherbe, Filippi et al. 2000). Two amino acids in the IgG2a hinge were mutated (L234/A235) to prevent the recombinant protein from binding to FcγR I/FcγR II (Lund, Winter et al. 1991; Wines, Powell et al. 2000). sIA^{g7}-Ig dimers were produced in stably transfected *Drosophila* S2 cells and purified by affinity chromatography.

SDS-PAGE analysis of purified sIA^{g7}-p217 dimers under denaturing conditions demonstrated two bands of 60 and 35 kD, which corresponded to the predicted molecular weights of the IA$^{d}\alpha$-Ig and peptide-IA$^{g7}\beta$ chains, respectively (Figure 2.1A). HPLC confirmed the molecular weight of the assembled sIA^{g7}-p217 dimer complex, in addition to showing that the purified preparations had no significant high molecular weight aggregates (Figure 2.1B).

Initially, the specificity of the sIA^{g7}-Ig recombinants was verified. Multimers of sIA^{g7}-mBDC, sIA^{g7}-p217 and sIA^{g7}-HEL were tested for binding to CD4$^+$ T cells found in peripheral blood lymphocytes (PBL) of BDC2.5 TCR Tg NOD mice, and the GAD65 p217-

specific T cell clone 11H11. To establish the multimers and detect binding via flow cytometry, sIA^{g7}-Ig dimers were preincubated with Protein A conjugated with the flurochrome Alexa 647. sIA^{g7}-mBDC multimers stained 88.7% of the BDC2.5 CD4^{+} T cells, whereas only 0.23% of the T cells were stained with the negative control sIA^{g7}-HEL multimers (Figure 2.2A). On the other hand, 98% and 0.3% of 11H11 CD4^{+} T cells stained with the sIA^{g7}-p217 and sIA^{g7}-HEL multimers, respectively (Figure 2.2B). These data demonstrate that the sIA^{g7}-Ig recombinants bind to T cells in a peptide-specific manner.

Next, the capacity of the sIA^{g7}-Ig dimers to stimulate CD4^{+} T cells *in vitro* was investigated. Ninety-six well plates were coated with sIA^{g7}-mBDC, sIA^{g7}-p217 or sIA^{g7}-HEL and the proliferative response of splenic BDC2.5 and 11H11 CD4^{+} T cells measured via [^{3}H] thymidine incorporation after 48 hours of culture. As demonstrated in Figure 2.3, significant proliferation of BDC2.5 CD4^{+} T cells was detected following stimulation with sIA^{g7}-mBDC, but not sIA^{g7}-p217 or sIA^{g7}-HEL. Furthermore a robust proliferative response was detected for 11H11 CD4^{+} T cells stimulated with sIA^{g7}-p217, but not sIA^{g7}-mBDC or sIA^{g7}-HEL (Figure 2.3). These results demonstrate that sIA^{g7}-Ig dimers stimulate CD4^{+} T cell proliferation in a peptide-specific manner.

The half-life of native peptides in blood is typically short, in the order of a few minutes. With this in mind, persistence of sIA^{g7}-Ig dimers in serum was examined. NOD.*scid* mice received a single i.v. injection of 50 µg of sIA^{g7}-Ig dimers prepared in PBS. Serum was harvested at varying times post-injection, and levels of sIA^{g7}-Ig dimers measured with an anti-mouse IgG2a specific ELISA. Results indicate that 50% of the maximum detectable

serum concentration of the sIA^{g7}-Ig dimers was observed 48 hours after injection, consistent with previous reports (Casares, Bona et al. 1997) (Figure 2.4).

sIA^{g7}-p217 and sIA^{g7}-p286 but not sIA^{g7}-mBDC dimers prevent diabetes in NOD mice

To assess the efficacy of sIA^{g7}-Ig dimers to suppress ongoing β cell autoimmunity and prevent diabetes, 12 week-old NOD female mice were treated with the respective recombinants. Specifically, NOD female mice received i.v. injections of 50 μg of sIA^{g7}-Ig dimers on 3 consecutive days; 3 weeks later a second course of sIA^{g7}-Ig dimers was administered. Diabetes was monitored weekly by measuring blood glucose levels. No significant difference in the time of onset or frequency of diabetes was detected in NOD mice left untreated or receiving sIA^{g7}-HEL, indicating that sIA^{g7}-Ig dimers do not prevent diabetes in a nonspecific manner (Figure 2.5). NOD mice treated with sIA^{g7}-mBDC developed diabetes at a similar rate and frequency as the sIA^{g7}-HEL treated and untreated control groups (Figure 2.5). In marked contrast, the majority of NOD mice treated with sIA^{g7}-p217 (8/10) or sIA^{g7}-p286 (9/10) dimers remained diabetes-free (Figure 2.5). Furthermore, histological analysis of pancreases showed a significantly reduced frequency of intra-insulitis in sIA^{g7}-p217 and sIA^{g7}-p286 dimer-treated mice compared to the sIA^{g7}-mBDC and sIA^{g7}-HEL treated groups (Figure 2.6). Interestingly, a comparison between sIA^{g7}-p217 and sIA^{g7}-p286 dimer-treated versus untreated 12 week-old NOD female mice showed no significant difference in the frequency of insulitis (Figure 2.6). This latter finding suggests that the progression of insulitis was effectively suppressed at the time sIA^{g7}-p217 and sIA^{g7}-p286 dimer treatment was initiated. Together, these results demonstrate that treatment with sIA^{g7}-

p217 and sIA^{g7}-p286 dimers can effectively prevent diabetes at a late preclinical stage of T1D, and that protection is epitope-specific.

Protection mediated by sIA^{g7}-p217 and sIA^{g7}-p286 dimers correlates with the induction of peptide-specific immunoregulatory Tr1 cells

To determine the nature of the T cell response induced by the respective sIA^{g7}-Ig dimers, 12 week-old NOD female were treated as above. Three weeks after the last injection, spleens were harvested and the frequency of IL-4, IL-10 and IFNγ-secreting T cells in response to the panel of peptides measured via ELISPOT. NOD mice treated with sIA^{g7}-mBDC dimers exhibited a significant increase in the frequency of T cells secreting IFNγ and IL-4 in response to mBDC, relative to the other groups (Fig. 2.7). On the other hand, cultures prepared from NOD mice vaccinated with the sIA^{g7}-p217 and sIA^{g7}-p286 dimers were characterized by an increased frequency of IL-10 secreting T cells and only a marginal (if any) increase in IL-4 and IFNγ-secreting T cells in response to the corresponding recall peptides (Fig. 2.7). Analyses of the frequency and absolute number of FoxP3-expressing CD4^{+}CD25^{+} Treg showed no significant difference in the spleen or pancreatic lymph nodes among the respective groups (data not shown).

The above results indicate that protection induced by the sIA^{g7}-p217 and sIA^{g7}-p286 dimers is mediated by immunoregulatory Tr1-like cells. To determine whether functional immunoregulation was indeed established by the sIA^{g7}-Ig dimers, a set of adoptive transfer experiments were carried out. Twelve week-old NOD female mice were treated with sIA^{g7}-p217 dimer as above, and splenocytes harvested 3 weeks after the last injection. These

splenocytes were then mixed with splenocytes prepared from diabetic NOD donor mice, injected into NOD.*scid* recipients, and diabetes monitored. As expected, transfer of diabetogenic splenocytes alone induced diabetes in all of the recipients (6/6) (Figure 2.8). In contrast, transfer of splenocytes prepared from sIA^{g7}-p217 dimer-treated mice alone failed to induce diabetes in NOD.*scid* recipients (0/6), indicating a lack of pathogenic T effectors (Figure 2.8). Notably, the onset of diabetes was significantly delayed in NOD.*scid* mice receiving the mixture of splenocytes from the two respective groups versus mice injected with diabetogenic splenocytes alone ($p=0.0015$, Kaplan-Meier Log Rank test) (Figure 2.8). These results demonstrate that the protection induced by sIA^{g7}-p217 dimer treatment is mediated by active immunoregulation.

2.4 Discussion

Peptide-based vaccination is one strategy of antigen-specific immunotherapy that is appealing for a number of reasons. For instance, peptides can be readily synthesized in bulk under conditions necessary for clinical application; the immunogenicity of peptides can be manipulated with greater ease than an intact antigen; the therapeutic efficacy of peptides has been demonstrated in experimental models of T1D and other tissue-specific autoimmune diseases; and clinical application of peptides for the treatment of allergies has shown promise (Daniel and Wegmann 1996; Wallner and Gefter 1996; Chao and McDevitt 1997; Tisch, Wang et al. 1999; Haselden, Kay et al. 2000). However, therapeutic efficacy continues to be limited by the short *in vivo* half-life, and the relatively inefficient *in vivo* presentation of exogenous peptides (Ishioka, Adorni et al. 1994). In addition, administration of high doses of soluble self-peptide has been reported to induce anaphylaxis (Liu, Moriyama et al. 2002; Pedotti, Sanna et al. 2003). The current study tested the hypothesis that administration of sIA^{g7}-Ig dimers is an effective approach to suppress β cell autoimmunity at late preclinical stages of T1D. The latter represents a more clinically relevant model since at risk individuals are the primary candidates for immunotherapy, who in turn are defined by ongoing β cell autoimmunity.

A key finding made in this study is that sIA^{g7}-p217 and sIA^{g7}-p286 dimers block the progression of insulitis (Figure 2.6) and prevent the onset of diabetes (Figure 2.5) at a late preclinical stage of T1D. Protection is achieved by two short courses of relatively low doses (e.g. 50 μg) of sIA^{g7}-Ig dimers in the absence of adjuvant. These results are in marked contrast to our earlier work in which a cocktail of p217 and p286 peptides prepared in IFA

and administered multiple times at high doses (e.g. 400 μg) was necessary to prevent diabetes in 12 week-old NOD female mice (Tisch, Wang et al. 1999). On a molar basis ~140-fold less peptide was required to suppress β cell autoimmunity by the sIA^{g7}-p217 and sIA^{g7}-p286 dimers versus the native GAD65-specific peptides. There are at least two mutually nonexclusive explanations for the increased therapeutic efficacy of the sIA^{g7}-Ig dimers versus "native" peptide. First, sIA^{g7}-Ig dimers are more efficient at eliciting a T cell response than the native peptides. For instance, sIA^{g7}-Ig dimers exhibit prolonged *in vivo* persistence (Figure 2.4), and directly stimulate T cells (Figure 2.3) therefore by-passing the need for APC to bind and present peptide. Second, sIA^{g7}-Ig dimers induce a more effective subset of immunoregulatory T cells. Treatment with p217 and p286 elicits a response dominated by IL-4 secreting Th2 cells (Tisch, Wang et al. 1999), whereas sIA^{g7}-p217 and sIA^{g7}-p286 dimers induce IL-10-secreting Tr1-like cells (Figure 2.7). The latter finding is consistent with work by Casares and colleagues who demonstrated in a TCR Tg model of T1D, that administration of sIE^{d}-Ig dimers tethered to an *influenza* hemagglutinin (HA) peptide blocked the diabetogenicity of HA-specific CD4^{+} T effectors via induction of HA-specific Tr1 cells (Casares, Hurtado et al. 2002). Tr1 cells are a particularly potent subset of immunoregulatory T cells that regulate the responses of naïve and memory T cells *in vitro* and *in vivo*, and suppress both Th1 and Th2 cell–mediated pathologies through bystander suppression mediated by local release of IL-10 (Groux, O'Garra et al. 1997; Roncarolo, Bacchetta et al. 2001). IL-10 not only affects T cells directly, but also indirectly by blocking the activation and function of APC such as dendritic cells (DC) (McGuirk, McCann et al. 2002). IL-10 treated DC gain a "tolergenic" phenotype and preferentially promote the development of immunoregulatory T cells (Wakkach, Fournier et al. 2003). The potency of

Tr1 cells may also explain why individually, sIA^{g7}-p217 and sIA^{g7}-p286 dimers prevent diabetes, whereas both p217 and p286 are required. For instance, a high frequency of IL-4 secreting Th2 cells (e.g. p217- plus p286-specific clonotypes) may be necessary to compensate for a reduced immunoregulatory function compared to Tr1 cells.

Another important observation made in this study is that sIA^{g7}-Ig dimer mediated protection is epitope-specific. Whereas sIA^{g7}-p217 and sIA^{g7}-p286 dimers suppressed β cell autoimmunity, sIA^{g7}-mBDC dimer failed to prevent diabetes (Figure 2.5). Interestingly, sIA^{g7}-mBDC dimer treatment induced "mixed" T cell reactivity in response to mBDC. Namely, a robust Th1 cell response and moderate but significant Th2 cell reactivity were detected in sIA^{g7}-mBDC dimer treated NOD mice (Figure 2.7). The lack of IL-10-secreting Tr1 cells in NOD mice receiving sIA^{g7}-mBDC dimer further argues that this subset of immunoregulatory T cells is necessary for the suppression of β cell autoimmunity. Currently, it is unclear why sIA^{g7}-mBDC dimer induced IFNγ and IL-4-secreting T cells, but not IL-10 producers. Significant induction of IFNγ-secreting Th1 cells may reflect expansion of existing effector and/or memory T cells or differentiation of naïve mBDC-specific T precursors into Th1 effectors. The TCR affinity/avidity of naïve mBDC-specific T precursors may also influence signaling events elicited by sIA^{g7}-mBDC dimer binding that promote (albeit weakly) differentiation of IL-4 versus IL-10-secreting T effectors. A better understanding of the biochemical and transcriptional signaling events transduced in CD4^{+} T cells by sIA^{g7}-Ig dimers would greatly aid in the interpretation of these results. Nevertheless, these findings underscore the need for selecting the "appropriate" self-antigen/peptide in order to suppress ongoing autoimmunity.

In summary, our data demonstrate that the efficacy of peptide-based immunotherapy to suppress ongoing β cell autoimmunity can be significantly enhanced through the use of sIA^{g7}-Ig dimers.

2.5 Materials and Methods

Mice

NOD/LtJ, NOD.BDC2.5 and NOD.CB17.Prkdc^{scid}/J (NOD.*scid*) mice were maintained and bred under specific-pathogen free conditions. Mice were diagnosed as diabetic when having blood glucose levels $\geq$250 mg/dl on two successive measurements as determined by an Autokit Glucose CII assay (WAKO). Animals were maintained at an American Association of Laboratory Animal Care-accredited animal facility. All procedures were reviewed and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Expression and purification of sIA^{g7}-Ig fusion proteins

The sIAg7-Ig dimers were engineered as previously described (Malherbe, Filippi et al. 2000). Briefly, IA^{g7} α and β chain extracellular domains were attached to fos and jun leucine zippers, respectively. Peptide epitopes were covalently linked to the NH_2-terminus of the IA$^{g7}\beta$ chain by a flexible thrombin-GGGGS linker. The dimerized IA^{g7} molecule replaces the original Ig heavy and light chains by adjoining to the Fc hinge via the leucine zipper. cDNAs encoding the respective sIA^{g7}-Ig recombinants were subcloned into the pMT-Bip vector and transgene expression driven by a metallothionein-inducible promoter. Expression vectors were co-transfected via calcium phosphate into *Drosophila* S2 cells with a vector containing the selectable marker pHygro, and stable transfectants selected in hygromycin containing medium. Expression of the sIA^{g7}-Ig dimers was confirmed using ELISA with antibody pairs specific for mouse IgG2a Fc (BD PharMingen, San Diego, CA) or a combination with the conformation-specific anti-IA^{g7} antibody, 10-2.16. sIA^{g7}-Ig dimer protein expression was induced by 500 μM $CuSO_4$ for 7-10 days and purified by affinity chromatography on a

Protein A column (Gebioscience). Bound molecules were eluted using 0.1 M Glycine-HCl (pH 2.5). Fractions were neutralized immediately with the addition of 1 M Tris (pH 9.0), dialyzed against PBS, and concentrated using Centricon concentrators (Millipore, Bedford, MA). Protein concentration was determined by a bicinchoninic acid assay (Pierce, Rockford, IL).

Flow Cytometry

To generate multimeric staining reagents, sIA^{g7}-Ig dimers were incubated with Alexa 647-coupled Protein A (Molecular Probes, Invitrogene, OR) as described (Malherbe, Filippi et al. 2000). Samples were also co-stained with anti-CD3 FITC, -CD19 PE, -CD4 PerCP, -CD11C Pacific blue, and -F4/80 PEcy7. Data was acquired on a Cyan flow cytometer (DakoCytomation), and analyzed using Summit software (DakoCytomation).

ELISPOT

ELISPOT plates (Millipore) were coated overnight at 4°C with purified rat anti-mouse cytokine antibodies in PBS (anti-IFN-γ, anti-IL-4, or anti-IL-10) (BD Pharmingen). Plates were seeded with splenocytes at 1 x 10^6 cells per well in HL-1 medium (BioWhittaker). Peptides were added at a final concentration of 20 μg/ml. Cultures were incubated for 48 hours at 37°C. Cells were removed by washing, and plates incubated with the appropriate biotinylated anti-mouse cytokine antibodies overnight at 4°C. Plates were then washed, incubated with streptavidin-HRP (BD Pharmingen) for 2 hours at room temperature, and developed using a 100-mM sodium acetate buffer containing 0.3 mg/ml 3-amino-9-

ethylcarbazole (Sigma-Aldrich) and 0.015% hydrogen peroxide. An ImmunoSpot plate reader (Cellular Technology) was used to count the spot-forming cells per well.

T cell proliferation assay

Proliferation assays were performed as previously described (Casares, Zong et al. 1999). Briefly, proliferation of $CD4^+$ T cells was assayed by incubating 2 x 10^5 cells with peptide at 30 μg/ml and irradiated (3000 rad) syngeneic splenocytes for 48 hours. One μCi per well of [^{3}H] thymidine was added during the last 16 hours of culture. Cells were then harvested on a Trilux 1450 Microbeta Wallac Harvester, and incorporated [^{3}H] thymidine was counted using the Microbeta 270.004 software (Wallac).

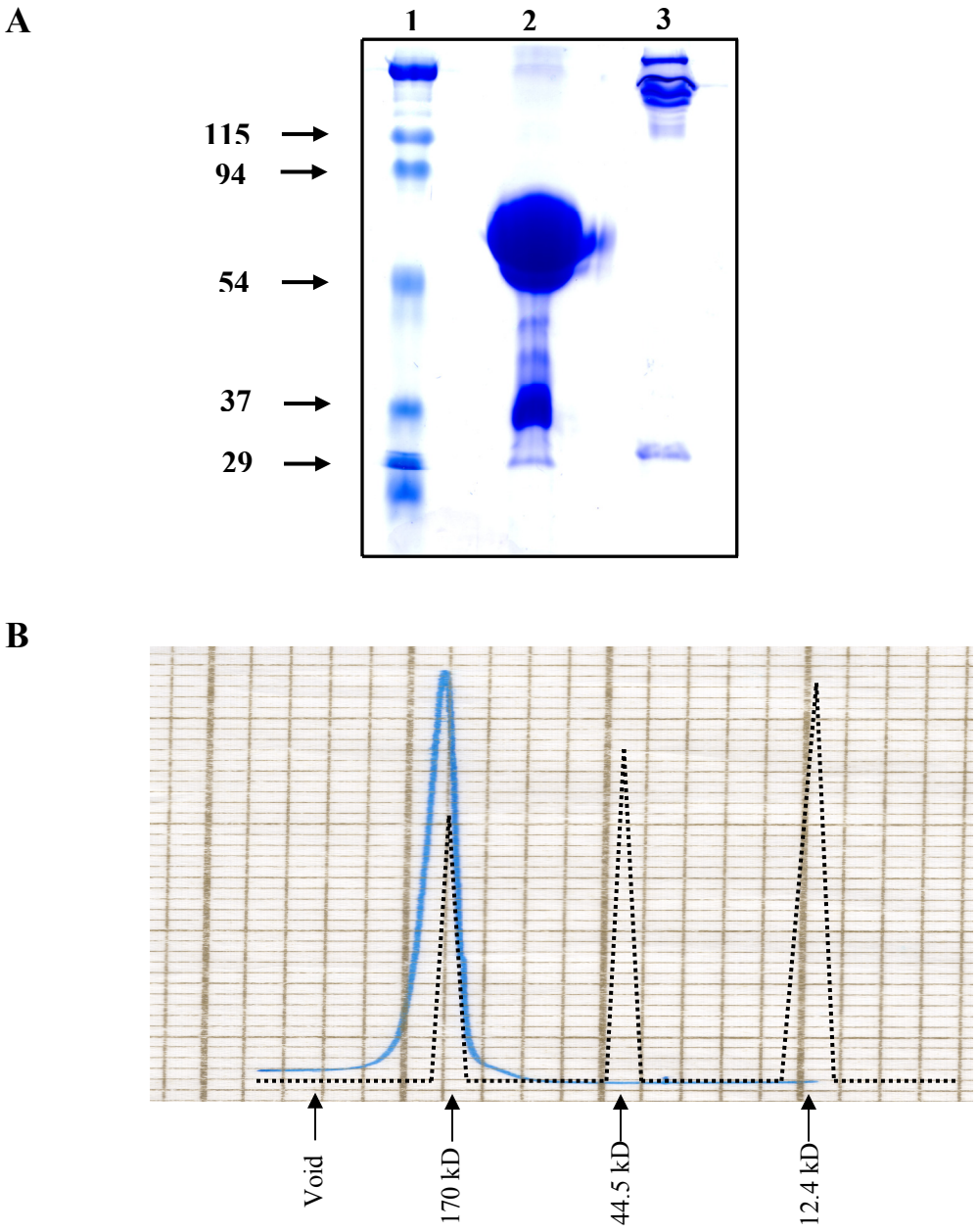
T cell adoptive transfers

Splenocytes prepared from diabetic (10 x 10^6) or sIA^{g7}-Ig dimer treated (10 x 10^6) NOD female mice were injected intraperitoneally either alone or mixed together into 5-8 week old NOD.*scid* mice. Recipient mice were monitored for diabetes incidence. Two consecutive readings of blood glucose levels of ≥250 mg/dl were indicative of diabetes.

Histopathology

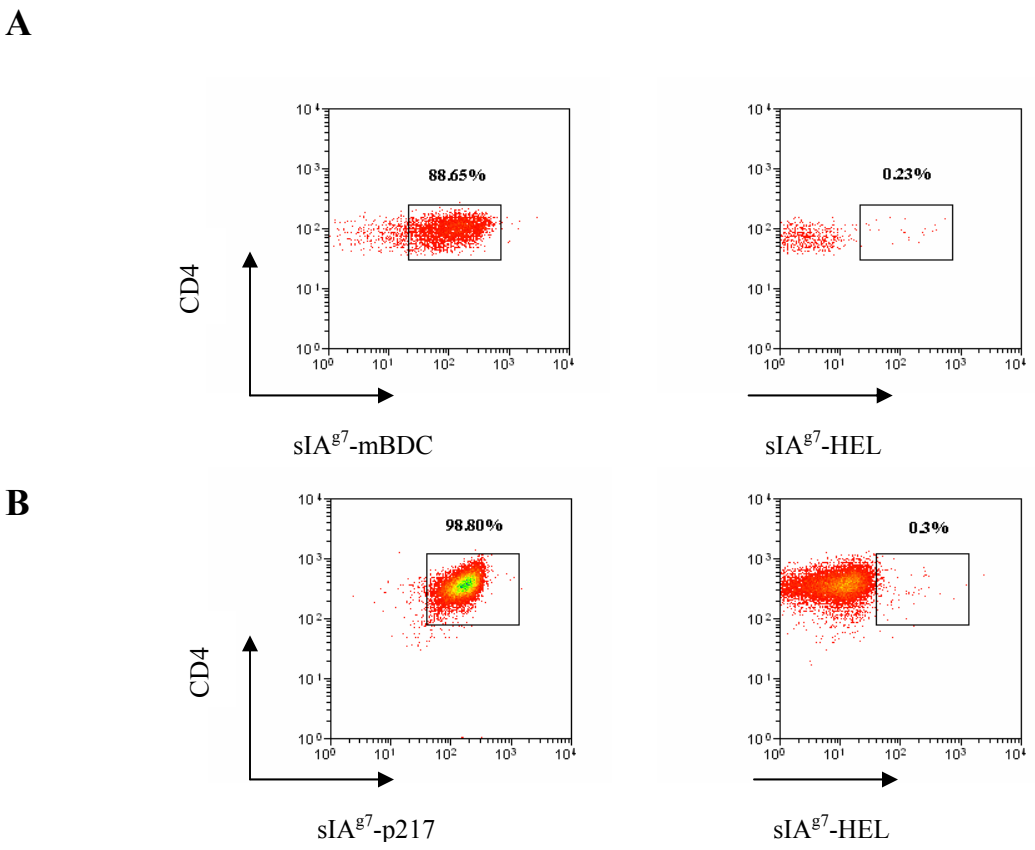
Pancreases were harvested from mice, and fixed with 10% formalin. Serial cross-sections (5 μm) were cut and stained with hematoxylin and eosin (H&E).

Figure 2.1



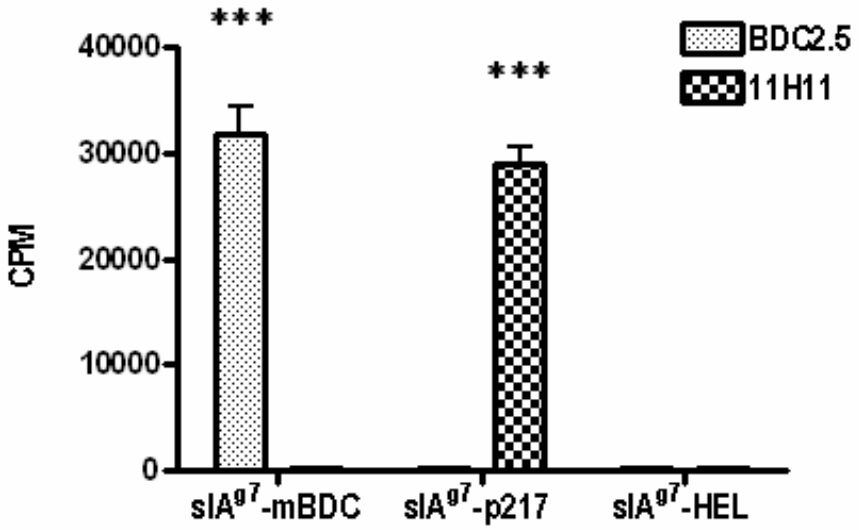
Characterization of sIA^{g7}-Ig dimers. A, SDS-PAGE analysis of sIA^{g7}-p217 dimers using a 10% polyacrylamide gel. Lane 1, protein standard; lane 2, reducing conditions plus boiling; lane 3, nonreducing conditions plus boiling. B, Size exclusion chromatography of affinity purified sIA^{g7}-p217 dimer.

Figure 2.2



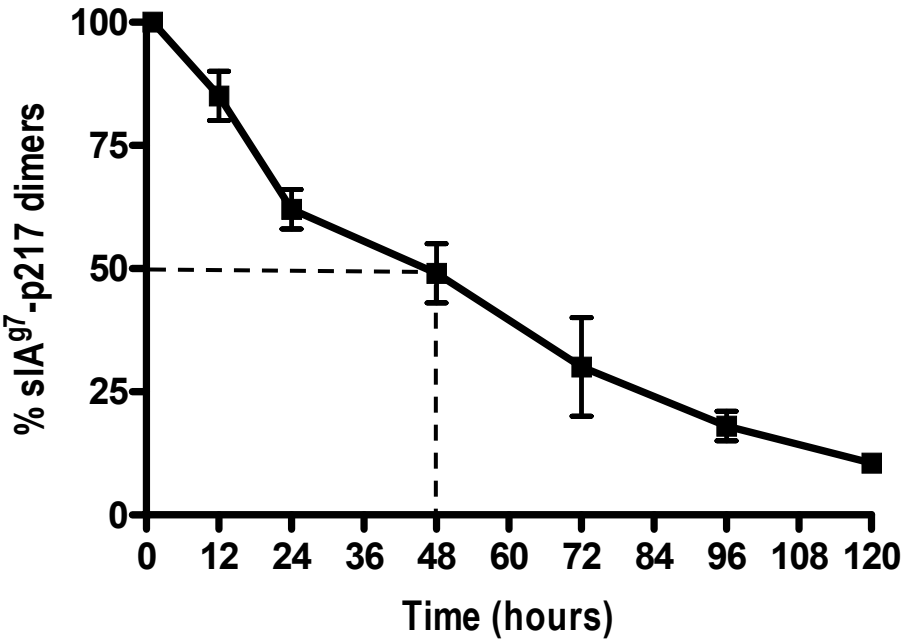
T cell binding by sIA^{g7}-Ig multimers is peptide specific. A, PBL from BDC2.5 Tg NOD mice or B, the p217-sepcific T cell clone (11H11) were co-stained with sIA^{g7}-Ig multimers and anti-CD3 and -CD4 antibodies.

Figure 2.3



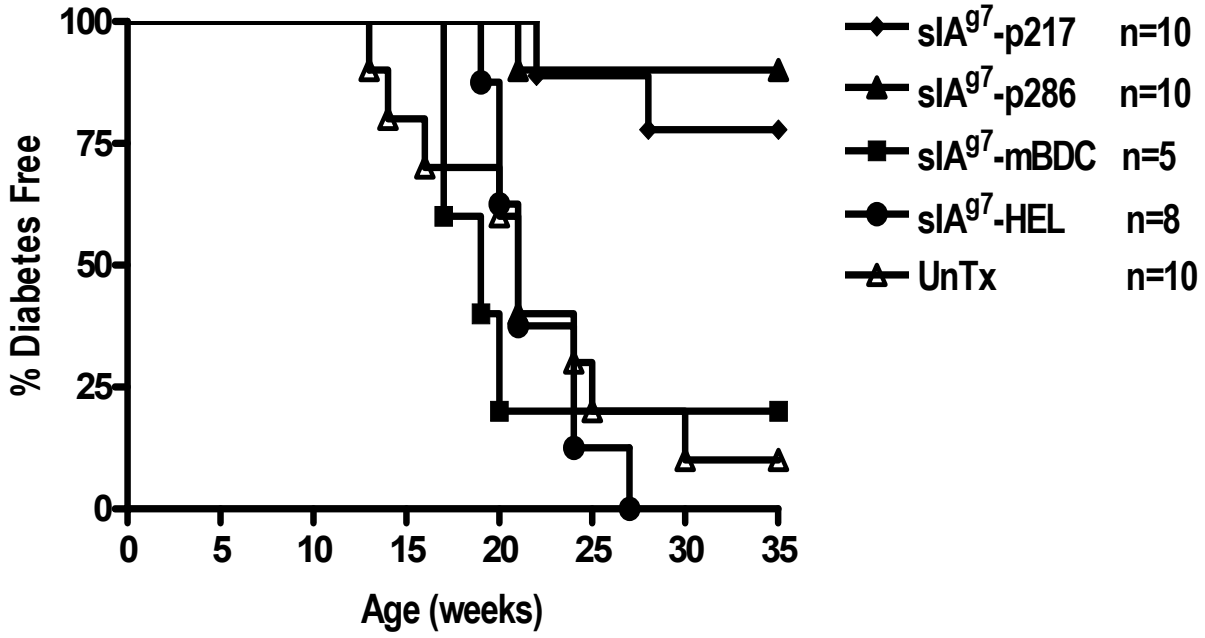
sIA^{g7}-Ig dimers induce T cell proliferation in a peptide-specific manner. BDC2.5 CD4^{+} T cells and the p217-specific T cell clone 11H11 were stimulated with plate-bound sIA^{g7}-BDC, sIA^{g7}-p217, or sIA^{g7}-HEL dimers and proliferation determined by the incorporation of [^{3}H] thymidine. *** $p<0.001$, Student's t test.

Figure 2.4



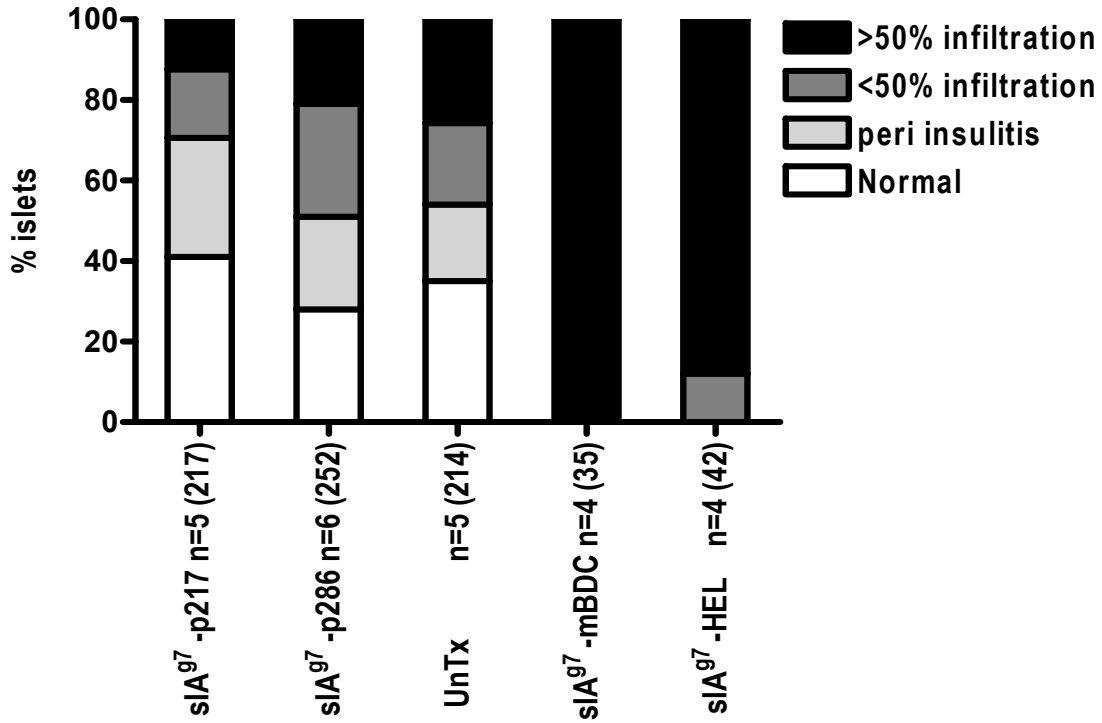
***In vivo* detection of sIA^{g7}-Ig dimers**. Two NOD.*scid* mice received an i.v. injection of 50 µg of sIA^{g7}-p217 dimers. Serum was harvested at different times post-injection and sIA^{g7}-p217 dimers detected using an anti-mouse IgG2a ELISA in triplicate. The amount of IgG2a detected at 1 hour post-injection is regarded as 100%. Data are representative of two separate experiments.

Figure 2.5



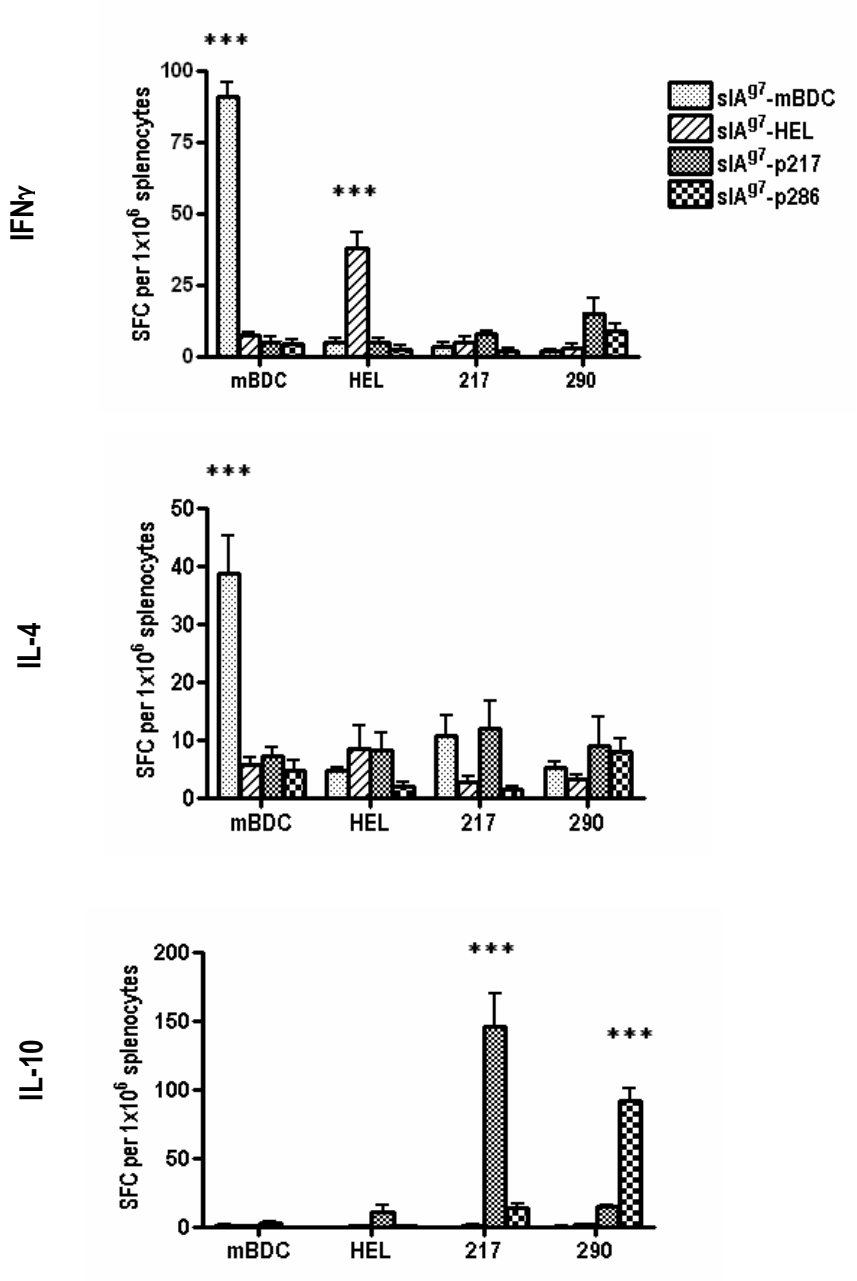
sIA^{g7}-p217 and sIA^{g7}-p286 but not sIA^{g7}-mBDC dimers prevent diabetes in NOD female mice. 12-week-old NOD female mice were injected i.v. with 50 μg of sIA^{g7}-p217, sIA^{g7}-p286, sIA^{g7}-mBDC, or sIA^{g7}-HEL for 3 consecutive days. Three weeks later, mice were similarly treated and diabetes incidence monitored. Mice were considered diabetic after 2 consecutive readings of $\geq$250 mg/dL. $p \leq 0.0003$, untreated or sIA^{g7}-HEL versus sIA^{g7}-p217; $p \leq 0.0001$, untreated or sIA^{g7}-HEL versus sIA^{g7}-p286, Kaplan-Meier Log Rank Test.

Figure 2.6



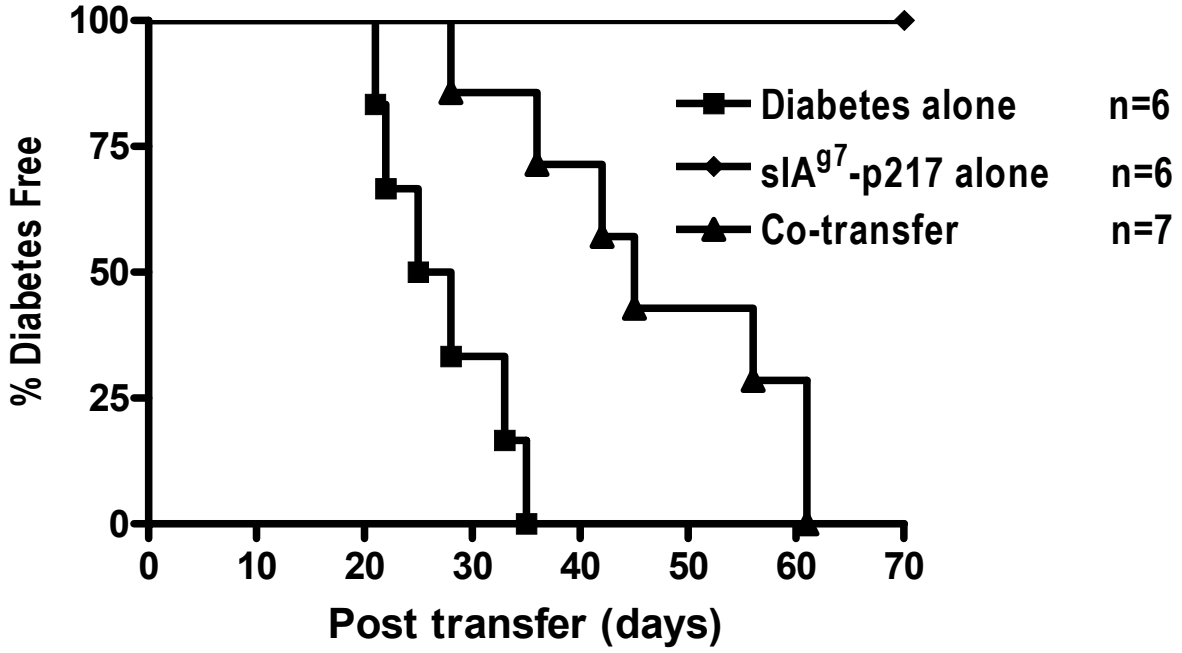
Treatment of sIA^{g7}-p217 or sIA^{g7}-p286 dimers blocks the progression of insulitis. The frequency of insulitis as determined by H&E staining was assessed for sIA^{g7}-p217 and sIA^{g7}-p286 treated nondiabetic NOD female mice 35 weeks of age, sIA^{g7}-BDC and sIA^{g7}-HEL treated diabetic NOD female mice, and unmanipulated 12-week-old NOD female mice. N=number of mice; total number of islets is indicated in parentheses. $p \leq 0.0011$, sIA^{g7}-mBDC or sIA^{g7}-HEL versus sIA^{g7}-p217 (>50% infiltration); $p \leq 0.002$, sIA^{g7}-mBDC or sIA^{g7}-HEL versus sIA^{g7}-p286 (>50% infiltration), Student's t test.

Figure 2.7



Protection mediated by sIA^{g7}-p217 and sIA^{g7}-p286 dimers correlates with the induction of peptide-specific immunoregulatory Tr1 cells. Splenocytes harvested from sIA^{g7}-p217, sIA^{g7}-p286, sIA^{g7}-mBDC, and sIA^{g7}-HEL dimer treated mice were examined via ELISPOT to determine the frequency of peptide-specific T cells secreting IFNγ, IL-4, and IL-10. Medium-only values were subtracted. *** $p< 0.001$, Student's t test.

Figure 2.8



sIA^{g7}-p217 dimer treatment induces immunoregulatory T cells. 10 x 10^6 splenocytes from diabetic or sIA^{g7}-p217 dimer treated NOD mice were transferred alone or co-transferred i.p. into 5-8 week old NOD.*scid* mice. The recipient mice were then followed for diabetes incidence. Two consecutive readings of blood glucose levels of ≥250 mg/dl was indicative of diabetes. p = 0.0005, diabetic alone versus sIA^{g7}-p217 alone; p =0.0015, diabetic alone versus cotransfer, Kaplan-Meier Log Rank Test.

2.6 References

Anderson, M. S. and J. A. Bluestone (2005). "The NOD mouse: a model of immune dysregulation." Annu Rev Immunol **23**: 447-85.

Appel, H., L. Gauthier, et al. (2000). "Kinetics of T-cell receptor binding by bivalent HLA-DR.Peptide complexes that activate antigen-specific human T-cells." J Biol Chem **275**(1): 312-21.

Appel, H., N. P. Seth, et al. (2001). "Anergy induction by dimeric TCR ligands." J Immunol **166**(8): 5279-85.

Babbitt, B. P., G. Matsueda, et al. (1986). "Antigenic competition at the level of peptide-Ia binding." Proc Natl Acad Sci U S A **83**(12): 4509-13.

Bach, J. F. (2001). "Immunotherapy of insulin-dependent diabetes mellitus." Curr Opin Immunol **13**(5): 601-5.

Balasa, B., T. Krahl, et al. (1997). "CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice." J Immunol **159**(9): 4620-7.

Bergman, B. and K. Haskins (1994). "Islet-specific T-cell clones from the NOD mouse respond to beta-granule antigen." Diabetes **43**(2): 197-203.

Cameron, M. J., G. A. Arreaza, et al. (1997). "IL-4 prevents insulitis and insulin-dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function." J Immunol **159**(10): 4686-92.

Casares, S., C. A. Bona, et al. (1997). "Engineering and characterization of a murine MHC class II-immunoglobulin chimera expressing an immunodominant CD4 T viral epitope." Protein Eng **10**(11): 1295-301.

Casares, S., A. Hurtado, et al. (2002). "Down-regulation of diabetogenic CD4+ T cells by a soluble dimeric peptide-MHC class II chimera." Nat Immunol **3**(4): 383-91.

Casares, S., C. S. Zong, et al. (1999). "Antigen-specific signaling by a soluble, dimeric peptide/major histocompatibility complex class II/Fc chimera leading to T helper cell type 2 differentiation." J Exp Med **190**(4): 543-53.

Chao, C. C. and H. O. McDevitt (1997). "Identification of immunogenic epitopes of GAD 65 presented by Ag7 in non-obese diabetic mice." Immunogenetics **46**(1): 29-34.

Chatenoud, L. (2005). "CD3-specific antibodies restore self-tolerance: mechanisms and clinical applications." Curr Opin Immunol **17**(6): 632-7.

Cochran, J. R., T. O. Cameron, et al. (2000). "The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers." Immunity **12**(3): 241-50.

Daniel, D. and D. R. Wegmann (1996). "Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23)." Proc Natl Acad Sci U S A **93**(2): 956-60.

Eisenbarth, G. S., H. Moriyama, et al. (2002). "Insulin autoimmunity: prediction/precipitation/prevention type 1A diabetes." Autoimmun Rev **1**(3): 139-45.

Groux, H., A. O'Garra, et al. (1997). "A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis." Nature **389**(6652): 737-42.

Hamad, A. R., S. M. O'Herrin, et al. (1998). "Potent T cell activation with dimeric peptide-major histocompatibility complex class II ligand: the role of CD4 coreceptor." J Exp Med **188**(9): 1633-40.

Haselden, B. M., A. B. Kay, et al. (2000). "Peptide-mediated immune responses in specific immunotherapy." Int Arch Allergy Immunol **122**(4): 229-37.

Hawa, M., R. Rowe, et al. (1997). "Value of antibodies to islet protein tyrosine phosphatase-like molecule in predicting type 1 diabetes." Diabetes **46**(8): 1270-5.

Ishioka, G. Y., L. Adorini, et al. (1994). "Failure to demonstrate long-lived MHC saturation both in vitro and in vivo. Implications for therapeutic potential of MHC-blocking peptides." J Immunol **152**(9): 4310-9.

Katz, J. D., B. Wang, et al. (1993). "Following a diabetogenic T cell from genesis through pathogenesis." Cell **74**(6): 1089-100.

Kurrer, M. O., S. V. Pakala, et al. (1997). "Beta cell apoptosis in T cell-mediated autoimmune diabetes." Proc Natl Acad Sci U S A **94**(1): 213-8.

Kuttler, B., K. Rosing, et al. (1999). "Prevention of autoimmune but not allogeneic destruction of grafted islets by different therapeutic strategies." J Mol Med **77**(1): 226-9.

Lenschow, D. J., S. C. Ho, et al. (1995). "Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse." J Exp Med **181**(3): 1145-55.

Liu, E., H. Moriyama, et al. (2002). "Anti-peptide autoantibodies and fatal anaphylaxis in NOD mice in response to insulin self-peptides B:9-23 and B:13-23." J Clin Invest **110**(7): 1021-7.

Lund, J., G. Winter, et al. (1991). "Human Fc gamma RI and Fc gamma RII interact with distinct but overlapping sites on human IgG." J Immunol **147**(8): 2657-62.

Malherbe, L., C. Filippi, et al. (2000). "Selective activation and expansion of high-affinity CD4+ T cells in resistant mice upon infection with Leishmania major." Immunity **13**(6): 771-82.

Maron, R., N. S. Melican, et al. (1999). "Regulatory Th2-type T cell lines against insulin and GAD peptides derived from orally- and nasally-treated NOD mice suppress diabetes." J Autoimmun **12**(4): 251-8.

McGuirk, P., C. McCann, et al. (2002). "Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis." J Exp Med **195**(2): 221-31.

Muller, S., L. Adorini, et al. (1990). "Selective in vivo inhibition of T cell activation by class II MHC-binding peptides administered in soluble form." J Immunol **145**(12): 4006-11.

Nicoletti, F., P. Zaccone, et al. (1999). "Early prophylaxis with recombinant human interleukin-11 prevents spontaneous diabetes in NOD mice." Diabetes **48**(12): 2333-9.

Nitta, Y., F. Tashiro, et al. (1998). "Systemic delivery of interleukin 10 by intramuscular injection of expression plasmid DNA prevents autoimmune diabetes in nonobese diabetic mice." Hum Gene Ther **9**(12): 1701-7.

Pedotti, R., M. Sanna, et al. (2003). "Severe anaphylactic reactions to glutamic acid decarboxylase (GAD) self peptides in NOD mice that spontaneously develop autoimmune type 1 diabetes mellitus." BMC Immunol **4**: 2.

Piccirillo, C. A., Y. Chang, et al. (1998). "TGF-beta1 somatic gene therapy prevents autoimmune disease in nonobese diabetic mice." J Immunol **161**(8): 3950-6.

Roncarolo, M. G., R. Bacchetta, et al. (2001). "Type 1 T regulatory cells." Immunol Rev **182**: 68-79.

Shizuru, J. A., C. Taylor-Edwards, et al. (1988). "Immunotherapy of the nonobese diabetic mouse: treatment with an antibody to T-helper lymphocytes." Science **240**(4852): 659-62.

Tian, J., M. A. Atkinson, et al. (1996). "Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes." J Exp Med **183**(4): 1561-7.

Tian, J., M. Clare-Salzler, et al. (1996). "Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice." Nat Med **2**(12): 1348-53.

Tisch, R. and H. McDevitt (1996). "Insulin-dependent diabetes mellitus." Cell **85**(3): 291-7.

Tisch, R., B. Wang, et al. (1999). "Induction of glutamic acid decarboxylase 65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope dependent." J Immunol **163**(3): 1178-87.

Tisch, R., X. D. Yang, et al. (1993). "Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice." Nature **366**(6450): 72-5.

Verge, C. F., R. Gianani, et al. (1996). "Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies." Diabetes **45**(7): 926-33.

Wakkach, A., N. Fournier, et al. (2003). "Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo." Immunity **18**(5): 605-17.

Wallner, B. P. and M. L. Gefter (1996). "Peptide therapy for treatment of allergic diseases." Clin Immunol Immunopathol **80**(2): 105-9.

Wang, B., A. Gonzalez, et al. (1996). "The role of CD8+ T cells in the initiation of insulin-dependent diabetes mellitus." Eur J Immunol **26**(8): 1762-9.

Wegmann, D. R., M. Norbury-Glaser, et al. (1994). "Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice." Eur J Immunol **24**(8): 1853-7.

Wines, B. D., M. S. Powell, et al. (2000). "The IgG Fc contains distinct Fc receptor (FcR) binding sites: the leukocyte receptors Fc gamma RI and Fc gamma RIIa bind to a region in the Fc distinct from that recognized by neonatal FcR and protein A." J Immunol **164**(10): 5313-8.

Yang, X. D., R. Tisch, et al. (1994). "Effect of tumor necrosis factor alpha on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process." J Exp Med **180**(3): 995-1004.

Yoshida, K., T. Martin, et al. (2002). "Evidence for shared recognition of a peptide ligand by a diverse panel of non-obese diabetic mice-derived, islet-specific, diabetogenic T cell clones." Int Immunol **14**(12): 1439-47.

Zaccone, P., J. Phillips, et al. (1999). "Interleukin-13 prevents autoimmune diabetes in NOD mice." Diabetes **48**(8): 1522-8.

Zechel, M. A., J. F. Elliott, et al. (1998). "Characterization of novel T-cell epitopes on 65 kDa and 67 kDa glutamic acid decarboxylase relevant in autoimmune responses in NOD mice." J Autoimmun **11**(1): 83-95.

Zuo, L., C. M. Cullen, et al. (2002). "A single-chain class II MHC-IgG3 fusion protein inhibits autoimmune arthritis by induction of antigen-specific hyporesponsiveness." J Immunol **168**(5): 2554-9.

CHAPTER 3

SELECTIVE EXPANSION OF β CELL-SPECIFIC T CELL RECEPTORS IN AUTOIMMUNE DIABETES

Li Li, Qiuming He, Rui Zhang, Bo Wang, Roland Tisch

Department of Microbiology and Immunology

School of Medicine

University of North Carolina, Chapel Hill

Chapel Hill, North Carolina

3.1 Abstract

It is well established that T cells are the primary mediators of β cell destruction in Type 1 diabetes (T1D). However, the relative role for different β cell-specific T cell clonotypes in the progression of the diabetogenic response, and the molecular basis for their expansion remain poorly understood. Accordingly, the current study exploits soluble (s) IA^{g7}-immunoglobulin (Ig) multimer technology to analyze BDC2.5 clonotypic $CD4^+$ T cells at the single cell level. $CD4^+$ T cells expressing the BDC2.5 clonotypic T cell receptor (TCR) and/or which recognize the mimetic BDC (mBDC) peptide are thought to have a key albeit undefined role in β cell autoimmunity of the nonobese diabetic (NOD) mouse. Using sIA^{g7}-mBDC multimers, BDC2.5 clonotypic $CD4^+$ T cells were tracked and/or isolated in a temporal manner. sIA^{g7}-mBDC-binding (g7-$mBDC^+$) $CD4^+$ T cells were detected in peripheral blood lymphocytes (PBL) and the islets at the onset of β cell autoimmunity in NOD female mice, with the frequency and/or number of g7-$mBDC^+$ T cells increasing with age. In contrast, a reduced frequency and number of g7-$mBDC^+$ T cells was observed in the PBL and islets of NOD male mice. A comparison of TCR gene usage by single, g7-$mBDC^+$ T cells isolated from 16 week-old NOD female mice demonstrated dominate usage of TRBV15 and monoclonality based on variable β (Vβ) gene complementary determinant region 3 (CDR3) sequences of BDC2.5 clonotypic $CD4^+$ T cells in the islets but not PBL. These data demonstrate that g7-$mBDC^+$ T cells are an early indicator of the development of destructive insulitis, and that both clonotypic expansion and preferential usage of TCR characterize islet infiltrating g7-$mBDC^+$ T cells.

3.2 Introduction

T1D is marked by the breakdown of self-tolerance to β cells within the T cell compartment (Tisch and McDevitt 1996; Haskins 2005). Defects in thymic selection coupled with aberrant peripheral immunoregulation are believed to contribute to the development and expansion of pathogenic, β cell-specific $CD4^+$ and $CD8^+$ T cells (Carrasco-Marin, Shimizu et al. 1996; Ridgway, Fasso et al. 1999). Nevertheless, the relative role of different β cell-specific clonotypes in the disease process, and the molecular basis underlying recognition of β cell autoantigens and subsequent expansion of pathogenic T effectors remain ill-defined. This is partly due to only partial knowledge of the identity of the key β cell autoantigens targeted in the diabetogenic response, and the low frequency of the corresponding T cell clonotypes *in vivo* thereby making analyses difficult. Groups have attempted to address some of these issues by establishing T cell clones from the islets of NOD mice, and then defining peptide specificity and TCR gene usage of these clones (Haskins, Portas et al. 1988; Katz, Wang et al. 1993; Gelber, Paborsky et al. 1994; Santamaria, Utsugi et al. 1995; Verdaguer, Yoon et al. 1996; Wong, Visintin et al. 1996; Quinn, McInerney et al. 2001). However, this approach is limited since *in vitro* expansion may promote out-growth of minor T cell clonotypes. NOD mice transgenic (Tg) for TCR of known β cell autoantigen specificities have proven to be valuable in studying the role of particular clonotypes in T1D (Katz, Wang et al. 1993; Verdaguer, Yoon et al. 1996; Jasinski, Yu et al. 2006). However, this approach is limited by the homogeneity of the peripheral T cell repertoire established in Tg mice. For instance, spontaneous β cell autoimmunity is driven by a large repertoire of T effectors in which clonotypes influence one another, as in the case of "epitope spread" (Zechel, Krawetz et al. 1998; Tian, Gregori et al. 2001). Targeting of certain epitopes leads to recognition of new

peptides, and subsequent "spreading" or recruitment of additional T cell clonotypes that amplify the autoimmune response (Kelemen, Wegmann et al. 2001; Olcott, Tian et al. 2005).

To track and define the clonotypic properties of β cell-specific $CD4^+$ T cells, we have in the current study exploited the use of sIA^{g7}-Ig multimer technology described in Chapter 2. Specifically, BDC2.5 clonotypic $CD4^+$ T cells recognizing mBDC have been analyzed in a temporal manner using sIA^{g7}-mBDC multimers. The BDC2.5 clonotype is defined by the BDC2.5 $CD4^+$ T cell clone, which was originally established from the spleen of diabetic NOD mice by Haskins and colleagues (Haskins, Portas et al. 1988). The BDC2.5 T cell clone is diabetogenic upon adoptive transfer into young NOD or NOD.*scid* recipients (Haskins and McDuffie 1990; Peterson, Pike et al. 1995; Peterson and Haskins 1996). In addition, NOD.*scid* mice transgenic for the BDC2.5 TCR α (Vα1)- and β (Vβ4) chains rapidly develop diabetes, further demonstrating the diabetogenic capacity and disease relevance of this T cell clonotype (Kurrer, Pakala et al. 1997). The β cell autoantigen recognized by BDC2.5 $CD4^+$ T cells has yet to be determined. However, the mimetic epitope mBDC has recently been defined thereby providing an important reagent to track and identify "BDC2.5-like" clonotypes *in vivo* (Yoshida, Martin et al. 2002).

In the current study we test the general hypothesis that diabetogenic $CD4^+$ T clonotypes are defined by common TCRs, and are selectively expanded in the islets. Specifically, three important questions will be addressed: 1) How does the temporal development of g7-$mBDC^+$ T cells correspond with the progression of β cell autoimmunity? 2) How does the repertoire

of g7-mBDC^{+} T cells compare between different tissues? 3) Is there preferential TCR usage by g7-mBDC^{+} T cells that in turn defines the diabetogenicity of this general clonotype?

3.3 Results

Detection of mBDC-specific $CD4^+$ T cells in PBL and the endogenous islets

To determine the association between BDC2.5 clonotypic $CD4^+$ T cells and the progression of β cell autoimmunity, the frequency of g7-$mBDC^+$ T cells was examined in PBL and the islets of NOD female and male mice of varying ages. Notably, the frequency of insulitis between NOD females and males is similar with age; however, only 20% of males develop diabetes compared to the 80% of female mice that become diabetic. To enhance the specificity of the analysis of $CD4^+$ T cells binding sIA^{g7}-mBDC, cells were also stained with antibodies specific for CD19, CD11c, and F4/80 in order to gate out B cells, dendritic cells and macrophages, respectively. Background levels of sIA^{g7}-Ig binding was typically 0.2-0.4% of $CD4^+$ T cells based on staining with a sIA^{g7}-Ig recombinant tethered to a hen egg lysozyme (HEL) epitope (sIA^{g7}-HEL) (Figure 3.1A).

Temporal analysis of purified islets in NOD female mice demonstrated that at 4 wks of age, the time at which insulitis is initiated, ~2.1% of $CD4^+$ T cells bound sIA^{g7}-mBDC (Figure 3.1B). By 6 weeks of age, the frequency of g7-$mBDC^+$ T cells was decreased by 2-fold and remained unchanged up to 24 weeks of age (Figure 3.1B). However, the absolute number of g7-$mBDC^+$ T cells was gradually increased with age (Figure 3.1C). In the islets of 16 and 24 week-old NOD male mice, a reduced frequency and a ~4-fold reduction in the number of g7-$mBDC^+$ T cells was observed relative to age matched NOD females (Figure 3.1B).

In the PBL of 4 week-old NOD female mice, ~0.35% of $CD4^+$ T cells bound sIA^{g7}-mBDC (Figure 3.1D). This frequency and the absolute number of g7-$mBDC^+$ T cells increased by 8 weeks of age and was maintained up to 24 weeks of age. In contrast, the frequency and number of g7-$mBDC^+$ T cells in the PBL of NOD male mice was markedly reduced compared to NOD female mice, especially in older animals (Figure 3.1 B and D). A profile of g7-$mBDC^+$ T cells similar to NOD males was detected in PBL from diabetes-resistant NOR female mice that develop only peri-insulitis (data not shown). Together, these findings demonstrate that g7-$mBDC^+$ T cells are detected in PBL and islets early in the disease process, and that the frequency and number of sIA^{g7}-mBDC binding $CD4^+$ T cells increases with age in NOD female mice.

The TCR Vβ repertoire of mBDC2.5-specific $CD4^+$ T cells is skewed in the islets

Next, we set out to gain insight into the TCR usage of mBDC-specific $CD4^+$ T cells, and in turn determine how the islets shape the repertoire of BDC2.5 clonotypic T cells. Accordingly, sIA^{g7}-mBDC binding $CD4^+$ T cell were sorted from the blood and islets of 10 individual 16 week-old NOD female mice, and TCR Vβ gene usage assessed in single cells via RT-PCR. The repertoire of the 197 g7-$mBDC^+$ T cells isolated from PBL was relatively diverse being distributed primarily among four TCR Vβ genes; namely TRBV13.2 (Vβ8.2; 18.8%) TRBV2 (Vβ4; 9.6%), TRBV15 (Vβ12; 10.7%), and TRBV5 (Vβ2; 9.1%) (Figure 3.2). In marked contrast, significant skewing of Vβ gene usage was detected among the g7-$mBDC^+$ T cells infiltrating the islets. Analysis of the 218 islet g7-$mBDC^+$ T cells showed that 62.4% expressed TCR β chains bearing TRBV15 (Figure 3.2A). In fact, ≥80% of islet g7-$mBDC^+$ T cells expressed TRBV15 in mouse #1 (19/22), #2 (12/15), #3 (47/51), #7 (38/43) and #10 (21/26) (Figure 3.2B). Even in mouse #4 (7/20), #5 (5/21), #6 (12/21) and #9 (6/20),

TRBV15 predominated among the other Vβ genes (Figure 3.2B). Furthermore, whereas 18.8% of PBL g7-mBDC$^+$ T cells expressed TRBV13.2, only 4.1% of islet T cells expressed this Vβ gene. Conversely, a six-fold increase in the frequency of TRBV15 gene usage was detected in islet versus PBL g7-mBDC$^+$ T cells.

Analysis of CDR3β sequences confirmed the monoclonality of the islet TRBV15$^+$ g7-mBDC$^+$ T cells in mouse #1 (KDSSYEQ; Table 3.1), #2 (LGWPGAEQ; Table 3.2), #3 (RPGGRDYAEQ; Table 3.3), #7 (LAQGQGYEQ; Table 3.7), and #10 (LAQGQGYEQ, Table 3.10). In mouse 6 the 12/21 TRBV15$^+$ islet g7-mBDC$^+$ T cells consisted of two clonotypes containing the PDRGQDTQ and LAQGQGYEQ CDR3β motifs. Furthermore, the LAQGQGYEQ CDR3β sequence was shared among mouse #6, #7, and #10, with this motif dominating in mouse #7 and #10. Moreover, no CDR3β sequence was shared among islet and PBL g7-mBDC$^+$ T cells within an individual mouse, with the exception of mouse #7 (LGQQDTQ; Table 3.7) and #8 (LANSQNTL; Table 3.8). Finally, no CDR3β sequence was shared among PBL g7-mBDC$^+$ T cells within an individual mouse with the exception of mouse #1 (LLPRATGQL, DRGPDTEV; Table 3.1). Together, these results demonstrate that PBL g7-mBDC$^+$ T cells exhibit a highly diverse repertoire, whereas the repertoire of islet g7-mBDC$^+$ T cells is skewed with a strong preference for TRBV15 usage. Finally, the repertoires of PBL versus islet g7-mBDC$^+$ T cells are largely distinct.

3.4 Discussion

A key question in T1D is the relative contribution of and the molecular basis for expansion of certain T clonotypes in mediating β cell destruction. To begin to address these issues we have used sIA^{g7}-Ig technology to study BDC2.5 clonotypic $CD4^+$ T cells. Based on evidence derived from T cell clones and TCR transgenic mice, the BDC2.5 clonotype is disease relevant (Haskins and McDuffie 1990; Kurrer, Pakala et al. 1997). However, the precise role of these β cell-specific $CD4^+$ T cells in the diabetogenic response remains ill defined.

The current work has made three major findings. First, expansion of g7-$mBDC^+$ T cells in the islets corresponds with the development of destructive insulitis. g7-$mBDC^+$ T cells are detected in PBL and/or islets at the time insulitis is initiated in NOD female and male mice (Figure 3.1). In NOD female mice, however, the number of g7-$mBDC^+$ T cells increase with age but not in NOD male mice (Figure 3.1). Notably, the majority of NOD male mice remain diabetes-free due to the lack of progression towards destructive insulitis. We propose that establishment of the destructive phase of insulitis is in part dependent on a sufficient number of g7-$mBDC^+$ T cells residing in the islets. In our model, islet g7-$mBDC^+$ T cells mediate β cell injury to recruit other clones specific for different β cell autoantigens via epitope spread. Continued expansion of islet g7-$mBDC^+$ T cells promotes further epitope spread thereby ensuring that appropriate numbers of pathogenic effectors are present to drive the destructive phase of insulitis. The fact that the frequency of islet g7-$mBDC^+$ T cells among $CD4^+$ T cells is maintained at a consistent level from 6 weeks of age and older, supports the idea that islet g7-$mBDC^+$ T cells are promoting the recruitment of other $CD4^+$ T clonotypes. The progressive increase in the frequency and number of g7-$mBDC^+$ T cells in PBL of NOD

female mice reflects the increasing numbers of g7-mBDC$^+$ T cells in the islets (Figure 3.1), suggesting a cause and effect association. Based on TCR usage, however, g7-mBDC$^+$ clonotypes in PBL are distinct compared to those infiltrating the islets (Figure 3.2; Tables 3.1-10). One possible explanation for this disparity is that the β cell autoantigen recognized by g7-mBDC$^+$ T cells is released systemically as the disease process continues, promoting the expansion of "nonpathogenic" (e.g. TRBV13.2$^+$) BDC2.5 clonotypic T cells in the periphery.

The second major observation made in this study is that the majority (>60%) of islet infiltrating g7-mBDC$^+$ T cells express TRBV15. In PBL there is a modest preference of TRBV13.2 usage, and TRBV15 is expressed by 10% of PBL g7-mBDC$^+$ T cells. On the other hand, in 50% of NOD female mice $\geq$80% of islet g7-mBDC$^+$ T cells expressed TRBV15. It is important to note that our analysis is at the single cell level and does not involve *in vitro* culturing, therefore ruling out the possibility that the observed skewing of TCR gene usage is an artifact of preferential *in vitro* expansion. Interestingly, preferential usage of TRBV15 has also been reported for islet-infiltrating CD4$^+$ T cells analyzed in bulk from early and late stages of disease progression in NOD female mice (Quinn, McInerney et al. 2001; Baker, Lee et al. 2002). It is tempting to speculate that the islet infiltrating TRBV15$^+$ CD4$^+$ T cells characterized by Baker *et al.* are the same g7-mBDC$^+$ T cells detected in our study. The simplest interpretation of the data is that TRBV15 provides islet-infiltrating g7-mBDC$^+$ T cells a selective advantage over clonotypes expressing other Vβ genes. For instance, TCR affinity/avidity may be increased by TRBV15. Experiments

examining binding kinetics of mBDC-specific TRBV15 versus TRBV13.2 TCR would directly address this possibility.

Our data also demonstrates that in most NOD female mice examined (Mouse #1, #2, #3, #4, #6, #7, #10), g7-mBDC$^+$ T cells are represented by one to two major clonotypes based on CDR3β sequences. This finding is analogous to earlier work by our group studying islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-specific CD8$^+$ T cells (Wong, Stevens et al. 2007). Here, IGRP-specific CD8$^+$ T cells infiltrating the islets also were represented by one to two dominant clonotypes that in turn were unique to individual NOD mice. Interestingly, mouse #7 and #10 shared the same islet-infiltrating immunodominant clonotype characterized by the LAQGQGYEQ CDR3β sequence. TRBV15$^+$ CD4$^+$ T cells bearing the LAQGQGYEQ motif may represent a more diabetogenic clonotype compared to other TRBV15$^+$ T cells. Together these data would suggest that pathogenic T effectors are preferentially expanded in the islets, and that relatively few clonotypes for a given epitope are necessary to drive the diabetogenic response at late stages of disease progression.

In summary, this work has demonstrated that BDC2.5 clonotypic CD4$^+$ T cells play a role early in the diabetogenic response by influencing the nature of insulitis, and that most islet infiltrating g7-mBDC$^+$ T cells are characterized by expression of TRBV15 and are represented by a small number of clonotypes.

3.5 Materials and Methods

Mice

NOD/LtJ mice were maintained and bred under specific-pathogen free conditions, and maintained at an American Association of Laboratory Animal Care-accredited animal facility. All procedures were reviewed and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Pancreatic islet isolation

Pancreases were perfused with 0.2 mg/ml Liberase (Roche) and digested for 30 minutes at 37°C. Islets were purified via Ficoll gradient, handpicked and counted. For flow cytometry analysis, freshly isolated islets were dissociated into a single-cell suspension using enzyme-free cell dissociation solution (Sigma-Aldrich) before staining. Lymphocytes infiltrating the islets were collected and cellular debris removed by 70-μm nylon filters.

Flow Cytometry

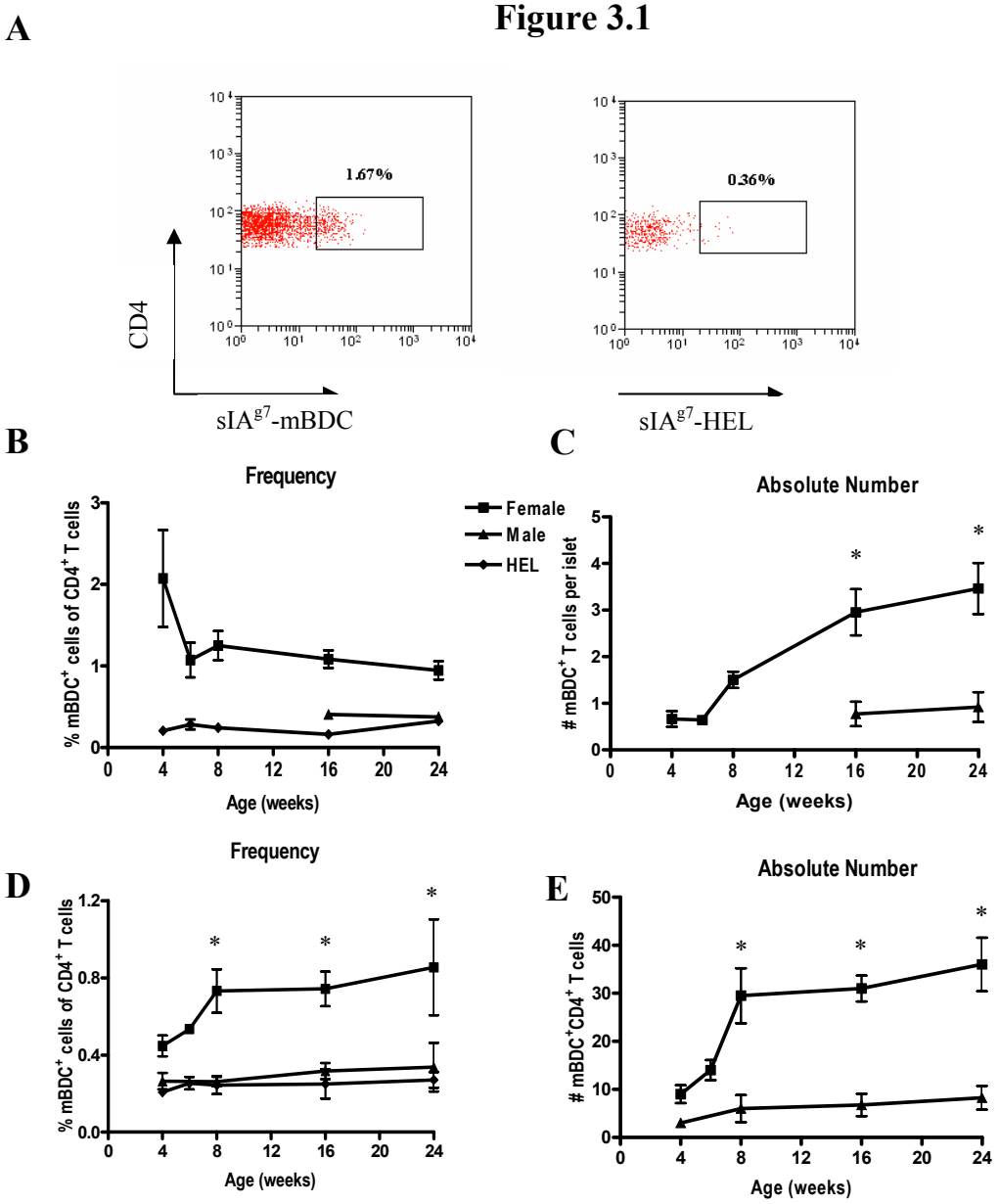
Single-cell suspensions from islets were prepared in PBS. Peripheral blood was collected via the tail vein and RBC lysed where appropriate. T cells were costained with sIA^{g7}-Ig multimers and Abs in PBS containing 3% FBS, 10 mM HEPES, and 1 mM EDTA for 1 hour at room temperature followed by CD3, CD4, CD19, CD11c, and F4/80 staining on ice for 30 minutes. Flow cytometry data were acquired on a Cyan instrument (DakoCytomation) and analyzed using Summit software (DakoCytomation). For all sIA^{g7}-Ig multimer analyses, $CD4^+$ T cells were gated based on forward and side scatter and CD3 and CD4 expression. For single-cell analyses, sIA^{g7}-mBDC multimer-binding $CD4^+$ T cells were sorted by a MoFlo high-speed sorter (DakoCytomation) into 4 μl of RT-PCR buffer at one cell per well of a 96-

well PCR plate (USA Scientific), and the RT-PCR was performed immediately. All flow cytometry analyses and single-cell sorting were performed at the University of North Carolina Flow Cytometry facility.

Single-cell RT-PCR and TCR repertoire analyses

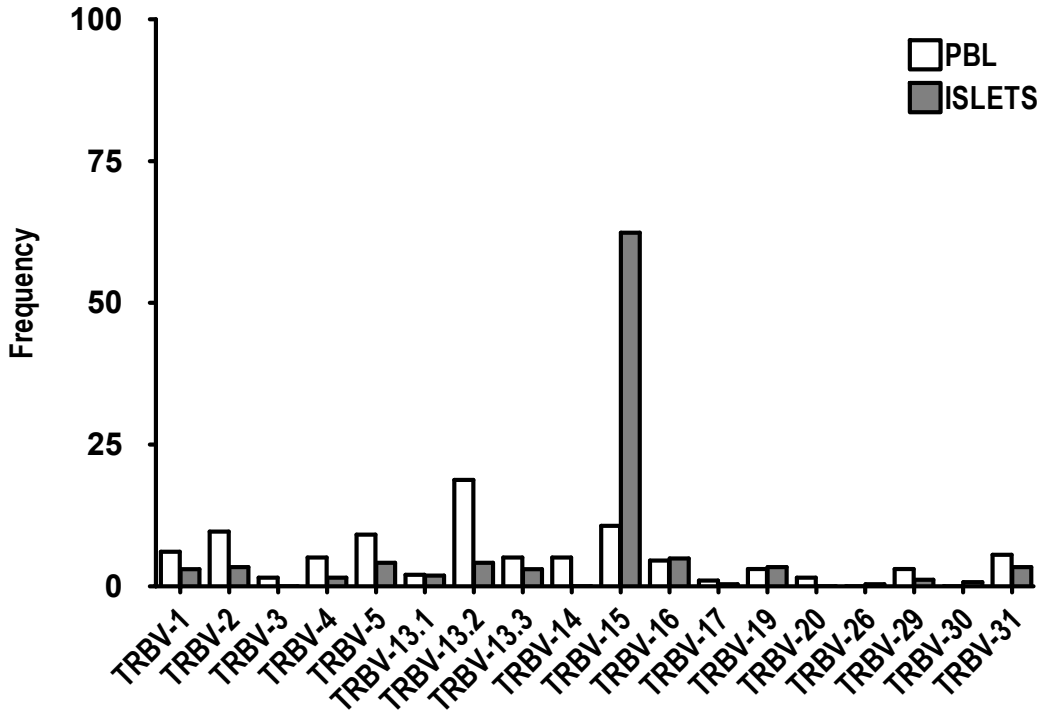
TCR usage was analyzed by a single-cell PCR protocol previously described (Baker, Lee et al. 2002) with the following modifications. Single-cell RT-PCR was performed using a Qiagen OneStep RT-PCR kit (Qiagen) according to the manufacturer's protocol. A panel of primers specific for all known TCR α- or β-chain variable regions and respective constant regions were used for reverse transcription and first-round PCR amplification. RT-PCR amplicons (2 μl) were used as templates for second-round PCR amplification using a panel of nested TCR α- or β-chain-specific primers. All oligonucleotides were synthesized at the Nucleic Acids Core Facility at the University of North Carolina. PCR products were treated with Exonuclease I (NEB Biolabs) and shrimp alkaline phosphatase (Roche), and sequenced at the University of North Carolina Genome Analysis Facility. TCR sequence alignments were performed using Sequencher software (Gene Codes). TCR β-chain (TRBV-D-J) gene family usage was identified and assigned using the SoDA software online (Volpe, Cowell et al. 2006) and former nomenclature based on Arden *et al.* (Arden 1992). CDR3 regions were analyzed using CLC Combined workbench software (CLCbio).

Figure 3.1

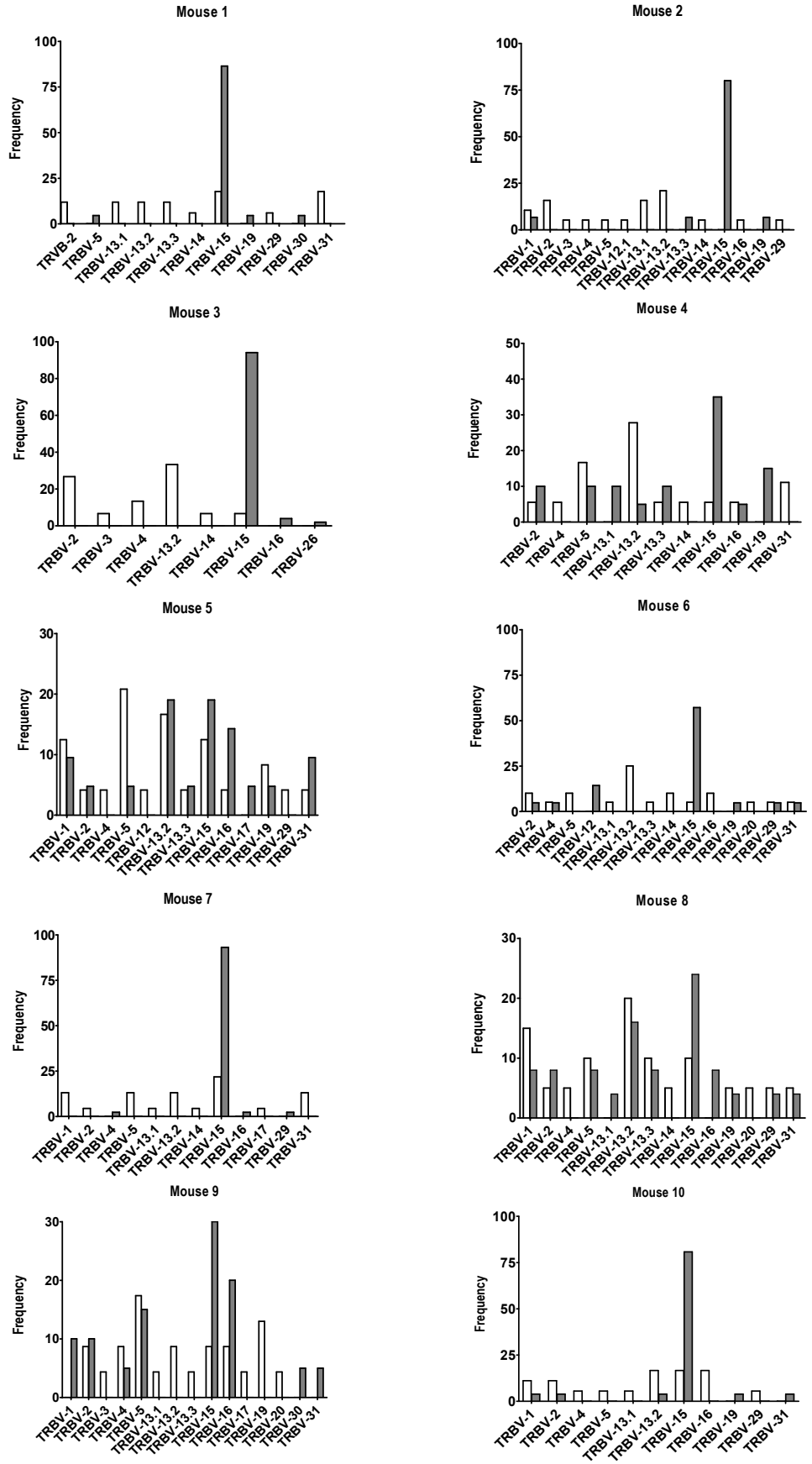


g7-mBDC$^+$ CD4$^+$ T cells are increased in PBL and the islets of NOD female mice. (A) Representative results for sIA^{g7}-mBDC and sIA^{g7}-HEL binding to PBL prepared from NOD female mice. CD4$^+$ and CD3$^+$ cells were gated on and assessed for sIA^{g7}-Ig multimer binding; cells staining with anti-CD19, -CD11c, and -F4/80 were excluded in a "dump channel". (B-C) Islets were isolated from individual NOD female or male mice of varying ages and the frequency (B) and the number per islet (C) of CD4$^+$ T cells binding sIA^{g7}-mBDC determined. (D-E) Similarly, PBL from individual NOD female and male mice were examined for the frequency (D) and number (E) of CD4$^+$ T cells binding sIA^{g7}-mBDC. Average absolute number of g7-mBDC$^+$ CD4$^+$ T cells were gated per 10,000 lymphocytes within the PBL of NOD female and NOD male mice (Student's t test, * $p \leq 0.024$). The average percentages of sIA^{g7}-mBDC$^+$ T cells ±SD calculated from five independent experiments are indicated.

A **Figure 3.2**


100
75
50
25
0
Frequency
PBL
ISLETS
TRBV-1
TRBV-2
TRBV-3
TRBV-4
TRBV-5
TRBV-13.1
TRBV-13.2
TRBV-13.3
TRBV-14
TRBV-15
TRBV-16
TRBV-17
TRBV-19
TRBV-20
TRBV-26
TRBV-29
TRBV-30
TRBV-31

B


Mouse 1
Frequency
TRVB-2
TRBV-5
TRBV-13.1
TRBV-13.2
TRBV-13.3
TRBV-14
TRBV-15
TRBV-19
TRBV-29
TRBV-30
TRBV-31
Mouse 2
Frequency
TRBV-1
TRBV-2
TRBV-3
TRBV-4
TRBV-5
TRBV-12.1
TRBV-13.1
TRBV-13.2
TRBV-13.3
TRBV-14
TRBV-15
TRBV-16
TRBV-19
TRBV-29
Mouse 3
Frequency
TRBV-2
TRBV-3
TRBV-4
TRBV-13.2
TRBV-14
TRBV-15
TRBV-16
TRBV-26
Mouse 4
Frequency
TRBV-2
TRBV-4
TRBV-5
TRBV-13.1
TRBV-13.2
TRBV-13.3
TRBV-14
TRBV-15
TRBV-16
TRBV-19
TRBV-31
Mouse 5
Frequency
TRBV-1
TRBV-2
TRBV-4
TRBV-5
TRBV-12
TRBV-13.2
TRBV-13.3
TRBV-15
TRBV-16
TRBV-17
TRBV-19
TRBV-29
TRBV-31
Mouse 6
Frequency
TRBV-2
TRBV-4
TRBV-5
TRBV-12
TRBV-13.1
TRBV-13.2
TRBV-13.3
TRBV-14
TRBV-15
TRBV-16
TRBV-19
TRBV-20
TRBV-29
TRBV-31
Mouse 7
Frequency
TRBV-1
TRBV-2
TRBV-4
TRBV-5
TRBV-13.1
TRBV-13.2
TRBV-14
TRBV-15
TRBV-16
TRBV-17
TRBV-29
TRBV-31
Mouse 8
Frequency
TRBV-1
TRBV-2
TRBV-4
TRBV-5
TRBV-13.1
TRBV-13.2
TRBV-13.3
TRBV-14
TRBV-15
TRBV-16
TRBV-19
TRBV-20
TRBV-29
TRBV-31
Mouse 9
Frequency
TRBV-1
TRBV-2
TRBV-3
TRBV-4
TRBV-5
TRBV-13.1
TRBV-13.2
TRBV-13.3
TRBV-15
TRBV-16
TRBV-17
TRBV-19
TRBV-20
TRBV-30
TRBV-31
Mouse 10
Frequency
TRBV-1
TRBV-2
TRBV-4
TRBV-5
TRBV-13.1
TRBV-13.2
TRBV-15
TRBV-16
TRBV-19
TRBV-29
TRBV-31

The Vβ repertoire of most islet infiltrating g7-mBDC^{+} CD4^{+} T cells is skewed. Single g7-mBDC^{+} CD4^{+} T cells sorted from PBL and islet infiltrates of 10 individual 16 week-old NOD female mice were sorted by MoFlo and Vβ gene usage determined by RT-PCR. (A) Vβ gene usage plotted as the average for the 10 NOD female mice. (B) Vβ gene usage plotted for individual mice.

Table 3.1

CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 1.

	VB CDR3		NUMBER	TRBV-D-J
MOUSE 1 ISLETS			22	
YLCASS	KDSSYEQ	YFG	19	15-D1.1-J2.7
YLCASS	RQDTQ	YFG	1	30-D1.1-J2.5
YFCASS	RTGGYAEQ	YFG	1	19-D2.1-J2.4
YFCASS	QRTGGGEQ	YFG	1	5-D2.1-J2.7
MOUSE 1 PBL			17	
YLCASS	DADRGTGNTL	YFG	1	13.1-D1.1-J1.3
YLCASS	LGTLTGQL	YFG	1	15-D1.1-J2.2
YFCASS	QDGGGEQ	YFG	1	2-D2.1-J2.7
YLCAWS	PGRDQNTL	YFG	1	31-D.11-J2.4
YLCAWS	LEGDTQ	YFG	1	31-D1.1-J2.5
YFCASS	DRGPDTEV	FFG	2	13.3-D1.1-J1.1
YLCASS	PGLGGLAETL	YFG	1	14-D2.1-J2.3
YLCAWS	LGQAGPYSDY	TFG	1	31-D2.1-J1.2
YCASS	LLPRATGQL	YFG	2	29-D1.1-J2.2
YLCASS	FGREGITNQAP	LFG	1	15-D1.1-J1.5
YLCASS	DAYRGTGNTL	FFG	1	13.1-D1.1-J1.3
YLCASS	LAQGQGYEQ	YFG	1	15-D1.1-J2.7
YFCASS	GDSDQNTL	YFG	1	13.2-D1.1-J2.4
YCASS	DRTISNERL	FFG	1	13.3-D1.1-J1.4
YFCASS	GEQITL	YFG	1	13.2-D1.1-J2.4

Table 3.2

CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 2.

	CDR3		NUMBER	TRBV-D-J
MOUSE 2 ISLETS			15	
YFCASS	LGWPGAEQ	FFG	12	15-D2.1-J2.1
FLCASS	IWQGDGNTL	YFG	1	19-D1.1-J1.3
YFCASS	GGQGSTL	YFG	1	13.3-D1.1-J2.4
YFCASS	AGTGGGYEG	YFG	1	1-D2.1-J2.7
MOUSE 2 PBL			19	
YCTCS	PYSFSNERL	FFG	1	1-D1.1-J1.4
YFCASS	HDSNNQAP	LFG	1	5-D1.1-J1.5
YFCASS	PLGGLNQDTQ	YFG	1	3-D2.1-J2.5
YFCAS	GEVGGQNTL	YFG	1	13.2-D2.1-J2.4
YFCAS	GDGGDYAEQ	FFG	1	13.2-D2.1-J2.1
YFCASS	QDRGVGAEQ	FFG	1	2-D2.1-J2.1
YFCASS	GDRDDWGGYEQ	YFG	1	13.2-D2.1-J2.7
YLCASS	LIWGGNQDTQ	YFG	1	16-D2.1-J2.5
YFCASS	DADSSAETL	YFG	1	13.1-D1.1-J2.3
YFCASS	HPDITSGNTL	YFG	1	2-D1.1-J1.3
YLCASS	THWGNYAEQ	FFG	1	4-D2.1-J2.1
YCTCS	ADRANTGQL	YFG	1	1-D1.1-J2.2
YFCASS	LSQQDTQ	YFG	1	29-D1.1-J2.5
YFCAS	GDAAGSGNTL	YFG	1	13.2-D1.1-J1.3
YFCASS	RRDRGVNTGQL	YFG	1	12.1-D1.1-J2.2
YFCASS	QPRTPSAETL	YFG	1	2-D1.1-J2.3
YLCASS	RDWGNQDTQ	YFG	1	14-D2.2-J2.5
YFCASS	AGGQDTQ	YFG	1	13.1-D2.1-J2.5
YFCAS	GDSWGAGDTQ	YFG	1	13.1-D2.1-J2.5

Table 3.3

CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 3.

	VB CDR3		NUMBER	TRBV-D-J
MOUSE 3 ISLETS			51	
YLCAS	RPGGRDYAEQ	FFG	47	15-D2.1-J1.1
YLCAS	NPHSSYEH	VLG	1	26-D1.1-J2.7
YLCAS	RLGGAQDTQ	YFG	1	16-D2.1-J2.5
YLCASS	FDRVEQ	YFG	1	16-D1.1-J2.7
YLCASS	TGGDTQ	YFG	1	15-D2.1-J2.5
Mouse 3 PBL			15	
YFCAS	GGGQGAGEQ	YFG	1	13.2-D1.1-J2.7
YLCASS	LGWQDTQ	YFG	1	15-D2.1-J2.5
YFCASS	QGGTTNSDY	TFG	2	2-D1.1-J1.2
YFCAS	DRLGGANTGQL	YFG	1	13.2-D2.1-J2.2
YLCASS	LGVESAETL	YFG	1	4-D2.1-J2.3
YLCASS	SRGSGNTL	YFG	1	4-D1.1-J1.3
YFCAS	GDHTEV	FFG	1	13.2-D1.1-J1.1
YFCASS	KLGYEQ	YFG	1	3-D2.1-J2.7
YFCAS	GDQSQNTL	YFG	1	13.2-D1.1-J2.4
YFCASS	QGQGTDY	TFG	1	2-D1.1-J1.2
YFCAS	GIETEQ	YFG	1	13.2-D1.1-J2.7
YLCAS	RRDRGNTEV	FFG	1	14-D1.1-J1.1
YFCASS	QEVGGHQDTQ	YFG	1	2-D2.1-J2.5
YFCASS	QGGTTNSDY	TFG	1	2-D1.1-J1.2

Table 3.4

CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 4.

	CDR3		NUMBER	TRBV-D-J
MOUSE 4 ISLETS			20	
YLCAS	RPGGRDYAEQ	FFG	3	15-D2.1-J1.1
YLCASS	LDASYEQ	YFG	2	15-D1.1-J2.7
YSCASS	IDGGRAETL	YFG	1	19-D2.1-J2.3
YLCAS	GEWDRGGNERL	FFG	1	13.2-D1.1-J1.4
YFCASS	LRGASAETL	YFG	1	19-D1.1-J2.3
FLCASS	IQADSAETL	YFG	1	19-D1.1-J2.3
YFCASS	QDWGPAETL	YFG	1	5-D2.1-J2.3
YFCASS	PGTNTEV	FFG	1	5-D1.1-J1.1
VYLCAS	GDAGTDDTQ	YFG	1	13.1-D2.1-J2.5
YFCASS	PSNSDY	TFG	1	2-D2.1-J1.2
YFCASS	HGGNYAEQ	FFG	1	2-D1.1-J2.1
YFCASS	DGTGEDTQ	YFG	1	13.1-D1.1-J2.5
YLCASS	RGAQDTQ	YFG	1	15-D2.1-J2.5
YFCASS	STNSQNTL	YFG	1	13.3-D1.1-J2.4
YLCASS	GDSYYNM	YFG	1	15-D1.1-J2.7
YFCASS	DLGASAETL	YFG	1	13.3-D1.1-J2.3
YLCASS	LDNERL	FFG	1	16-D1.1-J1.4
MOUSE 4 PBL			18	
YLCASS	QTTNSDY	TFG	1	4-D1.1-J1.2
YLCAS	RGHKYEQ	YFG	1	13.3-D1.1-J2.7
YFCAS	GGTGEDYAEQ	FFG	1	13.2-D1.1-J2.1
YFCASS	PRDWGGYEQ	YFG	1	5-D2.1-J2.7
YFCASS	RDGNYAEQ	FFG	1	5-D1.1-J2.1
YLCAWS	PDRGDTQ	YFG	1	31-D1.1-J2.5
YLCASS	PTHQDTQ	YFG	1	16-D1.1-J2.5
YFCASS	QEPSSGNTL	YFG	1	5-D1.1-J1.3
YLCAWS	PGTGGWQNTL	YFG	1	31-D2.1-J2.4
YLCAW	GNRDEQ	YFG	1	31-D1.1-J2.7
YLCAS	GYGWGGNTL	YFG	1	13.2-D2.1-J2.4
YLCASS	LELPL	YFG	1	15-D1.1-J1.6
YLCASS	GPTGEDTQ	YFG	1	13.2-D1.1-J2.5
YFCASS	RTGGAHEQ	YFG	1	2-D2.1-J2.7
YLCASS	PLGVYEQ	YFG	1	14-D2.1-J2.7
YFCASS	GDAGQNTL	YFG	1	13.2-D1.1-J2.4
YLCAWS	LGQAGPYSDY	TFG	1	31-D2.1-J1.2
YFCASS	GDQDTQ	YFG	1	13.2-D1.1-J2.5

Table 3.5

CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 5

	CDR3		NUMBER	TRBV-D-J
MOUSE 5 ISLETS			21	
YLCASS	LGQQDTQ	YFG	2	15-D1.1-J2.5
YLCAS	RPGGRDYAEQ	FFG	1	15-D2.1-J1.1
YLCASS	RLGETL	YFG	1	17-D2.1-J2.3
YLCASS	LNTEV	FFG	1	15-D1.1-J1.1
YCTCS	GTGVNSPL	YFG	1	1-D1.1-J1.6
YCTCS	AGADSGNTL	YFG	1	1-D1.1-J1.3
YLCASS	LGDREDTQ	YFG	1	16-D1.1-J2.5
YFCASS	PGGPDTQ	YFG	1	5-D2.1-J2.5
FLCASS	PDRDEQ	YFG	1	19-D1.1-J2.7
YLCAWS	LGGRAEQ	FFG	1	31-D2.1-J2.1
YFCAS	GDARGENTEV	FFG	1	13.2-D1.1-J1.1
YLCASS	LGGNTEV	FFG	1	15-D2.1-J1.1
YFCAS	GDRQEYEQ	YFG	1	13.2-D1.1-J2.7
YFCAS	GDGRQANERL	FFG	1	13.2-D2.1-J1.4
YFCASS	LPGGSTEV	FFG	1	2-D2.1-J1.1
YLCASS	TGGQYEQ	YFG	1	16-D1.1-J2.7
YFCASS	DGTGGTGQL	YFG	1	13.3-D1.1-J2.2
YFCAS	GDLGGRAEQ	FFG	1	13.2-D2.1-J2.1
YLCAW	GTGGTYEQ	YFG	1	31-D1.1-J2.7
YLCASS	FGLGGAEQ	FFG	1	16-D2.1-J2.1
MOUSE 5 PBL			24	
FLCASS	KNRPGGNYDEQ	FFG	1	19-D2.1-J2.1
YFCASS	PPGLGVYEQ	YFG	1	13.3-D2.1-J2.7
YCTCS	LWGGDEQ	YFG	1	1-D2.1-J2.7
YFCAS	GDKGADTQ	YFG	1	13.2-D1.1-J2.5
YFCAS	GDRISNERL	FFG	1	13.2-D1.1-J1.4
YCTCS	AGWGRMHEQ	FFG	1	1-D1.1-J2.1
YFCASS	QTGSGNERL	FFG	1	5-D1.1-J1.4
YLCASS	PGQGQ	YFG	1	15-D1.1-J2.7
YLCAS	RRDWGGFEQ	YFG	1	15-D2.1-J2.7
YLCAWS	SRDWGDEQ	YFG	1	31-D2.1-J2.7
YFCASS	QGGTTNSDY	TFG	1	2-D1.1-J1.2
YFCASS	PTGPNERL	FFG	1	12-D1.1-J1.4
YFCASS	DALGTGTNTGQL	YFG	1	5-D2.1-J2.1
YFCASS	QDLSYEQ	YFG	1	5-D2.1-J2.7
YFCASS	PGGYQDTQ	YFG	1	5-D1.1-J2.5
YFCAS	RDRNYNSPL	YFG	1	29-D1.1-J1.6
YFCASS	QPGQGGYEQ	YFG	1	5-D1.1-J2.7
FLCASS	PGTGSNTGQL	YFG	1	19-D1.1-J2.2
YLCASS	PGLGENTL	YFG	1	4-D2.1-J2.4
YFCAS	GDADYEQ	YFG	1	13.2-D1.1-J2.7
YLCASS	LGGENTL	YFG	1	16-D2.1-J2.4
YCTCS	APGTGVDTQ	YFG	1	1-D1.1-J2.5

Table 3.6

CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 6.

	CDR3		NUMBER	TRBV-D-J
MOUSE 6 ISLETS			21	
YLCASS	GTSNSDY	TFG	1	13.3-D1.1-J1.2
FLCASS	MGTGPNERL	FFG	1	19-D1.1-J1.4
YCASS	LSGSDY	TFG	1	29-D1.1-J1.2
YLCAWS	SLGGNYAEQ	FFG	1	31-D2.1-J2.1
YLCAS	RSKSSYEQ	YFG	1	4-D1.1-2.7
YFCASS	QDNFQRNI	FFG	1	2-D1.1-1.4
YFCASS	LGGAEQ	YFG	3	12-D2.1-J2.7
YLCASS	PDRGQDTQ	YFG	5	15-D1.1-J2.5
YLCASS	LAQGQGYEQ	YFG	7	15-D1.1-J2.7
MOUSE 6 PBL			20	
YLCASS	LGAETL	YFG	1	16-D1.1-J2.3
YLCASS	RTGGAAEQ	FFG	1	20-D2.1-J2.1
YLCASS	WDRGGTEV	FFG	1	16-D1.1-J1.1
YFCAS	GDATGSGNTL	YFG	1	13.2-D1.1-J1.3
YLCAWS	RGLGGYAEQ	FFG	1	31-D2.1-J2.1
YFCASS	DRGSAETL	YFG	1	5-D1.1-J2.3
YFCASS	QDSSYEQ	YFG	1	2-D1.1-J2.7
YLCASS	PLDWGDTQ	YFG	1	4-D2.1-J2.5
YFCASS	GDPGSYAEQ	FFG	1	2-D2.1-J2.1
YLCASS	PRDWGSEQ	FFG	1	15-D2.1-J2.1
YLCASS	GDRGAGNTL	YFG	1	13.2-D1.1-J1.3
YLCASS	GQGEQ	YFG	1	14-D1.1-J2.7
YFCASS	DSGNSDY	TFG	1	13.3-D1.1-J1.2
YFCASS	HSGTGRETQ	YFG	1	5-D1.1-J2.5
YFCAS	GGTGEATGQL	YFG	1	13.2-D1.1-J2.2
YFCASS	LWGELPDS	FFG	1	13.2-D2.1-J1.5
YLCASS	LRGGDAETL	YFG	1	14-D1.1-J2.3
YFCAS	GRGTGRQDTQ	YFG	1	13.2-D1.1-J2.5
YFCASS	LRGRGTEV	FFG	1	29-D1.1-J1.1
YFCASS	DLLVGPNQDTQ	YFG	1	13.1-D2.1-J2.5

Table 3.7

CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 7.

	CDR3		NUMBER	TRBV-D-J
MOUSE 7 ISLETS			43	
YLCASS	LAQGQGYEQ	YFG	38	15-D1.1-J2.7
YLCASS	AGGNQAP	LFG	1	4-D2.1-J1.5
YFCASS	TGVNQDTQ	YFG	1	29-D1.1-J2.5
YLCASS	LGQQDTQ	YFG	1	15-D1.1-J2.5
YLCASS	LDTGPNERL	FFG	1	16-D1.1-J1.4
YLCASS	LARGDGTGQL	YFG	1	15-D1.1-J2.2
MOUSE 7 PBL			23	
YLCASS	LDRDEQ	YFG	1	15-D1.1-J2.7
YLCASS	RETGGAEQ	FFG	1	17-D1.1-J2.1
YLCASS	TPGLGVEQ	YFG	1	15-D2.1-2.7
YFCASS	QDVWGGVEQ	YFG	1	5-D2.1-J2.7
YLCASS	LGQQDTQ	YFG	1	15-D1.1-J2.5
YLCAWS	LPGQQDTQ	YFG	1	31-D1.1-J2.5
YLCASS	PGLGGLAETL	YFG	1	14-D2.1-J2.3
YLCASS	PQGATNERL	FFG	1	4-D1.1-J1.4
YLCASS	RTGQEEQ	YFG	1	15-D1.1-J2.7
YFCASS	AGTPISNESL	FFG	1	13.1-D1.1-J1.4
YCTCS	AAWGYEQ	YFG	1	1-D2.1-J2.7
YLCAWS	LGLGGREQ	YFG	1	31-D2.1-J2.7
YFCASS	QAWGDYEQ	YFG	1	5-D2.1-J2.7
YFCASS	QTGDYAEQ	FFG	1	5-D2.1-J2.1
YLCAWS	PDWEQDTQ	YFG	1	31-D2.1-J2.5
YLCASS	LSGGAREQ	YFG	1	15-D2.1-2.7
YLCASS	GDAIQAP	LFG	1	13.2-D1.1-J1.5
YFCASS	QDGGALGNTL	YFG	1	2-D2.1-J1.3
YFCASS	GEPDSPL	YFG	1	13.2-D2.1-J1.6
YLCAS	GETGVAEQ	FFG	1	13.2-D1.1-J2.1
YFCASS	QETTGGNTGQL	YFG	1	2-D1.1-J2.2
YCTCS	AESGGGNSPL	YFG	1	1-D1.1-J1.6
YCTCS	ADWGSAETL	YFG	1	1-D2.1-J2.3

Table 3.8

CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 8.

	CDR3		NUMBER	TRBV-D-J
MOUSE 8 ISLETS			**25**	
YLCAS	GYRSNTGKL	YFG	1	13.2-D1.1-J2.2
YLCASS	QNTL	YFG	1	13.2-D2.1-J2.4
YFCAS	GVADQAP	LFG	1	13.2-D1.1-J1.5
YLCAS	GYAGGGGEQ	YFG	1	13.2-D2.1-J2.7
YFCASS	EGHTGQL	YFG	1	13.3-D1.1-2.2
YFCASS	DAKDRGHERL	FFG	1	13.3-D1.1-J1.4
YFCASS	QVRGDTQ	YFG	1	5-D1.1-J2.5
YFCASS	QSGNYAEQ	FFG	1	5-D1.1-J2.1
YLCASS	RDSSYEQ	YFG	4	15-D1.1-J2.7
YLCASS	IVGFQDTQ	YFG	1	16-D1.1-J2.5
YLCASS	LDEQGGYAEQ	FFG	1	16-D1.1-J2.1
YCTCS	ADPWTGGQDTQ	YFG	1	1-D2.1-J2.5
YCTCS	ADSDYEQ	YFG	1	1-D2.1-J2.7
YLCASS	LAQGQGYEQ	YFG	2	15-D1.1-J2.7
YLCAWS	LLAGGDTQ	YFG	1	31-D2.1-J2.5
YFCASS	LANSQNTL	YFG	1	29-D1.1-J2.4
FLCASS	DRGTGTGQL	YFG	1	19-D2.1-J2.2
YLCASS	DRGGSDY	TFG	1	13.1-D2.1-J1.2
YFCASS	HQDTQ	YFG	1	2-D2.1-J2.5
YFCASS	HRDNYEQ	YFG	1	2-D1.1-J2.7
FLCASS	DTSYNSPL	YFG	1	19-D1.1-J1.6
MOUSE 8 PBL			**20**	
YFCAS	GDIQDTQ	YFG	1	13.2-D1.1-J2.5
YFCASS	QKSGGCQNTL	YFG	1	2-D1.1-J2.4
YFCAR	GSGTGVEQ	YFG	1	13.2-D1.1-J2.7
YFCASS	VEGDKQ	YFG	1	13.3-D2.1-J2.7
YFCAS	GDGRDWGGATETL	YFG	1	13.2-D2.1-J2.3
YFCAS	GDAGANTGQL	YFG	1	13.2-D1.1-J2.2
YFCASS	LPGTGNTEV	FFG	1	13.3-D1.1-J1.1
YLCG	AIKNTGQL	YFG	1	20-D1.1-J2.2
YFCASS	QQYEQ	YFG	1	5-D1.1-J2.7
YFCASS	QKGTGGH	YFG	1	5-D2.1-J2.7
YCTCS	AMGGLEQ	YFG	1	1-D2.1-J2.7
YCTCS	DRDSQDTQ	YFG	1	1-D1.1-J2.5
YCTCS	GGQYEQ	YFG	1	1-D1.1-J2.7
YLCASS	LRTGQDTQ	YFG	1	14-D2.1-J2.5
YLCAWS	DRVDNQAP	LFG	1	31-D1.1-J1.5
YLCASS	LSGGALEQ	YFG	1	15-D2.1-J2.7
YLCASS	PRDRGAEQ	FFG	1	15-D1.1-J2.1
YFCASS	LANSQNTL	YFG	1	29-D1.1-J2.4
YLCASS	PGERL	FFG	1	4-D1.1-J1.4
FLCASS	IGRSSGNTL	YFG	1	19-D2.1-J1.3

Table 3.9

CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 9.

	CDR3		NUMBER	TRBV-D-J
MOUSE 9 ISLETS			20	
YLCASS	KTGGQNTL	YFG	1	15-D1.1-J2.4
YLCASS	FRDRKDTQ	YFG	1	16-D1.1-J2.5
YFCASS	QEGQLSNERL	FFG	1	5-D1.1-J1.4
YFCASS	QSLNGQNTL	YFG	1	2-D1.1-J2.4
YFCSS	RDLGGSTL	YFG	1	30-D1.1-J2.4
YLCASS	PGGAGSDY	TFG	1	15-D1.1-J1.2
YLCAW	RRGRGSYAEQ	FFG	1	31-D1.1-J2.1
YLCASS	FWGGQDTQ	YFG	1	16-D2.1-J2.5
YLCASS	LDNQDTQ	YFG	1	15-D2.1-J2.5
YCTCS	AGQGAPGNTL	YFG	1	1-D1.1-J1.3
YFCASS	RDWADTQ	YFG	1	5-D2.1-J2.5
YLCASS	RDNNNQAP	LFG	1	16-D1.1-J1.5
YLCASS	LLGGRDTQ	YFG	1	16-D2.1-J2.5
YLCASS	SGGDTL	YFG	1	15-D1.1-J2.4
YLCASS	LLGSYEQ	YFG	1	15-D2.1-J2.7
YFCASS	QDNYPGQL	YFG	1	2-D2.1-J2.2
YCTCS	APGQNTGQL	YFG	1	1-D1.1-J2.2
YFCASS	QDSQDTG	YFG	1	5-D1.1-J2.5
YLCASS	DRGAEV	FFG	1	4-D1.1-J1.1
YLCASS	LGGYAEQ	FFG	1	15-D1.1-D2.1
MOUSE 9 PBL			23	
YFCASS	QVRGHNERL	FFG	1	2-D1.1-J1.4
YLCASS	LPAKTGPL	YFG	1	15-D1.1-J2.2
YLCASS	PDWVNQDTQ	YFG	1	16-D2.2-J2.5
YLCASS	YGRGANTKV	FFG	1	4-D1.1-J1.1
YLCASS	GGARGYAEQ	FFG	1	20-D2.1-J2.1
YFCASS	QDRDQAP	LFG	1	5-D1.1-J1..5
YLCASS	PGQGHERL	FFG	1	14-D1.1-J1.4
YFCASS	RRGAQDTQ	YFG	1	3-D2.1-J2.5
YFCASS	VPGEDAEQ	LFG	1	13.3-D1.1-J2.1
YFCASS	QGTGGVSNERL	FFG	1	5-D1.1-J1.4
YFCASS	QDGGQGAETL	YFG	1	5-D1.1-J2.3
YLCASS	TGTGYEQ	YFG	1	4-D1.1-J2.7
FLCASS	IRISNERL	FFG	1	19-D1.1-J1.4
FLCASS	LTGGDDAETL	YFG	1	19-D1.1-J1.4
FLCASS	IRDKDTQ	YFG	1	19-D1.1-J2.5
YCTCS	ADGGAGERL	FFG	1	1-D2.1-J1.4
YLCAS	GEPGLGDQDTQ	YFG	1	13.2-D2.1-J2.5
YLCAS	RGVGTDQDTQ	YFG	1	13.2-D1.1-J2.5
YLCASS	QDADAEQ	FFG	1	13.1-D1.1-J2.1
YLCASS	LDPGNERL	FFG	1	16-D1.1-J1.4
YLCASS	GNYAEQ	FFG	1	15-D1.1-J2.1
YLCASS	RERANTEV	FFG	1	17-D1.1-J1.1
YFCASS	QDSAETL	YFG	1	5-D1.1-J2.3

Table 3.10

CDR3β analysis of g7-mBDC^{+} CD4^{+} T cells in PBL and islets from Mouse 10.

	CDR3		NUMBER	TRBV-D-J
MOUSE 10 ISLETS			26	
YLCASS	LAQGQGYEQ	YFG	21	15-D1.1-J2.7
FLCASS	QGLGAYEQ	YFG	1	19-D2.1-J2.7
YFCASS	QPTGGYEQ	YFG	1	2-D1.1-J2.7
YLCASS	GETGSAETL	YFG	1	13.2-D1.1-J2.3
YLCAW	AGLGGHQDTQ	YFG	1	31-D2.1-J2.5
YCTCS	GDRDAETL	YFG	1	1-D1.1-J2.3
MOUSE 10 PBL			18	
YLCASS	QDVSGNTL	YFG	1	15-D1.1-J1.3
YLCASS	LGGGGGERL	FFG	1	16-D1.1-J1.4
YFCASS	QDGTVYNSPL	YFG	1	2-D1.1-J1.6
YLCASS	DRNSDY	TFG	1	4-D1.1-J1.2
YLCASS	LDGTGRGNTL	YFG	1	16-D1.1-J1.3
YLCASS	LEGQAP	LFG	1	16-D1.1-J1.5
YCTCS	ATGTKDTQ	YFG	1	1-D1.1-J2.5
YLCASS	LAGNEQ	YFG	1	15-D1.1-J2.7
YLCAS	PGLYADTQ	YFG	1	29-D2.1-J2.5
YFCASS	RTGGNERL	FFG	1	5-D1.1-J1.4
YCTCS	LRDSYAEQ	FFG	1	1-D2.1-J2.1
YLCASS	LGRADTQ	YFG	1	15-D1.1-J2.5
YLCASS	LRDGVDTQ	YFG	1	15-1.1-J2.5
YLCAS	RTGNDTL	YFG	1	13.2-D1.1-J2.4
YFCAS	QGGWGEQ	YFG	1	13.2-D2.1-J2.7
YFCASS	AGTDQDTQ	YFG	1	13.1-D1.1-J1.4
YLCAS	QGGGRGVDTQ	YFG	1	13.2-D2.1-J2.5
YLCASS	RQGTNSDY	TFG	1	2-D1.1-J1.2

3.6 References

Arden, B. (1992). "Diversity of novel recombining elements suggests developmentally programmed expression of the T cell receptor alpha/delta locus." Eur J Immunol **22**(5): 1287-91.

Baker, F. J., M. Lee, et al. (2002). "Restricted islet-cell reactive T cell repertoire of early pancreatic islet infiltrates in NOD mice." Proc Natl Acad Sci U S A **99**(14): 9374-9.

Carrasco-Marin, E., J. Shimizu, et al. (1996). "The class II MHC I-Ag7 molecules from non-obese diabetic mice are poor peptide binders." J Immunol **156**(2): 450-8.

Gelber, C., L. Paborsky, et al. (1994). "Isolation of nonobese diabetic mouse T-cells that recognize novel autoantigens involved in the early events of diabetes." Diabetes **43**(1): 33-9.

Haskins, K. (2005). "Pathogenic T-cell clones in autoimmune diabetes: more lessons from the NOD mouse." Adv Immunol **87**: 123-62.

Haskins, K. and M. McDuffie (1990). "Acceleration of diabetes in young NOD mice with a CD4+ islet-specific T cell clone." Science **249**(4975): 1433-6.

Haskins, K., M. Portas, et al. (1988). "T-lymphocyte clone specific for pancreatic islet antigen." Diabetes **37**(10): 1444-8.

Jasinski, J. M., L. Yu, et al. (2006). "Transgenic insulin (B:9-23) T-cell receptor mice develop autoimmune diabetes dependent upon RAG genotype, H-2g7 homozygosity, and insulin 2 gene knockout." Diabetes **55**(7): 1978-84.

Katz, J. D., B. Wang, et al. (1993). "Following a diabetogenic T cell from genesis through pathogenesis." Cell **74**(6): 1089-100.

Kelemen, K., D. R. Wegmann, et al. (2001). "T-cell epitope analysis on the autoantigen phogrin (IA-2beta) in the nonobese diabetic mouse." Diabetes **50**(8): 1729-34.

Kurrer, M. O., S. V. Pakala, et al. (1997). "Beta cell apoptosis in T cell-mediated autoimmune diabetes." Proc Natl Acad Sci U S A **94**(1): 213-8.

Olcott, A. P., J. Tian, et al. (2005). "Antigen-based therapies using ignored determinants of beta cell antigens can more effectively inhibit late-stage autoimmune disease in diabetes-prone mice." J Immunol **175**(3): 1991-9.

Peterson, J. D. and K. Haskins (1996). "Transfer of diabetes in the NOD-scid mouse by CD4 T-cell clones. Differential requirement for CD8 T-cells." Diabetes **45**(3): 328-36.

Peterson, J. D., B. Pike, et al. (1995). "Induction of diabetes with islet-specific T-cell clones is age dependent." Immunology **85**(3): 455-60.

Quinn, A., B. McInerney, et al. (2001). "Regulatory and effector CD4 T cells in nonobese diabetic mice recognize overlapping determinants on glutamic acid decarboxylase and use distinct V beta genes." J Immunol **166**(5): 2982-91.

Ridgway, W. M., M. Fasso, et al. (1999). "A new look at MHC and autoimmune disease." Science **284**(5415): 749, 751.

Santamaria, P., T. Utsugi, et al. (1995). "Beta-cell-cytotoxic CD8+ T cells from nonobese diabetic mice use highly homologous T cell receptor alpha-chain CDR3 sequences." J Immunol **154**(5): 2494-503.

Tian, J., S. Gregori, et al. (2001). "The frequency of high avidity T cells determines the hierarchy of determinant spreading." J Immunol **166**(12): 7144-50.

Tisch, R. and H. McDevitt (1996). "Insulin-dependent diabetes mellitus." Cell **85**(3): 291-7.

Verdaguer, J., J. W. Yoon, et al. (1996). "Acceleration of spontaneous diabetes in TCR-beta-transgenic nonobese diabetic mice by beta-cell cytotoxic CD8+ T cells expressing identical endogenous TCR-alpha chains." J Immunol **157**(10): 4726-35.

Volpe, J. M., L. G. Cowell, et al. (2006). "SoDA: implementation of a 3D alignment algorithm for inference of antigen receptor recombinations." Bioinformatics **22**(4): 438-44.

Wong, C. P., R. Stevens, et al. (2007). "Identical beta cell-specific CD8(+) T cell clonotypes typically reside in both peripheral blood lymphocyte and pancreatic islets." J Immunol **178**(3): 1388-95.

Wong, F. S., I. Visintin, et al. (1996). "CD8 T cell clones from young nonobese diabetic (NOD) islets can transfer rapid onset of diabetes in NOD mice in the absence of CD4 cells." J Exp Med **183**(1): 67-76.

Yoshida, K., T. Martin, et al. (2002). "Evidence for shared recognition of a peptide ligand by a diverse panel of non-obese diabetic mice-derived, islet-specific, diabetogenic T cell clones." Int Immunol **14**(12): 1439-47.

Zechel, M. A., M. D. Krawetz, et al. (1998). "Epitope dominance: evidence for reciprocal determinant spreading to glutamic acid decarboxylase in non-obese diabetic mice." Immunol Rev **164**: 111-8.

CHAPTER 4

FUTURE PERSPECTIVES

4.1 sIA^{g7}-Ig dimer administration: A potent strategy of peptide vaccination.

Peptide-based vaccination is one approach of antigen-specific immunotherapy that has proven to be effective in experimental models of autoimmunity including the NOD mouse and Tg models of T1D. Nevertheless, various aspects of peptide immunotherapy need to be improved to enhance efficacy and safety for clinical application. With this in mind, we established an approach based on administration of peptide-sIA^{g7}-Ig recombinants. Recent work by the Bluestone group reported use of a similar sIA^{g7}-Ig recombinant tethered to the mimetic BDC2.5 peptide P31 (Masteller, Warner et al. 2003). Administration of sIA^{g7}-P31 dimers blocked the capacity of activated BDC2.5 CD4^{+} T cells to transfer diabetes to T cell deficient NOD recipients. However, treatment of young NOD female mice with sIA^{g7}-P31 failed to prevent diabetes. This finding is consistent with our observation that administration of sIA^{g7}-mBDC to 12 week-old NOD female mice also failed to block diabetes. On the other hand, sIA^{g7}-p217 and sIA^{g7}-p286 dimer vaccination of NOD female mice proved to be highly effective in suppressing the progression of insulitis and preventing diabetes at a late preclinical stage. Protection correlated with the induction of IL-10 secreting Tr1-like cells in agreement with work by Casares and colleagues, who used sIE^{d}-HA dimers to induce HA-specific Tr1 cells in a TCR Tg model of T1D (Casares, Hurtado et al. 2002).

A key issue that has yet to be addressed is the mechanism by which sIA^{g7}-p217 and sIA^{g7}-p286 dimers preferentially promote the differentiation of IL-10 secreting Tr1 cells. Noteworthy is that Tr1 cells can be generated *in vitro* and *in vivo* by chronic antigen exposure (Roncarolo, Bacchetta et al. 2001; Sundstedt, O'Neill et al. 2003; Wakkach, Fournier et al. 2003). The relatively long *in vivo* persistence of sIA^{g7}-Ig dimers coupled with

two courses of treatment may establish a similar state of chronic antigenic stimulation. Of particular interest will be the biochemical and transcriptional signals delivered by TCR following sIA^{g7}-Ig dimer binding, and how these events promote a Tr1 cell phenotype. One possibility is that different T cell phenotypes are established based on the affinity/avidity of sIA^{g7}-Ig binding, and in turn the quality and/or magnitude of the signaling events. The latter may explain why sIA^{g7}-mBDC dimers promote Th1 and Th2 cells, and Tr1 cells are induced by sIA^{g7}-p217 and sIA^{g7}-p286 dimers. In view of the fact that sIA^{g7}-Ig induced protection is dependent on the identity of the β cell peptide, it will be of interest to determine which other epitopes presented in the context of a sIA^{g7}-Ig dimer can mediate a sufficient immunoregulatory response. Of particular interest are the insulin B chain and proinsulin derived peptides which have been reported to induce protection in NOD mice via other modes of treatment (Liu, Abiru et al. 2002; Martinez, Augstein et al. 2003). Finally, experiments are needed to assess the general applicability of sIA^{g7}-Ig dimer vaccination. For instance, sIA^{g7}-p217 and sIA^{g7}-p286 dimer treatment can be tested for inducing islet graft protection, or be employed as part of a combinatorial approach to induce diabetes remission.

4.2 Characterization of autoreactive T cells in islet infiltrates.

A significant effort has gone on to define the specificities and TCR properties of diabetogenic T cells. Of particular interest has been the identity of the autoantigens and corresponding T clonotypes initiating β cell autoimmunity. Various observations suggest that insulin, proinsulin and GAD65 have key roles in the initial stages of β cell autoimmunity (Tisch, Yang et al. 1993; Wegmann, Norbury-Glaser et al. 1994; Krishnamurthy, Dudek et al. 2006). However, work by Davis and colleagues indicates that CD4$^+$ T cells recognizing other

β cell autoantigens and characterized by TCR expressing TRBV15 also reside in the islet infiltrates early in the diabetogenic response (Baker, Lee et al. 2002).

Our findings indicate that BDC2.5 clonotypic $CD4^+$ T cells are detected in the earliest islet infiltrates, consistent with observations that a surprisingly high frequency of BDC^+ $CD4^+CD8^-$ SP thymocytes are found in 2 week old NOD mice (Jang, Seth et al. 2003; Stratmann, Martin-Orozco et al. 2003). We argue that g7-$mBDC^+$ T cells establish conditions necessary for promoting the destructive phase of insulitis by mediating β cell injury needed for the recruitment of other diabetogenic T clonotypes. Two complementary approaches can be used to confirm this model. The first is to clonally delete g7-$mBDC^+$ T cells, using for instance sIA^{g7}-Ig molecules coupled to a toxin, and determine whether the progression of insulitis and the development of diabetes are inhibited. The second approach entails isolation of g7-$mBDC^+$ T cells and adoptively transferring these T cells into young NOD male recipients; insulitis should be exacerbated and diabetes readily induced in the recipient mice based on our model.

Another major conclusion from our study is that there is preferential TCR usage by, and clonal expansion of g7-$mBDC^+$ T cells residing in the islets but not PBL. Furthermore, the TCR repertoires are distinct between islet and PBL g7-$mBDC^+$ T cells. We propose that the affinity/avidity of the TCR determine the pathogenic potential of the respective BDC2.5 clonotypic T cells. For instance, g7-$mBDC^+$ T cells found in the periphery are expected to be nonpathogenic, and express TCR of relatively low affinity/avidity. In contrast, g7-$mBDC^+$ T cells infiltrating the islets are expected to be pathogenic, and express TCR of relatively high

affinity/avidity. This model can be tested in two ways. The first is to directly compare the diabetogenicity of g7-mBDC$^+$ T cells isolated from the periphery versus the islets upon adoptive transfer into NOD.*scid* recipients. Secondly, the binding kinetics of soluble TCR cloned from peripheral versus islet infiltrating g7-mBDC$^+$ T cells can be measured.

Our current analysis of TCR gene usage is limited to that found in 16 week-old NOD female mice. This analysis needs to be extended to NOD male mice, and to younger NOD female mice. In NOD male mice we would expect no or limited expansion of a given islet g7-mBDC$^+$ T clonotype. Interestingly, 3/10 NOD female mice examined in our study fall into this category; it is tempting to speculate that these 3 female mice were destined to remain diabetes-free. In young NOD female mice, it will be of keen interest to determine how clonal expansion evolves. For instance, in younger NOD females we would predict limited if any obvious selection of a given clonotype. However, with age clonal selection will become increasingly more evident. Finally, analysis of TCR Vα gene usage by islet g7-mBDC$^+$ T cells would aid in determining the relative contribution of Vβ gene segments such as TRBV15, in establishing the specificity of BDC2.5 clonotypic T cells.

4.3 References

Baker, F. J., M. Lee, et al. (2002). "Restricted islet-cell reactive T cell repertoire of early pancreatic islet infiltrates in NOD mice." Proc Natl Acad Sci U S A **99**(14): 9374-9.

Jang, M. H., N. P. Seth, et al. (2003). "Ex vivo analysis of thymic CD4 T cells in nonobese diabetic mice with tetramers generated from I-A(g7)/class II-associated invariant chain peptide precursors." J Immunol **171**(8): 4175-86.

Krishnamurthy, B., N. L. Dudek, et al. (2006). "Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP." J Clin Invest **116**(12): 3258-65.

Liu, E., N. Abiru, et al. (2002). "Induction of insulin autoantibodies and protection from diabetes with subcutaneous insulin B:9-23 peptide without adjuvant." Ann N Y Acad Sci **958**: 224-7.

Martinez, N. R., P. Augstein, et al. (2003). "Disabling an integral CTL epitope allows suppression of autoimmune diabetes by intranasal proinsulin peptide." J Clin Invest **111**(9): 1365-71.

Masteller, E. L., M. R. Warner, et al. (2003). "Peptide-MHC class II dimers as therapeutics to modulate antigen-specific T cell responses in autoimmune diabetes." J Immunol **171**(10): 5587-95.

Roncarolo, M. G., R. Bacchetta, et al. (2001). "Type 1 T regulatory cells." Immunol Rev **182**: 68-79.

Stratmann, T., N. Martin-Orozco, et al. (2003). "Susceptible MHC alleles, not background genes, select an autoimmune T cell reactivity." J Clin Invest **112**(6): 902-14.

Sundstedt, A., E. J. O'Neill, et al. (2003). "Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo." J Immunol **170**(3): 1240-8.

Tisch, R., X. D. Yang, et al. (1993). "Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice." Nature **366**(6450): 72-5.

Wakkach, A., N. Fournier, et al. (2003). "Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo." Immunity **18**(5): 605-17.

Wegmann, D. R., M. Norbury-Glaser, et al. (1994). "Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice." Eur J Immunol **24**(8): 1853-7.

Appendix I:

Early Autoimmune Destruction of Islet Grafts Is Associated with a Restricted Repertoire of IGRP-Specific $CD8^+$ T Cells in Diabetic Nonobese Diabetic Mice

Carmen P. Wong, Li Li, Jeffrey A. Frelinger, and Roland Tisch

Department of Microbiology and Immunology

School of Medicine

University of North Carolina, Chapel Hill

Chapel Hill, North Carolina

Abstract

β cell replacement via islet or pancreas transplantation is currently the only approach to cure type 1 diabetic patients. Recurrent β cell autoimmunity is a critical factor contributing to graft rejection along with alloreactivity. However, the specificity and dynamics of recurrent β cell autoimmunity remain largely undefined. Accordingly, we compared the repertoire of $CD8^+$ T cells infiltrating grafted and endogenous islets in diabetic nonobese diabetic mice. In endogenous islets, $CD8^+$ T cells specific for an islet-specific glucose-6-phosphatase catalytic subunit-related protein derived peptide ($IGRP_{206–214}$) were the most prevalent T cells. Similar $CD8^+$ T cells dominated the early graft infiltrate but were expanded 6-fold relative to endogenous islets. Single-cell analysis of the TCR α and β chains showed restricted variable gene usage by $IGRP_{206–214}$-specific $CD8^+$ T cells that was shared between the graft and endogenous islets of individual mice. However, as islet graft infiltration progressed, the number of $IGRP_{206–214}$-specific $CD8^+$T cells decreased despite stable numbers of $CD8^+$ T cells. These results demonstrate that recurrent β cell autoimmunity is characterized by recruitment to the grafts and expansion of already prevalent autoimmune T cell clonotypes residing in the endogenous islets. Furthermore, depletion of $IGRP_{206–214}$-specific $CD8^+$ T cells by peptide administration delayed islet graft survival, suggesting $IGRP_{206–214}$-specific $CD8^+$ T cells play a role early in islet graft rejection but are displaced with time by other specificities, perhaps by epitope spread.

Introduction

Type 1 diabetes (T1D) is an organ-specific autoimmune disease characterized by the destruction of the insulinproducing pancreatic β cells. The nonobese diabetic (NOD) mouse spontaneously develops T cell-dependent β cell destruction (1–3). $CD4^+$ T cells have an essential role in both regulating and mediating the diabetogenic response. It is also evident that autoreactive $CD8^+$ T cells play an important role in β cell destruction (4). $CD8^+$ T cell clones established from islet infiltrates of NOD mice mediate diabetes upon adoptive transfer, and diabetes is exacerbated in transgenic NOD mice expressing TCRs derived from pathogenic $CD8^+$ T cell clones (5–7). In addition, NOD mice that lack $CD8^+$ T cells, either by anti-CD8 Ab depletion (8) or a disrupted β_2-microglobulin gene (9 –12), fail to develop diabetes. Finally, pancreatic infiltrates (insulitis) of diabetic patients have significant numbers of $CD8^+$ T cells (13–16).

A concerted effort has been made to elucidate the β cell specificities of $CD8^+$ T cells involved in the pathogenesis of T1D. Early work showed that the TCR α-chain expressed by a high frequency of $CD8^+$ T cells infiltrating the islets of NOD mice was shared with the pathogenic 8.3 $CD8^+$ T cell clone (17). 8.3-like $CD8^+$ T cells are specific for an $H2K^d$-restricted epitope of isletspecific glucose-6-phosphatase catalytic subunit-related protein ($IGRP_{206–214}$) and are detected with $H2K^d$ (K^d) tetramers complexed with NRP mimotopes such as the high avidity NRP-A7 and NRP-V7 analogues (18 –20). Notably, selective expansion in peripheral blood and islets of high avidity/affinity NRP-A7- or NRPV7-specific clonotypes coincides with the onset of overt diabetes in NOD mice (19, 20). Peptides derived from the insulin B chain ($InsB_{15–23}$) (21) and dystrophia myotonica kinase ($DMK_{138–146}$) (22)

are also targeted in NOD mice by islet-infiltrating $H2K^d$- and $H2D^b$-restricted $CD8^+$ T cells, respectively. However, $IGRP_{206–214}$- specific clonotypes typically predominate in the islets relative to InsB- and DMK-specific $CD8^+$ T cells, especially at later stages of disease progression.

Islet or pancreas transplantation offers a permanent treatment for diabetic individuals. Analogous to other transplants, genetic differences in HLA between donor and recipient promote islet and pancreas graft rejection. In addition, successful β cell engraftment in diabetic patients is further complicated by recurrent autoimmunity (23, 24). The importance of β cell-specific $CD8^+$ T cells in recurrent autoimmunity is highlighted by studies demonstrating that MHC class I-deficient syngeneic islet grafts survive indefinitely in diabetic NOD mice (25, 26). However, the specificity of $CD8^+$ T cells associated with autoimmune-mediated destruction of islet grafts is undefined. One possibility is that T cell clonotypes involved in the destruction of endogenous islets are also recruited to the islet graft. Alternatively, "new" β cell specificities may be targeted in the islet graft due to "exhaustion" of clonotypes driving endogenous β cell destruction. Distinguishing between these and other possible scenarios is important for understanding the mechanism of recurrent autoimmunity and the development of strategies for inducing islet graft tolerance. Accordingly, the current study was initiated to gain insight into the nature of β cell-specific $CD8^+$clonotypes in autoimmune-mediated islet graft rejection.

Results

$IGRP_{206–214}$-specific $CD8^+$ T cells predominate the early infiltrates of syngeneic islet grafts

To gain insight into the mechanism of recurrent β cell autoimmunity, the specificity and frequency of $CD8^+$ T cells that infiltrate grafted vs endogenous islets were measured. Initially, the predominate $CD8^+$ clonotype(s) residing in the endogenous pancreas was assessed in nondiabetic 20-wk-old NOD female mice, which represent a late preclinical stage of T1D. ELISPOT was used to measure the relative frequency of IFN-γ-, IL-4-, and IL-10-secreting $CD8^+$ T cells specific for a panel of known β cell autoantigenic epitopes. This included $IGRP_{206–214}$ and the corresponding NRP-V7 mimotope, in addition to $InsB_{15–23}$, and $DMK_{138–146}$. $H2K^d$-restricted peptides derived from $ProInsB_{25\text{-}C34}$ (37) and GAD65 ($GAD65_{546–554}$) (38) were also tested. Pooled pancreatic islets from groups of four 20-wk-old NOD female mice were cultured for 3 days in IL-2-containing medium. Lymphocyte infiltrates were harvested and stimulated in vitro with the panel of peptides. IFN-γ-secreting $CD8^+$ T cells were detected in response to $IGRP_{206–214}$ and NRP-V7, but not $InsB_{15–23}$, $DMK_{138–146}$, $ProIns_{B25\text{-}C34}$, $GAD65_{546–554}$, or the control influenza NP peptide (Fig. 1*A*). No IL-4 or IL-10-secreting T cells were detected above background in response to any of the peptides tested. Similar results were obtained when lymphocyte infiltrates isolated from islets of individual 20 wk-old NOD female mice were examined (data not shown). Consistent with the ELISPOT data, $H2K^d$ tetramers complexed with NRP-V7 (K^d-NRPV7) bound $CD8^+$ T cells from islets prepared from four individual nondiabetic 20-wk old NOD female mice (Fig. 1*B*). K^d-NRPV7 bound 7.9 ± 2.8% of islet-infiltrating $CD8^+$ T cells, whereas only minimal binding was observed with K^d-InsB15–23 (0.7± 0.3%) or K^d-NP (0.4 ± 0.1%) (Fig. 1*B*). K^d-$NRPV7^+$

$CD8^+$ T cells were also detected in the pancreatic lymph nodes (PLN) (0.4 ± 0.1%) and spleen (0.5 ± 0.2%), albeit at lower frequencies than that seen in the islets (Fig. 1*B*). Because increased binding to $CD8^+$ T cells prepared from 8.3 TCR NOD transgenic mice was detected for K^d-NRPV7 compared with K^d tetramer complexed with $IGRP_{206–214}$ (K^d-IGRP) (data not shown), NRP-V7 tetramers were used in subsequent experiments to detect $IGRP_{206–214}$-specific clonotypes ex vivo.

The aforementioned results indicated that $IGRP_{206–214}$-specific $CD8^+$ T cells were the most prevalent of the known MHC class I-restricted β cell-specificities in the islets; therefore, efforts initially focused on K^d-NRPV7 binding in syngeneic islet grafts. Recent onset diabetic NOD female mice were transplanted with islets prepared from NOD.*scid* donor mice. Recurrent diabetes was typically detected ~2 wk postimplantation. The infiltrates from grafted and endogenous islets were compared 7 days postimplantation within individual recipients. Strikingly, a marked increase in the frequency of K^d-$NRPV7^+$ $CD8^+$ T cells was detected in islet grafts (42.1%) (Fig. 2*A*) compared with the endogenous islets (8.9%) (Fig. 2*B*). Few K^d-$NRPV7^+$ $CD8^+$ T cells were detected in the draining renal lymph node (0.7%), PLN (0.9%), or spleen (1.4%) of islet graft recipients (Fig. 2, *C–E*). In 10 recipients analyzed, a >6-fold increase in the frequency of K^d-$NRPV7^+$ $CD8^+$ T cells was detected in grafted vs endogenous islets ($p = 0.003$) (Fig. 2*F*). Minimal staining (0.6%) was observed using the control K^d-NP tetramer in all samples analyzed. Furthermore, no significant staining above background was detected with K^d-InsB (0.8 ± 0.3%) or K^d-ProIns (0.6 ± 0.2%) tetramers. Consistent with a role as effector cells, 98% of K^d-$NRPV7^+$ $CD8^+$ T cells infiltrating the islet graft were $CD62L^{low}CD44^{high}$ (data not shown).

The marked increase in K^d-NRPV7$^+$ CD8$^+$ T cells infiltrating the transplant was dependent on H2Kd expression by the islet graft. In diabetic NOD ($H2D^bK^d$) recipients of BALB/c ($H2D^dK^d$) islets, a 5-fold increase of K^d-NRPV7$^+$ CD8$^+$ T cells was detected in grafted (13.9 ± 0.9%) vs endogenous (2.8 ± 1.0%) islets ($p = 0.003$) (Fig. 3). In contrast, in NOD recipients of FVB ($H2D^qK^q$) islets, K^d-NRPV7$^+$ CD8$^+$ T cells were detected in the graft, but the frequency of tetramer binding CD8$^+$ T cells was equivalent to that detected in the endogenous islets (2.7 ± 0.6% vs 3.3 ± 1.9%, respectively) (Fig. 3). These results demonstrate that $IGRP_{206–214}$- specific CD8$^+$ T cells dominate the early infiltrate of syngeneic islet grafts, and that the frequency of this set of clonotypes is significantly expanded in grafts compared with the endogenous islets.

The TCR repertoire of $IGRP_{206–214}$-specific CD8$^+$ T cells in grafted and endogenous islet infiltrates is restricted and shared

To determine the diversity of $IGRP_{206–214}$-specific CD8$^+$ T cells residing in grafted vs endogenous islets, the TCR repertoire of K^d-NRPV7$^+$ CD8$^+$ T cells was examined in four individual recipients 7 days postimplantation via single-cell sorting and RT-PCR. A total of 53 Vα TCR sequences were analyzed from K^d- NRPV7$^+$ CD8$^+$ T cells isolated from grafted and endogenous islets, all of which used the Vα17-Jα42 segment (IMGT nomenclature, TRAV16-TRAJ42) characteristic of $IGRP_{206–214}$-specific clonotypes with a conserved N junction. Analysis of the TCR β -chain revealed preferential usage of Vβ 8.1 (TRBV13–3), and Jβ 2.4 (TRBJ2–4) and Jβ 2.7 (TRBJ2–7) (Fig. 4, *A* and *B*). Alignment of the CDR3β segments indicated a restricted number of T cell clones in each recipient, with one or two

dominant clonotypes comprising up to 87% of Kd-NRPV7$^+$ CD8$^+$ T cells analyzed (Fig. 4*C*). Notably, these clonotypes were found to be dominant in both grafted and endogenous islets of individual recipients (Fig. 4*C*). However, when the TCR repertoires of K^d-NRPV7$^+$ CD8$^+$ T cells were compared among the recipients, different sets of clones were detected in each recipient (Fig. 4*C*). The identity of the dominant clones also differed among the four recipient mice analyzed. Indeed, only two clonotypes with the respective CDR3β usage of SDSQNTL and SDGTYEQ were repeatedly observed (Fig. 4*C*). Taken together, these results indicate that in diabetic NOD mice, the TCR repertoire of IGRP$_{206–214}$-specific CD8$^+$ T cells infiltrating grafted and endogenous islets is shared and limited to a few dominant clonotypes. Furthermore, clonotypic variation exists within IGRP$_{206–214}$-specific CD8$^+$ T cells among individual recipient mice.

The specificity of CD8$^+$ T cells infiltrating an islet graft variesin a temporal manner

Next, the frequency of K^d-NRPV7$^+$ CD8$^+$ T cells was examined shortly before graft failure. The percentage of K^d-NRPV7$^+$ CD8$^+$ T cells present in the grafted islets was significantly reduced by day 13 postimplantation (Fig. 5*A*). An average of 4.7 ± 1.1% of CD8$^+$ T cells bound K^d-NRPV7 tetramers compared with 24.1 ± 4.3% in infiltrates of day 7 grafted islets ($p = 0.001$). The former was not significantly expanded compared with that detected in the endogenous islets (2.9 ± 1.6%). To determine whether this reduction was attributed to an influx of non-K^d-NRPV7$^+$ CD8$^+$ T cells, the number of CD4$^+$, CD8$^+$, and K^d-NRPV7$^+$ CD8$^+$ T cells present within the grafted and endogenous islets was analyzed. A 7-fold increase in CD4$^+$ T cells was observed in the islet graft infiltrates between days 7 and 13 ($p = 0.006$) (Table I). In comparison, the number of CD8$^+$ T cells increased only slightly (1.5-fold)

during this period. Strikingly, there was a 3-fold decrease in the number of K^d-NRPV7$^+$ CD8$^+$ T cells detected between days 7 and 13 in the grafted islets ($p = 0.02$) despite a relatively constant number of CD8$^+$ T cells in the islet graft. Furthermore, the number of K^d-NRPV7$^+$ CD8$^+$ T cells found in grafted and endogenous islets at 13 days postimplantation was equivalent (Table I). In contrast, at day 7 postimplantation, the number of K^d- NRPV7$^+$ CD8$^+$ T cells was increased >5-fold compared with the endogenous islets (Table I). No significant change in T cell numbers was observed in the endogenous islet infiltrates of the recipient mice between the two time points (Table I). The reduction of K^d-NRPV7$^+$ CD8$^+$ T cells in grafted islets could not be attributed to the influx of InsB-specific or ProIns-specific CD8$^+$ T cells, as staining with K^d-InsB (0.7 ± 0.1%) and K^d-ProIns (0.4 ± 0.3%) tetramers, respectively, was not significantly above that detected with Kd-NP tetramers (0.4 ± 0.04%). Similar to results observed at 7 days postimplantation, there was a preferential usage of Vβ8.1 (TRBV13–3) with Jβ2.4 (TRBJ2–4) or Jβ2.7 (TRBJ2–7) among K^d-NRPV7 binding CD8$^+$ T cells sorted from 13-day grafted and endogenous islets (Fig. 5, *B* and *C*). The TCR clonotypes of K^d-NRPV7 binding CD8$^+$ T cells detected in the grafted and endogenous islets were represented at similar frequencies (Fig. 5*D*), and the identity of the dominant clonotype(s) varied among the recipient mice. Collectively, these results demonstrate that the TCR repertoire of $IGRP_{206–214}$-specific CD8$^+$ T cells remains constant as islet graft destruction progresses, but that the number of these CD8$^+$ T cells declines.

Depletion of $IGRP_{206–214}$-specific CD8$^+$ T cells delays islet graft rejection

Because $IGRP_{206–214}$-specific CD8$^+$ T cells dominated the early pool of graft-infiltrating CD8$^+$ T cells, whether survival of the transplanted islets could be enhanced by depleting

these T cells was investigated. For this purpose, high doses of soluble peptide were administered. Injections of soluble $IGRP_{206–214}$ or NRP-V7 peptides were equally effective in near complete depletion of K^d- $NRPV7^+$ $CD8^+$ T cells (data not shown). Diabetic NOD mice were injected i.v. three times with soluble $IGRP_{206–214}$ in PBS on 2, 4, and 6 days before islet implantation. Two more peptide immunizations were given at 5 and 12 days postislet implantation to ensure continued depletion. Circulating levels of K^d-$NRPV7^+CD8^+$ T cells in peripheral blood before islet transplantation were significantly reduced after $IGRP_{206–214}$ (p = 0.002) but not HA peptide immunization (Table II). The frequency of Kd-$NRPV7^+$ $CD8^+$ T cells was also markedly reduced (0.3%) in graft infiltrates of $IGRP_{206–214}$-treated recipient mice examined 7 days postislet implantation. This indicates that $IGRP_{206–214}$ treatment effectively depleted K^d-$NRPV7^+$ $CD8^+$ T cells in peripheral blood and prevented infiltration of $IGRP_{206–214}$-specific $CD8^+$ T cells into the islet grafts.

The duration of graft survival in untreated and HA peptidetreated transplant recipients was not significantly different, with median graft survival of 15 and 12 days, respectively (Fig. 6). In contrast, islet graft survival in $IGRP_{206–214}$-treated mice was delayed with a median of 31 days (five mice per treatment group, p= 0.05, $IGRP_{206–214}$ vs untreated; p= 0.03, $IGRP2_{06–214}$ vs HA; log-rank test) (Fig. 6). One $IGRP_{206–214}$ treated-mouse remained euglycemic at 67 days postimplantation when the experiment was terminated. Recurrent diabetes in the remaining four $IGRP_{206–214}$-treated mice was not due to reappearance of K^d-$NRPV7^+$ $CD8^+$ T cells. For example, a reduced number of K^d-$NRPV7^+$ $CD8^+$ T cells was detected in islets implanted in $IGRP_{206–214}$ vs HA treated recipient mice (p = 0.04) at the time of onset of recurrent diabetes (Table II). No significant binding with K^d-InsB15–23 (0.5 ± 0.3%) or K^d-

ProInsB (0.2 ± 0.1%) tetramers was detected in the graft infiltrates of $IGRP_{206–214}$-treated recipients. These findings demonstrate that depletion of $IGRP_{206–214}$-specific $CD8^+$ T cells delays islet graft rejection.

Discussion

Established autoimmunity in diabetic islet (or pancreas) transplant recipients is an important factor contributing to the failure of subsequent β cell engraftment (23–26). $CD4^+$ and $CD8^+$ T cells have been reported to mediate autoimmune destruction of both allogeneic and syngeneic islet grafts (25, 26, 39–41). To develop effective strategies to induce and monitor islet transplantation tolerance in the clinic, knowledge of the β cell epitopes targeted by T cells and the dynamics of autoimmune-mediated destruction of an islet graft is needed. In the current study, these issues were examined by comparing the repertoire of β cell-specific $CD8^+$ T cells found infiltrating grafted and endogenous islets in diabetic NOD recipient mice.

A key observation made in this study is that autoimmune destruction of islet grafts is mediated by a restricted repertoire of β cell-specific $CD8^+$ T cells, which in turn evolves in a time-dependent manner. $IGRP_{206–214}$-specific $CD8^+$ T cells predominated in graft infiltrates 7 days postimplantation with up to 42% of infiltrating $CD8^+$ T cells binding K^d-NRPV7 tetramer (Fig. 2). Attempts to assess graft infiltrates at earlier posttransplantation times were unsuccessful due to insufficient T cell numbers. Detection of $IGRP_{206–214}$-specific $CD8^+$ T cells in the islet grafts is consistent with reports demonstrating the importance of this set of clonotypes in mediating the progression of β cell destruction in endogenous islets (19, 20). The frequency of K^d-$NRPV7^+$ $CD8^+$ T cells at 7 days postimplantation represented an >6-

fold increase in grafted vs endogenous islets (Fig. 2). Expansion of $IGRP_{206–214}$-specific $CD8^+$ T cells was dependent on $H2K^d$ expression by the transplanted islets. For example, a significant increase in K^d-$NRPV7^+$ $CD8^+$ T cells compared with endogenous islets was detected in BALB/c ($H2K^d$) but not FVB ($H2K^q$) islets (Fig. 3). This increase in K^d-$NRPV7^+$ $CD8^+$ T cells is likely due to direct and indirect presentation of the $IGRP_{206–214}$ epitope by $H2K^d$ expressing donor β cells and APC residing in the graft, respectively. Albeit reduced relative to NOD and BALB/c islets, a significant frequency of K^d-$NRPV7^+$ $CD8^+$ T cells was also detected in infiltrates of MHC mismatched FVB islets (Fig. 3). This result suggests that, in fully MHC mismatched islet grafts, autoimmunemediated destruction occurs via cross-presentation and –priming by recipient APC. Notably, the frequency and number of K^d-$NRPV7^+$ $CD8^+$ T cells varied in a temporal manner despite a relatively constant number of $CD8^+$ T cells during infiltration and destruction of syngeneic islet grafts. For instance, a >3-fold reduction in the number of K^d-$NRPV7^+$ $CD8^+$ T cells was detected in NOD islet grafts 13 vs 7 days postimplantation (Table I). The progressive loss of K^d-$NRPV7^+$ $CD8^+$ T cells suggests that $IGRP_{206–214}$-specific $CD8^+$ T cells are recruited into the islet graft from a finite pool, and undergo expansion and subsequent contraction. A similar profile of expansion and contraction was detected in islet grafts after adoptive transfer of $CD8^+$ T cells isolated from 8.3 TCR NOD transgenic mice (C. P. Wong and R. Tisch, unpublished results). The above findings also suggest that inter- (and intra-) molecular epitope spread occurs in an ordered progression during islet graft destruction. By 13 days postimplantation, $IGRP_{206–214}$-specific $CD8^+$ T cells are displaced as a major set of clonotypes in the islet graft by other $CD8^+$ T cells that, however, do not include either $InsB_{15–23}$- and $ProIns_{B25\text{-}C34}$-specific $CD8^+$ T cells. The specificity and diversity of these additional clonotypes are of obvious interest, and need

to be defined. These results suggest a scenario in which $IGRP_{206–214}$-specific $CD8^+$ T cells promote early autoimmune destruction of islet grafts and subsequent epitope spread. Indeed, a delay (albeit short-lived) was detected in the onset of recurrent diabetes in islet graft recipient mice treated with high doses of soluble peptide (Fig. 6) and depleted of $IGRP_{206–214}$-specific $CD8^+$ T cells (Table II). This delay in islet graft rejection may reflect the recruitment and/or differentiation of sufficient numbers of other pathogenic effectors. These results also indicate that islet graft rejection can be mediated in the absence of $IGRP_{206–214}$-specific $CD8^+$ T cells.

Single-cell analysis of TCR Vα and Vβ gene usage by K^d-$NRPV7^+$ $CD8^+$ T cells demonstrated that the immunodominant clonotypes mediating β cell destruction in the endogenous islets were also recruited to the islet grafts. All of the sorted K^d-$NRPV7^+$ $CD8^+$ T cells expressed the canonical Vα17-Jα42 element characteristic of $IGRP_{206–214}$-specific clonotypes (17, 36). However, as determined by CDR3β sequences, up to two dominant clonotypes were detected in the endogenous islets that, in turn, were also found to dominate the islet graft of an individual recipient (Figs. 4 and 5). The diversity of these immunodominate clonotypes may in fact be greater based on recent findings by Santamaria and colleagues (42) showing that three different Vα17 elements are used by $IGRP_{206–214}$-specific clonotypes. Due to the positioning of primers used in our study, the sequence spanning CDR1α that contains the respective substitutions in the Vα17 elements could not be determined. These findings indicate that the $IGRP_{206–214}$-specific $CD8^+$ T cells driving early islet graft infiltration are recruited from an already established pool of effector and/or memory T cells as opposed to naive precursors. Immunodominance within the islet graft is

likely to be established by clonotypes found at a relatively high frequency and/or exhibiting increased avidity/affinity. Indeed, progression toward overt diabetes in NOD mice corresponds with the expansion of $IGRP_{206–214}$-specific $CD8^+$ T cells having increased vidity/affinity (20). However, whether recruitment of other β cell-specific clonotypes to the islet graft follow the same "rules" as $IGRP_{206–214}$-specific $CD8^+$T cells remains to be determined.

In summary, autoimmune destruction of islet grafts is characterized by a restricted repertoire of β cell-specific $CD8^+$ T cells, and an apparent ordered progression of epitopes that are targeted. Early infiltrates are dominated by established effector and/or memory $IGRP_{206–214}$-specific $CD8^+$ T cells that are needed for efficient islet graft rejection. Finally, tolerogenic strategies targeting graft-infiltrating β cell-specific $CD8^+$ T cells may prove to be of significant clinical value in preventing recurrent autoimmunity in islet transplantation.

Materials and Methods

Mice

NOD/LtJ, NOD.Cg-Tg(TcraTcrbNY8.3)1Pesa (8.3 TCR transgenic), and NOD.CB17.Prkdc*scid*/J (NOD.*scid*) mice were bred and housed under specific pathogen-free conditions. Diabetes was monitored weekly by measuring urine glucose levels with Diastix (Bayer). Mice were diagnosed as diabetic when the level of urine glucose exceeds 0.25% for two successive measurements according to manufacturer's guidelines. A urine glucose level of 0.25% is equivalent to a blood glucose value of >250 mg/dl as determined by an Autokit Glucose CII assay (WAKO) (data not shown). BALB/c and FVB/J mice were bred and

housed in filter-covered isolator cages. Animals were maintained at an American Association of Laboratory Animal Care-accredited animal facility. All procedures were reviewed and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Peptides

MHC class I peptides NRP-V7 (KYNKANVFL), IGRP206–214 (VYLKTNVFL), GAD546–554 (SYQPLGDKV), InsB15–23 (LYLVCGERG), InsBG9V(LYLVCGERV), proinsulin (ProInsB25-C34; FYTPMSRREV), DMK138–146 (FQDENYLYL), influenza-derived hemagglutinin (HA512–520,IYSTVASSL), and nucleoprotein (NP147–155, TYQRTRALV) were synthesized at the University of North Carolina Peptide Synthesis Core Facility. The InsB-G9V peptide was modified from its native sequence to increase MHC class I stability (27).

Tetramers, Abs, and flow cytometry

$H2K^d$ tetramers were prepared as described (28). Briefly, peptide/MHC monomers were purified by HPLC and biotinylated using biotin-protein ligase (Avidity). Tetramers were assembled by conjugating MHC monomers with streptavidin-PE (Molecular Probes). Fluorescent-conjugated antimouse mAbs used for cell surface staining include anti-CD4 purchased from BD Pharmingen, and anti-CD3, anti-CD8, anti-CD62L, and anti-CD44 purchased from eBioscience. Single-cell suspensions from spleens, lymph nodes, islets, and islet grafts were prepared in PBS. Peripheral blood was collected via the tail vein and RBC lysed where appropriate. T cells were costained with tetramers and Abs in PBS containing 3% FBS, 10 mM HEPES, and 1 mM EDTA for 1 h on ice. Flow cytometry data were

acquired on FACSCalibur (BD Biosciences) and analyzed using Summit software (DakoCytomation). For all tetramer analyses, $CD8^+$ T cells were gated based on forward and side scatter and CD3 and CD8 expression. For single-cell analyses, K^d-NRPV7 tetramer-binding $CD8^+$ T cells were sorted by a MoFlo high-speed sorter (DakoCytomation) into 25 ul of RT-PCR buffer at one cell per well of a 96-well PCR plate (USA Scientific), and the RT-PCR was performed immediately (see third paragraph below). All flow cytometry analyses and single-cell sorting were performed at the University of North Carolina Flow Cytometry facility.

Pancreatic islet isolation

Pancreases were perfused with 0.2 mg/ml Liberase (Roche) and digested for 30 min at 37°C. Islets were purified via Ficoll gradient and handpicked. For flow cytometry analysis, freshly isolated islets were dissociated into a single-cell suspension using enzyme-free cell dissociation solution (Sigma-Aldrich) before staining. Alternatively, islets were cultured overnight in RPMI 1640 containing 10% FBS and 4 ng/ml recombinant murine IL-2 (PeproTech). Lymphocytes infiltrating the islets were collected and cellular debris was removed by 70-um nylon filters. For ELISPOT, islets were cultured up to 3 days in IL-2-containing medium before use.

Islet transplantation and graft harvest

Recent onset diabetic NOD female mice received daily insulin injections until the day of transplantation. Recipients were transplanted within 2 wk of glycosuria. Five hundred freshly isolated syngeneic (NOD.*scid*) or allogeneic (BALB/c or FVB) islets were transplanted under

the renal capsule of the left kidney. Urine glucose levels were monitored daily posttransplantation. Successful islet engraftment was defined as restoration of glycemic control for a minimum of 7 days. Graft failure was defined as glycosuric values exceeding 0.25% (>250 mg/dl blood glucose) for two successive measurements. At 7 or 13 days posttransplantation, or shortly after graft failure, the area of kidney containing the visible islet graft was dissected. A single-cell suspension of the islet graft was prepared by lysing RBC, removing debris using a 70-um nylon filter, and resuspending in FACS buffer for flow cytometric analysis. For a negative control, a similar sized tissue sample was dissected from the nontransplanted kidney and processed accordingly.

Single-cell RT-PCR and TCR repertoire analyses

TCR usage was analyzed by a single-cell PCR protocol previously described (29) with the following modifications. Single-cell RT-PCR was performed using a Qiagen OneStep RT-PCR kit (Qiagen) according to the manufacturer's protocol. A panel of primers specific for all known TCR α-or β-chain variable regions and respective constant regions were used for reverse transcription and first-round PCR amplification. RT-PCR amplicons (2.5 ul) were used as templates for second-round PCR amplification using a panel of nested TCR α- or β-chain-specific primers. All oligonucleotides were synthesized at the Nucleic Acids Core Facility at the University of North Carolina. PCR products were treated with Exonuclease (NEB Biolabs) and shrimp alkaline phosphatase (Roche), and sequenced at the University of North Carolina Genome Analysis Facility. TCR sequence alignments were performed using Sequencher software (Gene Codes). TCR α- and β-chain (TRA and TRB, respectively) gene family usage was identified and assigned using the international ImMunoGeneTics (IMGT)

information system (http://imgt.cines.fr; Refs. 30–35) and former nomenclature based on Arden et al. (36).

ELISPOT

ELISPOT plates (Millipore) were coated overnight at 4°C with purified rat anti-mouse cytokine Abs in PBS (anti-IFN-γ, anti-IL-4, or anti-IL-10) (BD Pharmingen). Plates were seeded with islet-infiltrating lymphocytes at 1 x 10^4 cells per well in HL-1 medium (BioWhittaker), and 5 x 10^5 irradiated splenocytes were added. Peptides were added at a final concentration of 10 ug/ml. Cultures were incubated for 24 h at 37°C. Cells were removed by washing, and the plates were incubated with the appropriate biotinylated anti-mouse cytokine Abs overnight at 4°C. Plates were then washed, incubated with streptavidin-HRP (BD Pharmingen) for 2 h at room temperature, and developed using a 100-mM sodium acetate buffer containing 0.3 mg/ml 3-amino-9-ethylcarbazole (Sigma-Aldrich) and 0.015% hydrogen peroxide. An ImmunoSpot plate reader (Cellular Technology) was used to count the spot-forming cells (SFC) per well.

Peptide immunization

Diabetic NOD mice were immunized i.v. with 200 ug of IGRP or HA peptide in PBS. A total of five immunizations were given at 2, 4, and 6 days before islet implantations, and at 5 and 12 days postimplantation. Levels of $IGRP_{206–214}$-specific $CD8^+$ T cells in peripheral blood were determined by flow cytometry before the first peptide immunization and after the third injection before islet transplantation using K^d-NRPV7 tetramer. Alternatively, peptide-treated diabetic NOD mice received islet grafts, and islet

infiltrates were analyzed 7 days postimplantation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software). Values of p were calculated using Student's t test. Survival curves were compared using Kaplan Meier log-rank test.

Acknowledgments

We thank Dr. Pere Santamaria for providing 8.3-NOD mice used in this study, and Carrie Barnes for technical assistance.

Disclosures

The authors have no financial conflict of interest.

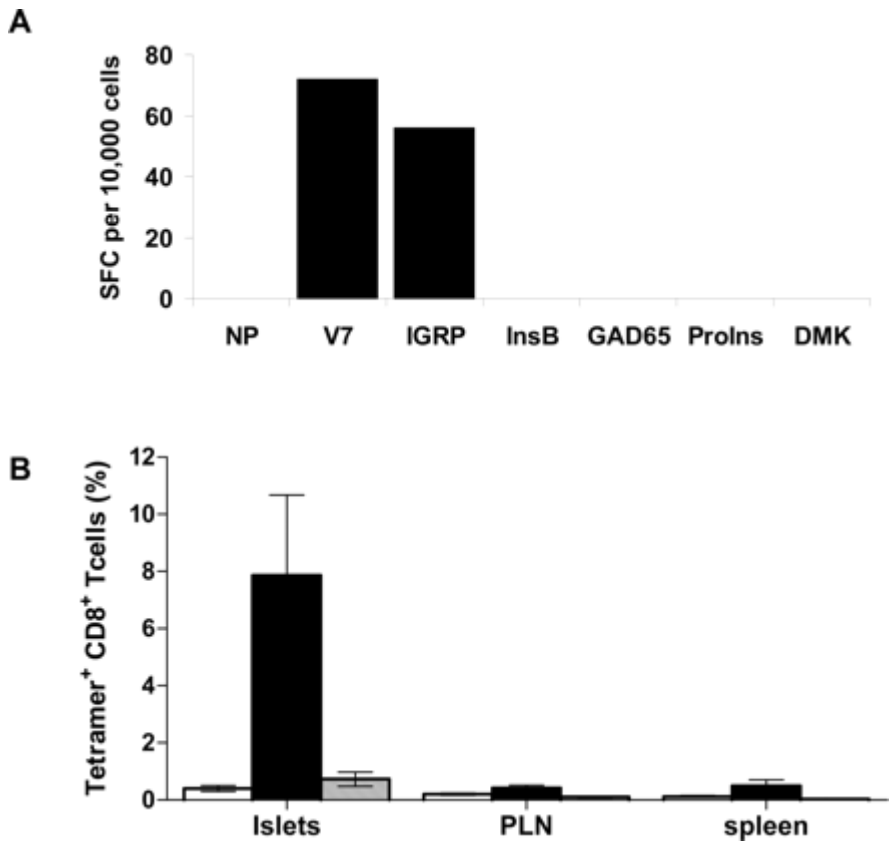


FIGURE 1. $IGRP_{206–214}$-specific $CD8^+$ T cells are the prevalent β cell-specific clonotypes in the islets of 20-wk-old NOD female mice. *A*, Pooled islet T cell infiltrates from four 20-wk-old NOD female mice were expanded in IL-2-containing medium. ELISPOT was used to measure the frequency of IFN-γ-secreting T cells upon restimulation with a panel of MHC class I-restricted peptides (NP, NRP-V7, $IGRP_{206–214}$, $InsB_{15–23}$, $GAD_{546–554}$, $ProIns_{B25\text{-}C34}$, and $DMK_{138–146}$). IFN-γ-specific SFC per 10,000 islet-infiltrating lymphocytes is shown after subtraction of background (approximately six SFC) in medium-only wells. Data are representative of four separate experiments. *B*, The average percentage (±SEM) of K^d-$NRPV7^+$ (■) and K^d-$InsB_{15–23}^+$ (▩) $CD8^+$ T cells isolated from islet infiltrates, PLN, and spleens of four individual 20-wk-old NOD female mice was determined. K^d-NP served as a negative control (□).

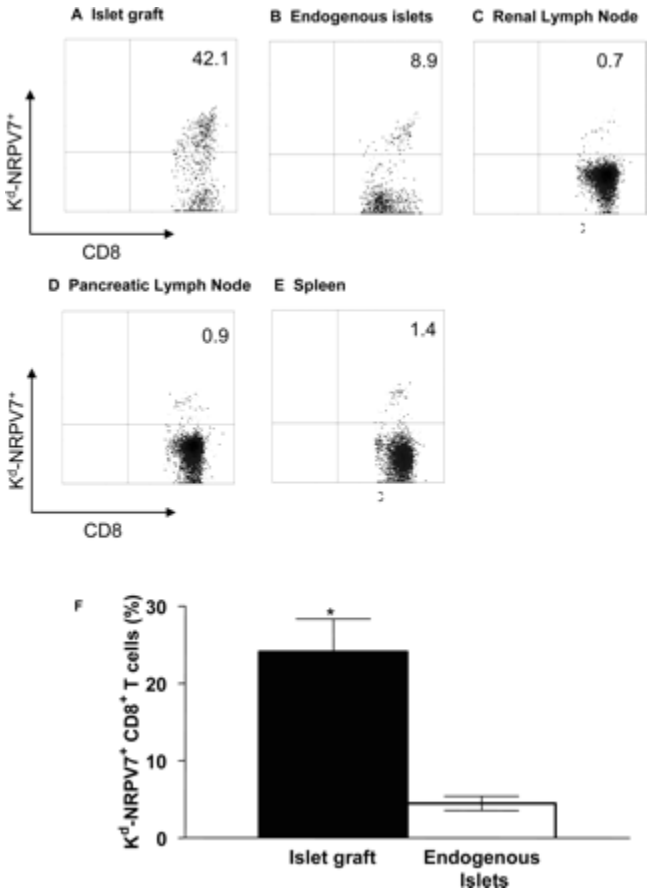


FIGURE 2. K^d-NRPV7$^+$ CD8$^+$ T cells dominate the early infiltrates of syngeneic islet grafts. Representative K^d-NRPV7 staining profiles in a diabetic NOD female mouse transplanted with a syngeneic islet graft. The frequencies of K^d-NRPV7$^+$ CD8$^+$ T cells in islet graft (*A*), endogenous islets (*B*), draining renal lymph node (*C*), PLN (*D*), and spleen (*E*) were analyzed 7 days postimplantation. Numbers indicate percentage of tetramer-positive CD8$^+$ T cells. The percentage of staining using control K^d-NP tetramer was <0.6%. *F*, Average percentage (±SEM) of K^d-NRPV7$^+$ CD8$^+$ T cells in grafted islets (■) and endogenous islets (□) in 10 diabetic transplant recipients (∗, $p = 0.003$, Student's *t* test).

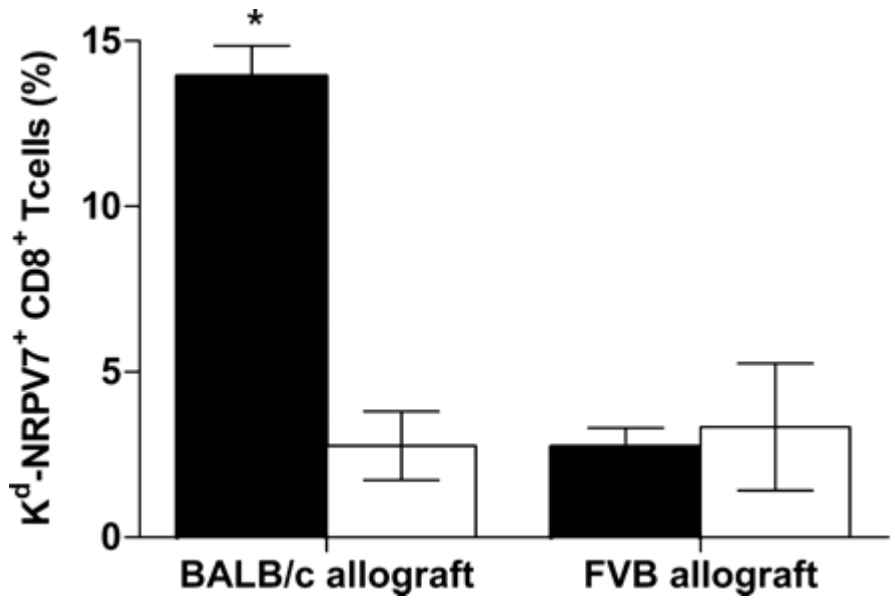


FIGURE 3. K^d-NRPV7$^+$ CD8$^+$ T cells are detected in islet allografts. Diabetic NOD female mice were transplanted with partially mismatched BALB/c islets ($H2D^dK^d$) or fully mismatched FVB islets ($H2D^qK^q$). The frequencies of K^d-NRPV7$^+$ CD8$^+$ T cells in endogenous (□) and grafted (■) islets were analyzed 7 days postimplantation. Numbers indicate the average percentage (±SEM) of K^d-NRPV7$^+$ CD8$^+$ T cells from three recipients per group. The percentage of staining using control K^d-NP was <0.6%. $*$, $p = 0.0013$, grafted BALB/c islets vs endogenous islets, Student's *t* test.

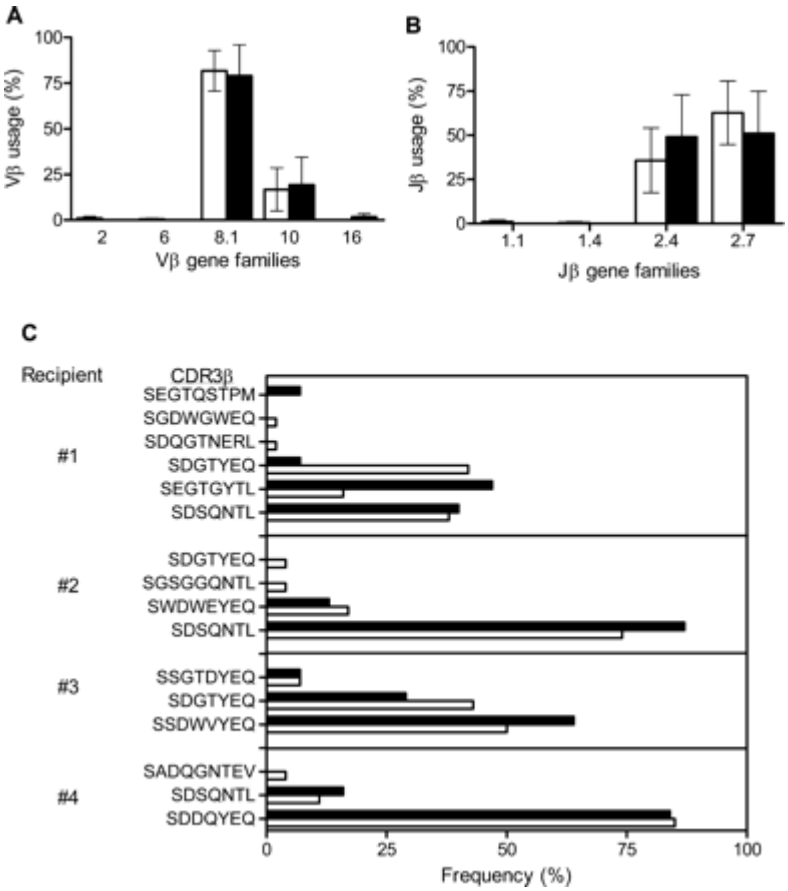


FIGURE 4. A restricted TCR repertoire of K^d-NRPV7$^+$ CD8$^+$ T cells is detected in grafted and endogenous islets. The TCR β-chain repertoire of K^d-NRPV7$^+$ CD8$^+$ T cells present in the endogenous and grafted islets in individual transplant recipients were compared using single-cell RT-PCR. K^d-NRPV7$^+$ CD8$^+$ T cells in endogenous islets (□) and grafted islets (■) were analyzed 7 days postimplantation for V β (*A*) and J β (*B*) gene usage. Vβ 2, Vβ 6, Vβ 8.1, Vβ 10, and Vβ 16 correspond to IMGT nomenclature of TRBV1, TRBV19, TRBV13–3, TRBV4, and TRBV3, respectively. Data represent averaged percentages derived from four islet recipients. *C*, Comparison of CDR3β usage of K^d-NRPV7$^+$ CD8$^+$ T cells in the endogenous islets and grafted islets from four individual transplant recipients. A total of 27, 14, 23, and 50 CDR3β sequences were analyzed from the endogenous islets of recipients 1–4, respectively. TCR sequences were compared with a total of 25, 14, 23, and 15 CDR3β sequences derived from the grafted islets of the respective recipients.

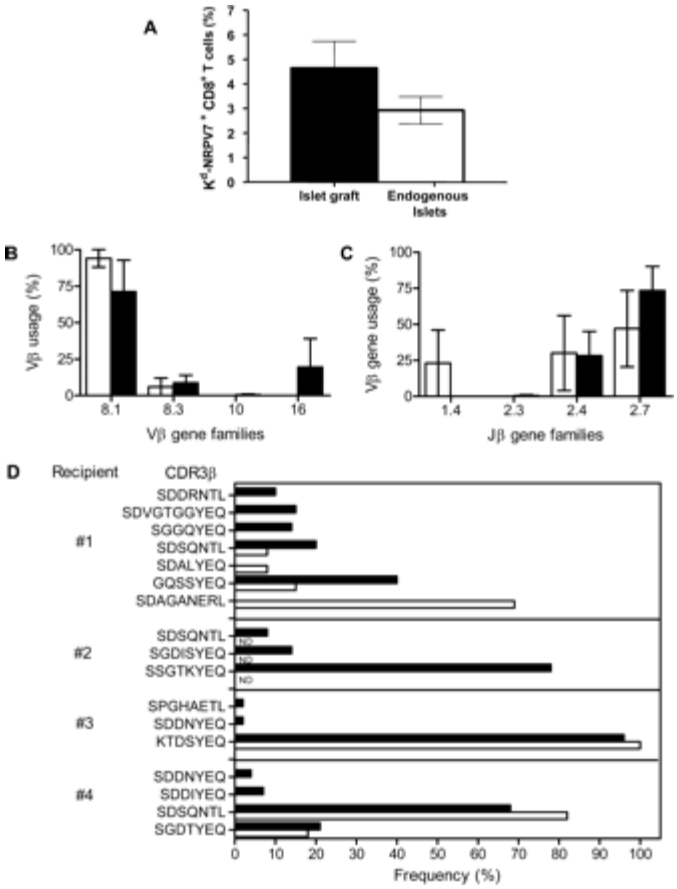


FIGURE 5. The frequency of K^d-NRPV7$^+$ CD8$^+$ T cells decreases as islet graft destruction progresses but the TCR repertoire remains constant. The frequency of K^d-NRPV7$^+$ CD8$^+$ T cells in endogenous (□) and grafted (■) islets in diabetic islet recipients was analyzed 13 days postimplantation (n = 8) *A*, The percentage of staining using control K^d-NP was <0.5%. The TCR β-chain repertoire of K^d-NRPV7$^+$ CD8$^+$ T cells present in grafted and endogenous islets from individual transplant recipients was determined using single-cell RT-PCR at 13 days postimplantation. Averaged frequencies of Vβ (*B*), Jβ (*C*), and CDR3β (*D*) gene usage of sorted K^d-NRPV7$^+$ CD8$^+$ T cells from four recipients are shown. Vβ8.1, Vβ8.3, Vβ10, and Vβ16 correspond to IMGT nomenclature of TRBV13–3, TRBV13–1, TRBV4, and TRBV3, respectively. A total of 11, 14, and 13 CDR3β sequences were analyzed from the endogenous islets of recipients 1, 3, and 4, respectively. TCR sequences were compared with a total of 28, 51, 36, and 20 CDR3β sequences derived from the grafted islets of the recipients 1–4, respectively. ND, Not done, islets were not recovered from recipient two for analysis.

Table I. *$CD4^+$, $CD8^+$, and K^d-$NRPV7^+$ $CD8^+$ T cells present in islet grafts and endogenous islets at 7 and 13 days posttransplantation*

Days Posttransplantation	Islet Graft (absolute number per 10,000 gated events)[a]			Endogenous Islets (absolute number per 10,000 gated events)[a]		
	$CD4^+$	$CD8^+$	$CD8^+$ K^d-$NRPV7^+$	$CD4^+$	$CD8^+$	$CD8^+$ K^d-$NRPV7^+$
Day 7	655 (±241)	826 (±207)	153 (±29)	3679 (±690)	1390 (±340)	31 (±8)
Day 13	4731 (±707)	1275 (±130)	45 (±3)	3410 (±808)	1390 (±259)	38 (±7)

[a] Data represents averaged events (±SEM) from three recipient mice at each time point. A total of 10,000 events were analyzed within the lymphocyte gate based on forward and side scatter, and subsequently were gated on $CD4^+$, $CD8^+$ and $CD8^+$ K^d-$NRPV7^+$ T cells.

Table II. *Frequency of K^d-NRPV7$^+$ CD8$^+$ T cells present in peripheral blood and islet graft infiltrates of diabetic transplant recipients treated with IGRP or HA peptides*

Peptide treatment	K^d-NRPV7$^+$ CD8$^+$ T Cells in Peripheral Blood Pretransplantation (%)[a]		Graft-Infiltrating CD8$^+$ T Cells (absolute number per 10,000 gated events)[b]	
	Prepeptide tx	Postpeptide tx	CD8	CD8$^+$$K^d$-NRP-V7$^+$
IGRP (*n* = 4)[c]	0.89 (±0.14)	0.11 (±0.03)	491 (±145)	2 (±1)[d]
HA (*n* = 5)	0.48 (±0.16)	0.43 (±0.13)	1220 (±330)	24 (±8)

[a] Averaged percentage (±SEM) of K^d-NRPV7$^+$ CD8$^+$ T cells in peripheral blood from diabetic mice pre- and postpeptide treatments prior to receiving islet grafts.

[b] Averaged data (±SEM) from peptide-treated, transplanted mice analyzed upon the onset of recurrent diabetes. A total of 10,000 events were analyzed within the lymphocyte gate based on forward and side scatter, and subsequently were gated on CD8 and K^d-NRPV7$^+$ T cells.

[c] Five mice were treated with IGRP peptide. One recipient mouse was used for histological assessment upon diagnosis of recurrent diabetes

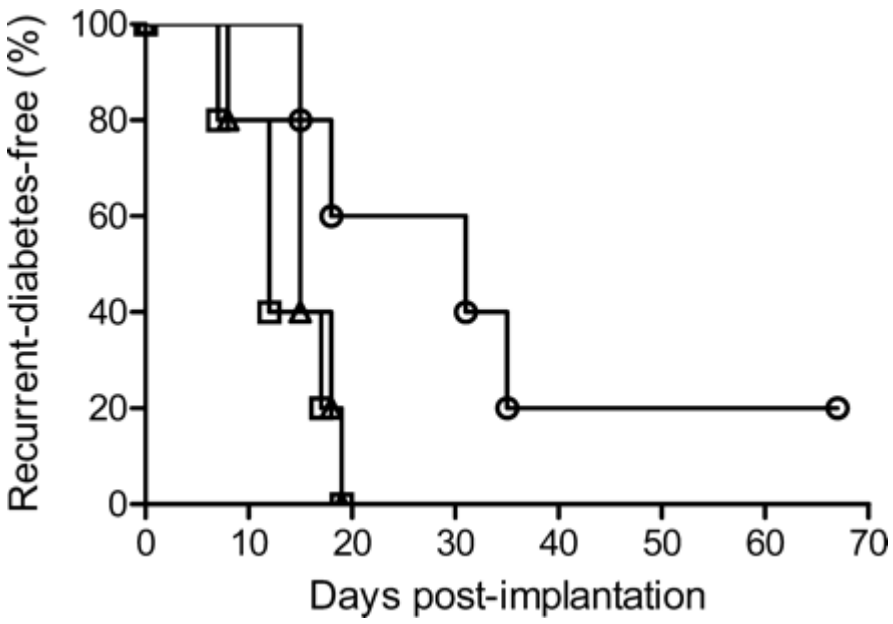


FIGURE 6. Islet graft rejection is delayed in diabetic NOD mice depleted of $IGRP_{206–214}$-specific $CD8^+$ T cells. Groups of five diabetic NOD female mice were left untreated (△), treated with 200 µg soluble $IGRP_{206–214}$ (○), or HA peptide (□) at 2, 4, and 6 days before islet transplantation. Depletion was verified by checking circulating levels of K^d-$NRPV7^+$ $CD8^+$ T cells in peripheral blood pre- and postpeptide treatment. Mice were transplanted with 500 NOD.*scid* islets, and received two additional doses of peptide at 5 and 12 days postimplantation. (Log-rank test, untreated vs $IGRP_{206–214}$, $p = 0.05$; HA vs $IGRP_{206–214}$, $p = 0.03$).

References

1. Anderson, M. S., and J. A. Bluestone. 2005. The NOD mouse: a model of immune dysregulation. *Annu. Rev. Immunol.* 23: 447–485.

2. Delovitch, T. L., and B. Singh. 1997. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity* 7: 727–738.

3. Tisch, R., and H. McDevitt. 1996. Insulin-dependent diabetes mellitus. *Cell* 85: 291–297.

4. DiLorenzo, T. P., and D. V. Serreze. 2005. The good turned ugly: immunopathogenic basis for diabetogenic CD8_ T cells in NOD mice. *Immunol. Rev.* 204: 250–263.

5. Graser, R. T., T. P. DiLorenzo, F. Wang, G. J. Christianson, H. D. Chapman, D. C. Roopenian, S. G. Nathenson, and D. V. Serreze. 2000. Identification of a CD8 T cell that can independently mediate autoimmune diabetes development in the complete absence of CD4 T cell helper functions. *J. Immunol.* 164: 3913–3918.

6. Wong, F. S., I. Visintin, L. Wen, R. A. Flavell, and C. A. Janeway, Jr. 1996. CD8 T cell clones from young nonobese diabetic (NOD) islets can transfer rapid onset of diabetes in NOD mice in the absence of CD4 cells. *J. Exp. Med.* 183: 67–76.

7. Verdaguer, J., D. Schmidt, A. Amrani, B. Anderson, N. Averill, and P. Santamaria. 1997. Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. *J. Exp. Med.* 186: 1663–1676.

8. Wang, B., A. Gonzalez, C. Benoist, and D. Mathis. 1996. The role of CD8_ T cells in the initiation of insulin-dependent diabetes mellitus. *Eur. J. Immunol.* 26: 1762–1769.

9. Sumida, T., M. Furukawa, A. Sakamoto, T. Namekawa, T. Maeda, M. Zijlstra, I. Iwamoto, T. Koike, S. Yoshida, H. Tomioka, et al. 1994. Prevention of insulitis and diabetes in _2-microglobulin-deficient non-obese diabetic mice. *Int. Immunol.* 6: 1445–1449.

10. Serreze, D. V., E. H. Leiter, G. J. Christianson, D. Greiner, and D. C. Roopenian. 1994. Major histocompatibility complex class I-deficient NOD-B2mnull mice are diabetes and insulitis resistant. *Diabetes* 43: 505–509.

11. Wicker, L. S., E. H. Leiter, J. A. Todd, R. J. Renjilian, E. Peterson, P. A. Fischer, P. L. Podolin, M. Zijlstra, R. Jaenisch, and L. B. Peterson. 1994. _2-Microglobulin- deficient NOD mice do not develop insulitis or diabetes. *Diabetes* 43:500–504.

12. Hamilton-Williams, E. E., S. E. Palmer, B. Charlton, and R. M. Slattery. 2003. _cell MHC class I is a late requirement for diabetes. *Proc. Natl. Acad. Sci. USA* 100: 6688–6693.

13. Hanninen, A., S. Jalkanen, M. Salmi, S. Toikkanen, G. Nikolakaros, and O. Simell. 1992. Macrophages, T cell receptor usage, and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes mellitus. *J. Clin.Invest.* 90: 1901–1910.

14. Itoh, N., T. Hanafusa, A. Miyazaki, J. Miyagawa, K. Yamagata, K. Yamamoto, M. Waguri, A. Imagawa, S. Tamura, M. Inada, et al. 1993. Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. *J. Clin. Invest.* 92: 2313–2322.

15. Somoza, N., F. Vargas, C. Roura-Mir, M. Vives-Pi, M. T. Fernandez-Figueras, A. Ariza, R. Gomis, R. Bragado, M. Marti, D. Jaraquemada, et al. 1994. Pancreas in recent onset insulin-dependent diabetes mellitus. Changes in HLA, adhesion molecules and autoantigens, restricted T cell receptor V_ usage, and cytokine profile. *J. Immunol.* 153: 1360–1377.

16. Santamaria, P., R. E. Nakhleh, D. E. Sutherland, and J. J. Barbosa. 1992. Characterization of T lymphocytes infiltrating human pancreas allograft affected by isletitis and recurrent diabetes. *Diabetes* 41: 53–61.

17. Anderson, B., B. J. Park, J. Verdaguer, A. Amrani, and P. Santamaria. 1999. Prevalent CD8_ T cell response against one peptide/MHC complex in autoimmune diabetes. *Proc. Natl. Acad. Sci. USA* 96: 9311–9316.

18. Lieberman, S. M., A. M. Evans, B. Han, T. Takaki, Y. Vinnitskaya, J. A. Caldwell, D. V. Serreze, J. Shabanowitz, D. F. Hunt, S. G. Nathenson, et al.
2003. Identification of the _ cell antigen targeted by a prevalent population of pathogenic CD8_ T cells in autoimmune diabetes. *Proc. Natl. Acad. Sci. USA* 100: 8384–8388.

19. Trudeau, J. D., C. Kelly-Smith, C. B. Verchere, J. F. Elliott, J. P. Dutz, D. T. Finegood, P. Santamaria, and R. Tan. 2003. Prediction of spontaneous
autoimmune diabetes in NOD mice by quantification of autoreactive T cells in peripheral blood. *J. Clin. Invest.* 111: 217–223.

20. Amrani, A., J. Verdaguer, P. Serra, S. Tafuro, R. Tan, and P. Santamaria. 2000. Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. *Nature* 406: 739–742.

21. Wong, F. S., J. Karttunen, C. Dumont, L. Wen, I. Visintin, I. M. Pilip, N. Shastri, E. G. Pamer, and C. A. Janeway, Jr. 1999. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. *Nat. Med.* 5: 1026–1031.

22. Lieberman, S. M., T. Takaki, B. Han, P. Santamaria, D. V. Serreze, and T. P. DiLorenzo. 2004. Individual nonobese diabetic mice exhibit unique patterns of CD8_ T cell reactivity to three islet antigens, including the newly identified widely expressed dystrophia myotonica kinase. *J. Immunol.* 173: 6727–6734.

23. Stegall, M. D., K. J. Lafferty, I. Kam, and R. G. Gill. 1996. Evidence of recurrent autoimmunity in human allogeneic islet transplantation. *Transplantation* 61: 1272–1274.

24. Okitsu, T., S. T. Bartlett, G. A. Hadley, C. B. Drachenberg, and A. C. Farney 2001. Recurrent autoimmunity accelerates destruction of minor and major histoincompatible islet grafts in nonobese diabetic (NOD) mice. *Am. J. Transplant.*1: 138–145.

25. Prange, S., P. Zucker, A. M. Jevnikar, and B. Singh. 2001. Transplanted MHC class I-deficient nonobese diabetic mouse islets are protected from autoimmune injury in diabetic nonobese recipients. *Transplantation* 71: 982–985.

26. Young, H. Y., P. Zucker, R. A. Flavell, A. M. Jevnikar, and B. Singh. 2004. Characterization of the role of major histocompatibility complex in type 1 diabetes recurrence after islet transplantation. *Transplantation* 78: 509–515.

27. Wong, F. S., A. K. Moustakas, L. Wen, G. K. Papadopoulos, and C. A. Janeway, Jr. 2002. Analysis of structure and function relationships of an autoantigenic peptide of insulin bound to H-2Kd that stimulates CD8 T cells in insulin-dependent diabetes mellitus. *Proc. Natl. Acad. Sci. USA* 99: 5551–5556.
28. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996.
Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94–96.

29. Baker, F. J., M. Lee, Y. H. Chien, and M. M. Davis. 2002. Restricted islet-cell reactive T cell repertoire of early pancreatic islet infiltrates in NOD mice. *Proc. Natl. Acad. Sci. USA* 99: 9374–9379.

30. Lefranc, M. P., V. Giudicelli, C. Ginestoux, J. Bodmer, W. Muller, R. Bontrop, M. Lemaitre, A. Malik, V. Barbie, and D. Chaume. 1999. IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res.* 27: 209–212.

31. Lefranc, M. P. 2001. IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res.* 29: 207–209.

32. Lefranc, M. P. 2003. IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res.* 31: 307–310.

33. Lefranc, M. P., V. Giudicelli, C. Ginestoux, N. Bosc, G. Folch, D. Guiraudou, J. Jabado-Michaloud, S. Magris, D. Scaviner, V. Thouvenin, et al. 2004. IMGTONTOLOGY for immunogenetics and immunoinformatics. *In Silico Biol.* 4:17–29.

34. Lefranc, M. P., V. Giudicelli, Q. Kaas, E. Duprat, J. Jabado-Michaloud, D. Scaviner, C. Ginestoux, O. Clement, D. Chaume, and G. Lefranc. 2005.
IMGT, the international ImMunoGeneTics information system. *Nucleic Acids Res.* 33: D593–D597.

35. Ruiz, M., V. Giudicelli, C. Ginestoux, P. Stoehr, J. Robinson, J. Bodmer, S. G. Marsh, R. Bontrop, M. Lemaitre, G. Lefranc, et al. 2000. IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res.* 28: 219–221.

36. Arden, B., S. P. Clark, D. Kabelitz, and T. W. Mak. 1995. Mouse T-cell receptor variable gene segment families. *Immunogenetics* 42: 501–530.

37. Martinez, N. R., P. Augstein, A. K. Moustakas, G. K. Papadopoulos, S. Gregori, L. Adorini, D. C. Jackson, and L. C. Harrison. 2003. Disabling an integral CTL epitope allows suppression of autoimmune diabetes by intranasal proinsulin peptide. *J. Clin. Invest.* 111: 1365–1371.

38. Quinn, A., M. F. McInerney, and E. E. Sercarz. 2001. MHC class I-restricted determinants on the glutamic acid decarboxylase 65 molecule induce spontaneous CTL activity. *J. Immunol.* 167: 1748–1757.

39. Mottram, P. L., L. J. Murray-Segal, W. Han, J. Maguire, A. Stein-Oakley, and T. E. Mandel. 1998. Long-term survival of segmental pancreas isografts in NOD/Lt mice treated with anti-CD4 and anti-CD8 monoclonal antibodies. *Diabetes*47: 1399–1405.

40. Makhlouf, L., S. T. Grey, V. Dong, E. Csizmadia, M. B. Arvelo, H. Auchincloss, Jr., C. Ferran, and M. H. Sayegh. 2004. Depleting anti-CD4 monoclonal antibody cures new-onset diabetes, prevents recurrent autoimmune diabetes, and delays allograft rejection in nonobese diabetic mice. *Transplantation* 77: 990–997.

41. Kupfer, T. M., M. L. Crawford, K. Pham, and R. G. Gill. 2005. MHC-mismatched islet allografts are vulnerable to autoimmune recognition in vivo. *J. Immunol.* 175: 2309–2316.

42. Han, B., P. Serra, A. Amrani, J. Yamanouchi, A. F. Maree, L. Edelstein-Keshet, and P. Santamaria. 2005. Prevention of diabetes by manipulation of anti-IGRP autoimmunity: high efficiency of a low-affinity peptide. *Nat. Med.* 11: 645–652.

Appendix II:

Identical β Cell-Specific $CD8^+$ T Cell Clonotypes Typically Reside in Both Peripheral Blood Lymphocyte and Pancreatic Islets

Carmen P. Wong, Rosemary Stevens, Brian Long, Li Li, Yaming Wang, Mark A. Wallet, Kevin S. Goudy, Jeffrey A. Frelinger, and Roland Tisch

Department of Microbiology and Immunology

School of Medicine

University of North Carolina, Chapel Hill

Chapel Hill, North Carolina

Abstract

A major issue regarding T cell responses in autoimmunity is how the repertoire compares between the periphery and target organ. In type 1 diabetes, the status of at-risk or diabetic individuals can be monitored by measuring β cell-specific T cells isolated from PBL, but whether these T cells accurately reflect the repertoire residing in the pancreatic islets is unclear. The TCR repertoire of disease-relevant, tetramer-sorted $CD8^+$ T cells was examined at the single-cell level in PBL, pancreatic lymph nodes (PLN), and the islets of individual NOD mice. CDR3α and CDR3β sequences demonstrated that the same repertoire of T cells in PBL was detected in the islets and PLN, although the frequency of specific clonotypes varied. Albeit infrequent, clonotypes that were prevalent in the islets but not found in PBL were also detected. β cell Ag immunization expanded immunodominant PBL clonotypes present in the islets and PLN. These results show that insight into repertoire profiles of islet-infiltrating T cells can be obtained from PBL.

Introduction

A key question in T cell immunology is how the repertoire at one site reflects the repertoire at distinct anatomical locations. This is of considerable importance in both tissue-targeted infections and in organ-restricted autoimmune diseases such as type 1 diabetes (T1D). Insight into the nature of an immune response can be gained by knowing whether T cells found in blood represent 1) a broad spectrum of clonotypes some of which are involved in mediating a localized response, or 2) a selected population identical with those effectors residing in the target tissue.

Autoimmune destruction of the insulin-producing β cells in T1D is mediated by $CD4^+$ and $CD8^+$ T cells (1– 4). Several β cell autoantigens are targeted by T cells in diabetic patients and the NOD mouse, a model for T1D (1– 4). An $H2K^d$-restricted peptide derived from islet-specific glucose-6-phosphatase catalytic subunit-related protein $(IGRP)_{206–214}$ is recognized by a high frequency of $CD8^+$ T cells residing in islet infiltrates of NOD mice (5, 6). Several lines of evidence suggest that $IGRP_{206–214}$-specific $CD8^+$ T cells play a critical role in T1D. First, the frequency and TCR avidity of $IGRP_{206–214}$-specific $CD8^+$ T cells increase in the islet infiltrates during disease progression (7, 8). Second, diabetes onset is accelerated in NOD mice that express a transgenic $IGRP_{206–214}$-specific TCR (9). Third, depletion of $IGRP_{206–214}$-specific $CD8^+$ T cells via treatment with the mimetic peptide NRP-A7 protects NOD mice from diabetes (8, 10). Finally, elevated $IGRP_{206–214}$-specific $CD8^+$ T cells in PBL of prediabetic NOD mice correlates with the progression to overt diabetes (11).

Tracking changes in β cell-specific T cells may provide an accurate readout for disease progression in at-risk or diabetic patients. β cell-specific T cells have been detected in PBL of patients and rodent models using tetramer technology or sensitive ELISPOT assays (12–14). Nevertheless, whether autoreactive T cell clones detected in PBL are identical with those found in the islets and/or at sites of T cell activation such as the draining pancreatic lymph nodes (PLN) has yet to be ascertained (15). We used a single cell-based PCR sequencing method to determine the α- and β-TCR repertoire of $IGRP_{206–214}$-specific $CD8^+$ T cells (16, 17) in PBL, the PLN, and islet infiltrates of unmanipulated or immunized NOD mice.

Results

$IGRP_{206–214}$-specific $CD8^+$ TCR repertoire is similar in PBL, PLN, and islets

Because islet-infiltrating T cells are inaccessible in humans, it is critical to know whether peripheral T cell clones are also found in the islets. To address this question, the TCR repertoire of $IGRP_{206–214}$-specific $CD8^+$ T cells residing in PBL, draining PLN, and islets of recent onset diabetic or euglycemic 20-wk-old NOD female mice was compared. At 20 wk of age, a high frequency of intrainsulitis is detected in the islets of nondiabetic NOD female mice. $H2K^d$ (K^d) tetramers complexed with the high-affinity NRP-V7 mimetic peptide (K^d-V7) were used to identify $IGRP_{206–214}$-specific $CD8^+$ T cells. Consistent with previous reports, K^d-V7 bound $IGRP_{206–214}$-specific $CD8^+$ T cells isolated from 8.3 TCR NOD transgenic mice with increased avidity relative to K^d tetramers complexed with native $IGRP_{206–214}$ (data not shown) (17). In PBL, PLN, and islets, 1.67 ± 0.74, 0.51 ± 0.27, and 2.21 ± 0.94% of $CD8^+$ T cells bound K^d-V7, respectively, whereas staining with control K^d tetramers complexed with an influenza-derived nucleoprotein peptide $NP_{147–155}$ (K^d-NP) was minimal (Fig. 1). Single

K^d-$V7^+$ $CD8^+$ T cells were sorted from PBL, PLN, and islets of five individual mice, and TCR α-chain usage was determined via single-cell RT-PCR. In agreement with earlier work, K^d-$V7^+$ $CD8^+$ T cells exclusively used TCRα-chain *Vα17* and *Jα42* elements, with the former further divided into three distinct genes, namely *Vα17.4*, *Vα17.5*, and *Vα17.6* (Table I) (7).

In each mouse, no significant difference in the frequency of *Vα17* genes was detected among K^d-$V7^+$ $CD8^+$ T cells isolated from PBL, islets, or PLN (Table I). Furthermore, examination of CDR3α sequences showed no significant difference in the frequency of specific junctional sequences among tetramer$^+$ $CD8^+$ T cells isolated from the three tissues in NOD mice #1, #4, and #5 (Fig. 2). In these mice, the canonical MRD motif associated with TCRα-chain $IGRP_{206–214}$ specificity (7, 19, 20) was dominant (Fig. 2). In NOD mice #2 and #3, however, differences in the frequency for a particular motif were observed. For example, in NOD mouse #2, an increased frequency of K^d-$V7^+$ $CD8^+$ T cells using the MRD motif was found in the islets (32 of 36) vs PBL (15 of 27) ($p = 0.004$, Fisher's exact test) (Fig. 2). In addition, the MRV motif (7) in NOD mouse #2 was used by 41% (11 of 27) of PBL but not by any of the islet (0 of 36) tetramer$^+$ $CD8^+$ T cells ($p < 10^{-3}$, Fisher's exact test). In NOD mouse #3, in which the MRV motif was dominant, no difference was detected between islet (26 of 31) and PBL (40 of 42) K^d-$V7^+$ $CD8^+$ T cells, but a significant decrease in the pool of PLN tetramer$^+$ $CD8^+$ T cells (20 of 42) ($p \leq 0.002$, Fisher's exact test) was observed (Fig. 2). Collectively, these data demonstrate that the major V_ genes are found in PBL, PLN, and the islets of individual NOD mice, although the frequency of these clonotypes may vary among the respective tissues.

Because the TCR α-chain repertoire of $IGRP_{206–214}$-specific $CD8^+$ T cells is limited, it was possible that clonotypic variation within the PBL, PLN, and islets was obscured. The Santamaria (5, 19, 20) group and others demonstrated that the TCR β-chain repertoire of $IGRP_{206–214}$-specific $CD8^+$ T cells is heterogeneous, and therefore can provide a more "sensitive" readout for potential repertoire differences. Therefore, TCRβ-chain usage of K^d-$V7^+$ $CD8^+$ T cells from the same NOD mice and an additional three 20-wk-old nondiabetic NOD female mice were assessed. Analogous to earlier work (17), ≥80% of IGRP-specific $CD8^+$ T cells in PBL, PLN, and the islets used *V*β*8.1* in combination with *J*β*1.3*, *J*β*2.4*, or *J*β*2.7* elements. CDR3β analysis revealed a high degree of heterogeneity in which 50 distinct motifs were detected with only five CDR3β sequences shared among some of the individual mice (SDSQNTL, SDPGNTL, STDWGYEQ, SSDTYEQ, SNDTYEQ) (Table II). Up to four prevalent clonotypes (e.g., >10%) were found in a mouse (Table II). Between each mouse prevalent clonotypes typically differed, with the exception of mice #6 and #7, which shared the immunodominant SSDTYEQ motif (Table II). In six of eight NOD mice (mice #1, #2, and #4 –7), the same immunodominant T cell clones detected in PBL were also found in the islets and PLN (Table II). In NOD mice #4 –7, no significant difference in the frequency of CDR3β sequences was detected between PBL and islet K^d-$V7^+$ $CD8^+$ T cells (Table II). In fact, a single clonotype with the CDR3β sequence SDAQNTL was detected in all three tissues of NOD mouse #4 (Table II). In NOD mice #1 and 2, identical clonotypes were detected in PBL and islets (and PLN) although at varying frequencies. For instance in NOD mice #1 and 2, immunodominant motifs (e.g., SDPGNTL and SDSQNTL, respectively) were shared between PBL and islet K^d-$V7^+$ $CD8^+$ T cells but with significantly different frequencies ($p \leq 0.05$, Fisher's exact test) (Table II). Furthermore, in NOD mouse #1, the

SDPRNTL motif was prevalent in islet (8 of 33) but absent in PBL (0 of 22) tetramer$^+$ CD8$^+$ T cells. In NOD mouse #3, an interesting profile was observed for the two immunodominant CDR3β motifs that were detected. SAERGANSDYT usage was significantly increased in the islet (11 of 18) vs PBL (0 of 22; $p < 10^{-3}$, Fisher's exact test) or PLN (2 of 25; $p< 10^{-3}$, Fisher's exact test), whereas STDWGYEQ was prevalent in the PBL (17 of 22) but not the islets (1 of 18; $p < 10^{-3}$, Fisher's exact test) or PLN (1 of 25; $p < 10^{-3}$, Fisher's exact test) (Table II). Similarly, in NOD mouse #8 SSGDNYEQ usage dominated islet (9 of 25) but not PBL (1 of 14) tetramer$^+$ CD8$^+$ T cells (Table II), although this difference was not statistically significant. Collectively, these results demonstrate that immunodominant clonotypes as defined by CDR3β usage are generally shared among IGRP$_{206–214}$-specific CD8$^+$ T cells in PBL and islets (and PLN) (e.g., mouse #1-SDPGNTL; mouse #2-SDSQNTL; mouse #4-SDAQNTL; mouse #5-SDPAYEQ and SGDDYEQ; mouse #6-SSDTYEQ; mouse #7-SSDTYEQ). In addition, the relative distribution of specific clonotypes can vary in the respective tissues, particularly with less dominant T cell clones. Finally, only few mice contain clonotypes that are prevalent in the islets but not found in PBL (e.g., mouse #3-SAERGANSDYT; mouse #8-SSGDNYEQ).

IGRP$_{206–214}$-specific CD8$^+$ T cell clonotypes expanded in the periphery post-IGRP$_{206–214}$ immunization are detected in the islets

The T cell repertoire following immunization with IGRP$_{206–214}$ was assessed. The goal was to determine whether distinct clonotypes expanded in the periphery were also found in the islets. A possible clinical scenario would be the monitoring of PBL for β cell-specific T effectors induced by Ag-specific immunotherapy. Nondiabetic NOD female mice 15 wk of age

received a single injection of nonreplicating VRP-IGRP encoding an $IGRP_{206-214}$-Ig fusion molecule (22, 23). Preliminary studies indicate that diabetes is prevented in female NOD mice by targeting $IGRP_{206-214}$-specific $CD8^+$ T cells via Ag-encoding VRP vaccines (R. Tisch, unpublished results). Ten days later, the frequency of K^d-$V7^+$ $CD8^+$ T cells in PBL was compared with prevaccination levels. As expected, expansion of $IGRP_{206-214}$-specific $CD8^+$ T cells was readily induced by VRP-IGRP (Fig. 3, *A* and *B*). For the five NOD mice, an average increase of 14.1-fold was detected in postvaccination PBL. K^d-$V7^+$ $CD8^+$ T cells increased from 0.36 ± 0.09 to 5.06 ± 0.52% of $CD8^+$ T cells ($p = 10^{-3}$, Student's *t* test). Importantly, expansion of K^d-$V7^+$ $CD8^+$ T cells was $IGRP_{206-214}$-specific. No significant increase in $IGRP_{206-214}$-specific $CD8^+$ T cells was detected in PBL from three age-matched NOD mice vaccinated with VRP-HA encoding an $HA_{512-520}$-Ig fusion (Fig. 3*A*). In pre- and post-VRP-HA PBL samples, 0.58 ± 0.14 and 0.83 ± 0.17%, respectively, of $CD8^+$ T cells were K^d-$V7^+$.

To determine whether vaccination altered the α- and β-TCR repertoire among the tissues, cDNA from K^d-$V7^+$ $CD8^+$ T cells sorted from prevaccination PBL, and from PBL, islets and PLN 10 days post-VRP-IGRP was sequenced. No significant difference in the frequency of *Vα17.4*, *Vα17.5*, or *Vα17.6* genes was detected among pre- or postvaccination PBL, islets, or PLN (Fig. 3*C*). Furthermore, CDR3α usage was invariant in which all of the 244 TCRα-chain sequences examined from individual mice contained the canonical MRD motif. Near uniform usage of *Vβ8.1* regardless of the immunization status or tissue sampled was detected, with *Jβ2.4* being used in the majority of K^d-$V7^+$ $CD8^+$ T cells analyzed (Fig. 3, *D* and *E*). CDR3β usage revealed that in four of five mice (NOD mice #1–4) examined, there was no

significant difference in the frequency of immunodominant clonotypes in PBL pre- vs postvaccination (Table III) despite robust expansion upon VRP-IGRP administration (Fig. 3), indicating equivalent expansion of these clonotypes. This was most evident in NOD mice #1 and #2 in which immunodominant clonotypes using the SDWGTNTGQL and SDAQNTL motifs, respectively, were found at similar frequencies pre- and post-VRP-IGRP vaccination (Table III).

In 5 of 5 treated NOD mice, the most prevalent CDR3β motif used by K^d-$V7^+$ $CD8^+$ T cells in PBL prevaccination was also immunodominant in the islets and PLN (Table III). In NOD mouse #3, however, the SDAQNTL motif was detected at a notable frequency (7 of 25) in the islets but not in pre- or postvaccination PBL ($p \leq 0.03$, Fisher's exact test) (Table III). Nevertheless, SDSQNTL, which was dominant in pre- (16 of 21) and post- (10 of 17) vaccination PBL, was also the dominant motif in the islets (12 of 25). Noteworthy is the fact that, despite marked induction in PBL, no significant difference in the frequency of K^d-$V7^+$ $CD8^+$ T cells was detected in the islets or PLN of VRP-IGRP (6.2 ±1.2% (islets); 0.66 ± 0.04% (PLN)) vs VRP-HA (4.2 ± 1.0% (islets); 0.45 ± 0.1% (PLN)) vaccinated NOD mice. This suggests that the frequency of immunodominant clonotypes found in the islets and PLN was not significantly affected by VRP-IGRP treatment, and that clonotypes in prevaccination PBL represented the TCR repertoire of $IGRP_{206–214}$-specific $CD8^+$ T cells established in the islets and PLN.

Discussion

The analysis of β cell-specific T cell reactivity in at-risk or diabetic individuals is dependent on T cells prepared from blood. However, it is unclear how accurate clonotypes residing in blood reflect those targeting the islets. Insight into this issue would aid in our understanding of disease progression, in addition to the development of novel strategies to monitor the efficacy of immunotherapies applied in the clinic. Accordingly, the current study was conducted to define the TCR repertoire of disease-relevant $IGRP_{206–214}$-specific $CD8^+$ T cells residing in PBL, islets, and PLN of female NOD mice. $CD8^+$ T cells were sorted from the respective tissues using K^d tetramers complexed with the mimetic NRP-V7 peptide, and CDR3α and CDR3β segment heterogeneity was determined at a single-cell level. K^d tetramers complexed with NRP-V7 rather than $IGRP_{206–214}$ were used in the study because a broader spectrum of clonotypes was typically detected in K^d-$V7^+$ vs $IGRP_{206–214}{}^+$ $CD8^+$ T cells sorted from the same individual NOD mice (C. P. Wong and R. Tisch, unpublished results). Noteworthy is the fact that prevalent clonotypes were shared; however, only a subset of the overall repertoire of K^d-$V7^+$ $CD8^+$ T cells was detected in K^d-$IGRP_{206–214}{}^+$ $CD8^+$ T cells, likely reflecting the higher binding avidity of K^d-V7 compared with K^d-$IGRP_{206–214}$ (C. P. Wong and R. Tisch, unpublished results).

An important observation made in this study is that the same immunodominant clonotypes detected in PBL were also prevalent in the islets (and PLN) of six of eight untreated NOD female mice (e.g., NOD mice #1, #2, and #4–7; Table II), although the frequency of these T cell clones varied depending on the tissue. T cell clonotype distribution was independent of disease status because both diabetic (mice #2, #4, #5) and 20-wk-old nondiabetic (mice #1,

#6, #7) NOD female mice contained the same immunodominant clones in PBL and islets (and PLN) (Table II). Untreated NOD mouse #4 provided an extreme example in which Kd-$V7^+$ $D8^+$ T cells consisted of a single clonotype (based on both CDR3α and CDR3β motif usage) that was detected in all three tissues (Fig. 2 and Tables I and II). Exceptions to the general trend were nevertheless observed. For instance, in untreated NOD mice #3 and #8, the CDR3β sequences STDWGYEQ and SSLDRVEQ, respectively, were prevalent in PBL but not the islets (Table II). In contrast, the CDR3β sequences SAERGANSDYT and SSGDNYEQ dominated the islets but not PBL in untreated NOD mice #3 and #8, respectively (Table II). Fluctuation in the number of K^d-$V7^+$ $CD8^+$ T cells residing in PBL may account for differences detected in the few mice in which there was a significant disparity with a T cell clone found in the islets. For example, the number of K^d-$V7^+$ $CD8^+$ T cells in PBL has been shown to undergo continual rounds of expansion and contraction, which in turn is thought to indicate waves of clonal proliferation of $IGRP_{206-214}$-specific $CD8^+$ T cells undergoing avidity maturation in the islets and/or PLN (11). Nevertheless, in the majority of untreated NOD mice, K^d-$V7^+$ $CD8^+$ T cell clonotypes prevalent in PBL represent a "selected" repertoire that is involved in islet-infiltration. This interpretation is consistent with work by Trudeau et al. (11), which demonstrated that detection of K^d-$V7^+$ $CD8^+$ T cells in PBL of euglycemic NOD mice provides a relatively accurate predictive marker for the progression to overt diabetes. We recently demonstrated that K^d-$V7^+$ $CD8^+$ T cell clones prevalent in the endogenous islets are also dominant in early infiltrates of islet grafts implanted in diabetic NOD recipients (17). This observation coupled with the findings made in the current study, suggest that T cell clones mediating islet graft destruction are directly recruited from PBL. Further evidence indicating that clonotypes found in PBL

exhibit a diabetogenic capacity is provided by Roep and colleagues (24) who showed that β cell-specific $CD4^+$ T cell clones prepared from PBL of diabetic patients readily traffick to the islets after transfer into NOD.*scid* mice.

The association between T cell clonotypes residing in PBL and the target tissue is a key issue for several autoimmune diseases including T1D. Addressing this issue has proven to be problematic in large part due to the lack of knowledge of the critical autoantigens and corresponding epitopes driving the respective autoimmune responses. Celiac disease (CD), however, is an exception in which HLA-DQ2-restricted, transglutaminase-modified wheat gliadin peptides have been identified as major targets of $CD4^+$ T cells in the intestinal mucosa (25). Evidence indicates that gliadin-specific T cells in PBL vs the gut of CD patients differ in terms of HLA-restriction, epitope specificity, and a requirement for transglutaminase-mediated deamidation of the epitopes (26). Nevertheless, disease-relevant $CD4^+$ T cell clones have been reported in PBL of CD patients under certain conditions such as challenging individuals with Ag (27) or using a sensitive CFSE-based assay to measure proliferation in response to deamidated gliadin in vitro (28). These data suggest that in CD and possibly other tissuespecific autoimmune diseases, only a low frequency of pathogenic effectors exists in PBL of patients that may vary depending on disease progression. In contrast, our findings indicate that the frequency of β cell-specific $CD8^+$ T cells can be relatively high in PBL (i.e., >4% of $CD8^+$ T cells) and that these T cell clones are also generally found dominating the islets of NOD mice. An important question that still needs to be addressed, however, is whether $CD8^+$ and $CD4^+$ T cells specific for other β cell

autoantigens also exhibit a similar clonotypic distribution between the periphery and islets of NOD mice, and ultimately in T1D patients.

Vaccination of NOD female mice with VRP-IGRP provided further insight into the TCR repertoire of $IGRP_{206–214}$-specific $CD8^+$ T cells in the respective tissues. First, the repertoire for $IGRP_{206–214}$-specific $CD8^+$ T cells in the periphery is relatively limited. For example, immunodominant clonotypes detected in PBL before vaccination were also prevalent in the PBL of four of five NOD mice treated with VRP-IGRP despite expansion of up to 40-fold in K^d-$V7^+$ $CD8^+$ T cells (Fig. 3, *A* and *B*). Novel clonotypes were detected in postvaccination PBL of some of the NOD mice (mice #1, #3, #5); however, these T cell clones were found only at a low frequency (Table III). Notably, VRP-IGRP vs VRP-HA vaccination had no significant effect on the number of K^d-$V7^+$ $CD8^+$ T cells detected in the islets (or PLN), indicating that expansion of $IGRP_{206–214}$-specific $CD8^+$ T cells occurred predominately in the periphery (e.g., PBL and spleen). Importantly, in five of five mice the same immunodominant clonotypes detected in pre- and postvaccination PBL also were prevalent in the islets and PLN, although again the frequency of these clonotypes varied in the respective tissues (Table III). This observation further supports the conclusion that the repertoire of $IGRP_{206–214}$-specific $CD8^+$ T cells in PBL is similar to islet-infiltrating T cell clones, even after immunization.

TCR analysis in patients with tissue-specific autoimmune diseases such as rheumatoid arthritis or multiple sclerosis have shown variation in immunodominant clonotypes among individuals, and within the targeted tissues (i.e., joints or brain plaques) of the same

individual (29, 30). Interestingly, Carnaud and colleagues (31) have also demonstrated that a high degree of heterogeneity of TCR CDR3β gene usage exists at an early age among islets in NOD mice, although the specificity of these T cells was not determined. These studies are consistent with the high degree of variability detected in CDR3β sequences between individual NOD mice in the current work (Tables II and III) and in an earlier study investigating the repertoire of single K^d-$V7^+$ $CD8^+$ T cells isolated from grafted vs endogenous islets (17). Interestingly, the CDR3β motif SDSQNTL was detected either as a dominant or minor clone in two of eight untreated (mice #2, #3; Table II), four of five VRP-IGRP-treated mice (mice #2–5; Table III), and in six of eight mice examined in our earlier study (17). The marked heterogeneity observed in the CDR3β segment would suggest that specificity and affinity associated with $IGRP_{206–214}$ clonotypes is largely due to the canonical Vα17-Jα42 elements (7). However, in view of the relatively high frequency among individual NOD mice, the CDR3β SDSQNTL motif may also contribute to TCR specificity and/or affinity.

This study extends previous work (11, 17) by providing novel insight into the clonotypic composition and distribution of diseaserelevant $CD8^+$ T cells at the single-cell level in individual, unmanipulated, and immunized NOD mice. Specifically, fine TCR repertoire analysis of single $IGRP_{206–214}$-specific $CD8^+$ T cells demonstrates that a limited number of immunodominant clonotypes found in PBL typically reside in the islets. Furthermore, clones prevalent in PBL are preferentially expanded following immunization, and it is these clonotypes that typically reside in the islets. Finally, this study shows that insight into the

clonotypic nature of disease-relevant T cells (e.g., islet infiltrating) can be gained by analysis of T cells isolated from PBL.

Materials and Methods

Mice

NOD/LtJ mice were bred and housed under specific pathogen-free conditions. Each mouse used in this study was derived from an independent litter. Animal protocols were approved by the University of North Carolina Institutional Animal Care Committee.

Tetramers, Abs, and FACS

H2Kd monomers (18) were complexed with NRP-V7 (KYNKANVFL) or NP147–155 (TYQRTRALV). Tetramers were assembled by conjugating H2Kd monomers with streptavidin-PE (Molecular Probes). Anti-CD3- FITC and anti-CD8-allophycocyanin mAbs were purchased from eBioscience. T cells were costained with tetramers and Abs in PBS containing 3% FBS, 10 mM HEPES, and 1 mM EDTA for 1 h on ice. FACS data were acquired on a FACSCalibur (BD Biosciences) and analyzed using Summit software (DakoCytomation). For all tetramer analyses, CD8_ T cells were gated based on forward and side scatter, and CD3 and CD8 expression. For single-cell analyses, tetramer_ CD8_ T cells were sorted by a MoFlo highspeed sorter (DakoCytomation) at 1 cell/well into a 96-well PCR plate (USA Scientific), each well containing 4 ul buffer of 0.5x PBS, 10 mM DTT, and 8 U RNaseOUT RNase inhibitor (Invitrogen Life Technologies). Plates were kept frozen at -80°C.

Islet isolation

Islets were purified (17) and cultured overnight in RPMI 1640 containing 10% FBS and 4 ng/ml recombinant murine IL-2 (PeproTech). Lymphocytes infiltrating the islets were collected and cellular debris removed by 70-um nylon filters before FACS.

Single-cell RT-PCR and TCR repertoire analyses

TCR usage was analyzed by a single-cell RT-PCR protocol (16, 17). TCR α-chain analysis was performed using a Vα17-specific primer in combination with an α-chain constant region primer for RT-PCR because all $IGRP_{206–214}$-related clonotypes have an invariant *Vα17* gene usage (19, 20). For TCR β-chain analysis, a panel of primers specific for all known TCR β-chain variable regions in combination with a β-chain constant region primer was used. RT-PCR amplicons were used as templates for a second round of PCR amplification using a panel of nested TCR α- or β-chain-specific primers. Efficiency of RT-PCR for Vα and Vβ gene segments was ≥95 and 60–95%, respectively. PCR products were treated with Exonuclease I (NEB Biolabs) and shrimp alkaline phosphatase (Roche) and sequenced by the UNC Sequencing Core Facility. TCR sequence alignments were performed using Sequencher software (Gene Codes).

Generation of Venezuelan equine encephalitis virus replicon particles (VRP)

To generate Ag-expressing, nonreplicating VRP, cDNAs encoding murine IGRP206–214 (VYLKTNVF), or influenza hemagglutinin (HA)512–520 (IYSTVASSL)) fused to IgGFc were subcloned into pVR21. RNA was transcribed from plasmids encoding VRP-IGRP or VRP-HA and capsid and envelope glycoprotein using mMESSAGE mMACHINE T7 Kit

(Ambion). In vitro-transcribed RNAs were electroporated into baby hamster kidney cells that were cultured for 24 h at 37°C at 5% CO2. VRP were harvested, concentrated, titered, and stored in PBS plus 1% FCS at -80°C (21). Mice were immunized via footpad with 5 _ 105 VRP-IGRP or VRP-HA infectious units in PBS. Ten days after VRP immunization, PBL, PLN, and islets were harvested and tetramer^{+} CD8^{+} T cells were isolated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad). Values of *p* were calculated using Student's *t* test or Fisher's exact test.

Disclosures

The authors have no financial conflict of interest.

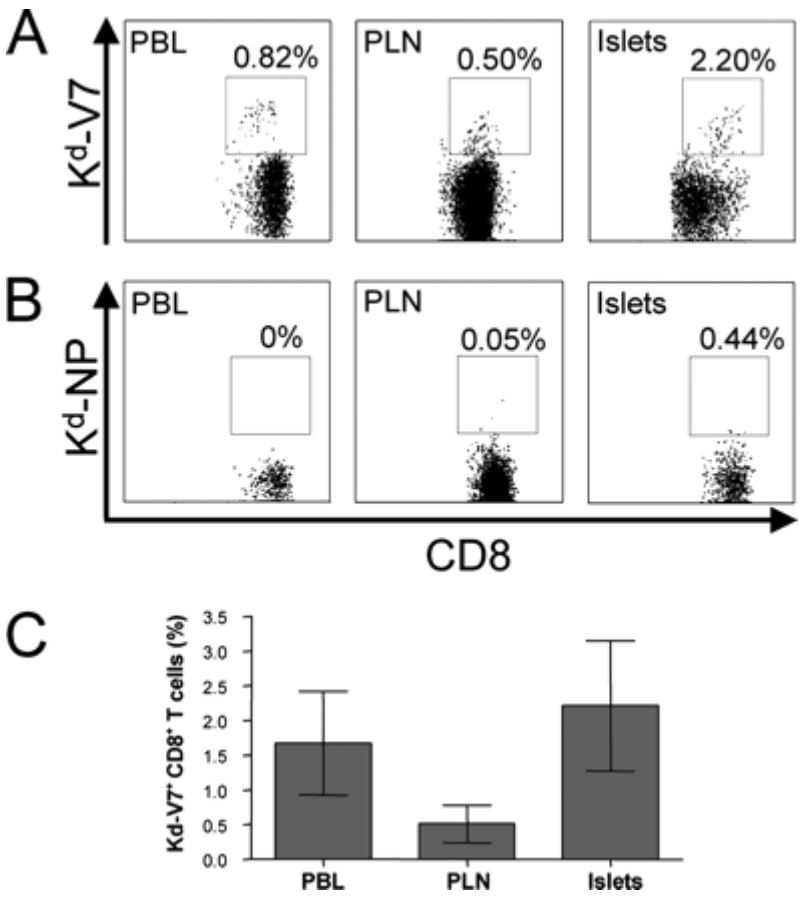


FIGURE 1. IGRP$_{206–214}$-specific CD8$^+$ T cells are detected in PBL, PLN, and islets of NOD mice. *A*, Representative FACS plots showing K^d-V7$^+$ and (*B*) control K^d-NP$^+$ CD8$^+$ T cells in PBL (mouse #2), PLN (mouse #3), and islet infiltrates (mouse #4). Events were gated on CD3$^+$ and CD8$^+$ T cells. *C*, Summary of K^d-V7$^+$ CD8$^+$ cells in PBL, PLN, and islets of five individual female NOD mice 20 wk old. Data represent average tetramer$^+$ CD8$^+$ T cells (±SEM).

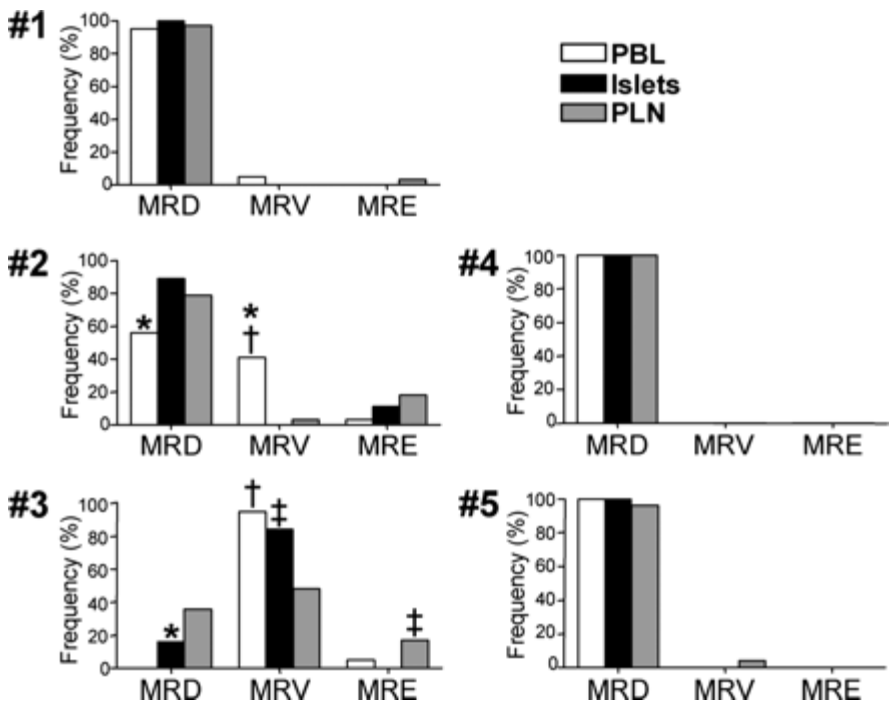


FIGURE 2. Comparison of TCR CDR3α repertoire of $IGRP_{206–214}$-specific $CD8^+$ T cells in PBL, islets, and PLN. CDR3α usage of $IGRP_{206–214}$-specific $CD8^+$ T cells in PBL, islets, and PLN were determined by single-cell RT-PCR in five individual NOD female mice 20 wk old that were diabetic (#2, #4, #5) or euglycemic (#1, #3). The number of CDR3α sequences analyzed in PBL, islets, and PLN, respectively, were as follows: mouse #1 22, 28, 31; mouse #2 27, 36, 34; mouse #3 42, 31, 42; mouse #4 12, 34, 33; mouse #5 11, 19, and 24. *, $p \leq 0.05$, PBL vs islet; †, $p \leq 0.01$, PBL vs PLN; ‡, $p \leq 0.02$, islets vs PLN; Fisher's exact test.

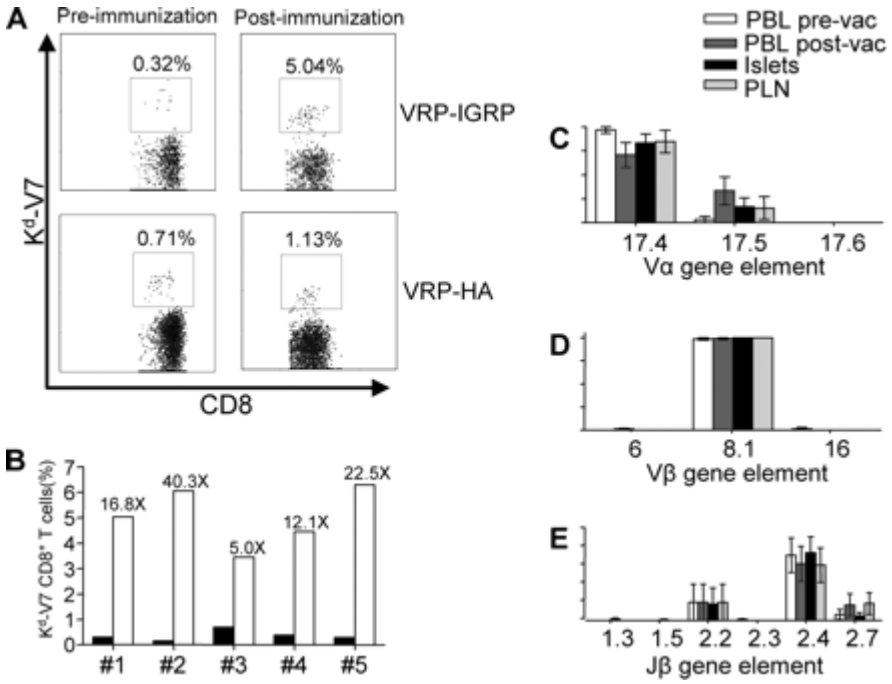


FIGURE 3. Expansion of $IGRP_{206–214}$-specific $CD8^+$ T cells in PBL of VRP-IGRP-vaccinated mice. *A*, Representative FACS plots showing expansion of $IGRP_{206–214}$-specific $CD8^+$ T cells in PBL of 15-wk-old NOD mice vaccinated with 5 x 10^5 VRP-IGRP, or VRP-HA via footpad. $IGRP_{206–214}$-specific $CD8^+$ T cells in PBL were stained with K^d-V7 pre- and 10 days post-VRP vaccination. Events were gated on $CD3^+$ and $CD8^+$ T cells. *B*, Five NOD mice 15–20 wk old each received a single footpad vaccination of 5 x 10^5 VRP-IGRP infectious units. Frequencies of K^d-$V7^+$ $CD8^+$ T cells were determined pre- (■) and postvaccination (□) in PBL of each mouse. Numbers represent the fold increase of K^d-$V7^+$ $CD8^+$ T cells in each mouse (percentage of K^d-$V7^+$ $CD8^+$ T cells in PBL post- vs prevaccination). *V*α (*C*), *V*β (*D*), and *J*β (*E*) usage were determined by single-cell RT-PCR analysis using K^d-$V7^+$ $CD8^+$ T cells sorted from PBL of individual mice before VRP-IGRP vaccination or from PBL, islets, and PLN 10 days after VRP vaccination. Data represent averaged frequencies of *V*α, *V*β, and *J*β gene families detected from each sample (±SEM).

Table I. *V α17 gene element usage in female NOD mice*

Mouse	Disease Status	Vα Gene Elements	PBL[a]	Islets[a]	PLN[a]
#1	Nondiabetic	17.4	2 (9%)	6 (21%)	0
		17.5	20 (91%)	22 (79%)	31 (100%)
		17.6	0	0	0
#2	Diabetic	17.4	16 (59%)	23 (64%)	27 (79%)
		17.5	11 (41%)	13 (36%)	6 (18%)
		17.6	0	0	1 (3%)
#3	Nondiabetic	17.4	0	1 (3%)	0
		17.5	42 (100%)	30 (97%)	42 (100%)
		17.6	0	0	0
#4	Diabetic	17.4	12 (100%)	34 (100%)	33 (100%)
		17.5	0	0	0
		17.6	0	0	0
#5	Diabetic	17.4	0	0	0
		17.5	5 (45%)	4 (21%)	11 (46%)
		17.6	6 (55%)	15 (79%)	13 (54%)

[a] Represents the number of TCR sequences identified using the respective Vα gene elements in single-cell-sorted K^d-$V7^+$ $CD8^+$ T cells in five individual mice. Percentage of $CD8^+$ T cells staining with K^d-V7 for PBL, islets, and PLN, respectively, were as follows: mouse #1, 0.43, 0.9, and 0.2%; mouse #2, 0.82, 1.61, and 0.4%; mouse #3, 0.6, 1.2, and 0.5%; mouse #4, 1.5, 2.2, and 0.5%; and mouse #5, 4.4, 5.94, and 1.6%.

Table II. *TCR CDR3β repertoire in single-cell-sorted K^d-$V7^+$ $CD8^+$ T cells in female NOD mice*

	Disease	CDR3β	PBL[a]	Islets[a]	PLN[a]
#1	Nondiabetic	SDPGNTL	15 (68.2%)	14 (42.4%)[b]	22 (75.8%)[d]
		SDPRNTL	0	8 (24.2%)[b]	6 (20.7%)
		SGDNYEQ	2 (9.1%)	3 (9.1%)	1 (3.4%)
		SDPKYEQ	2 (9.1%)	5 (15.2%)	0
		SLGDWGYEQ	1 (4.5%)	0	0
		SPGDTGQL	1 (4.5%)	0	0
		GDARDWGGRDTQ	1 (4.5%)	0	0
		SDTKNTL	0	1 (3%)	0
		SDPENTL	0	1 (3%)	0
		RTGGSQNTL	0	1 (3%)	0
		Total	22	33	29
#2	Diabetic	SDSQNTL	10 (38.5%)	23 (65.7%)[b]	10 (43.5%)
		KGSSYEQ	6 (23.1%)	5 (14.3%)	8 (34.8%)
		STDWGYEQ	3 (11.5%)	0	0
		SGDKYEQ	3 (11.5%)	0	0
		SDFAEQ	1 (3.8%)	1 (2.9%)	0
		SSDRFEQ	1 (3.8%)	3 (8.6%)	4 (17.4%)
		SSGTDYEQ	1 (3.8%)	0	0
		SPQGWEQ	1 (3.8%)	0	0
		SETVYEQ	0	1 (2.9%)	0
		SEGQKYTL	0	2 (5.6%)	1 (4.3%)
		Total	26	35	23
#3	Nondiabetic	STDWGYEQ	17 (77.3%)	1 (5.6%)[b]	1 (4%)[c]
		NRDSAETL	3 (13.6%)	3 (16.7%)	6 (24%)
		SQVWGASQNTL	1 (4.5%)	0	0
		SDRGSSAETLY	1 (4.5%)	0	0

		SAERGANSDYT	0	11 (61.1%)[b]	2 (8%)[d]
		SDPGNTL	0	1 (5.6%)	4 (16%)
		SDSQNTL	0	1 (5.6%)	5 (20%)
		SHRDNYEQ	0	1 (5.6%)	1 (4%)
		SDAQYEQ	0	0	3 (12%)
		AGDSYEQ	0	0	2 (8%)
		GQSSYEQ	0	0	1 (4%)
		Total	22	18	25
#4	Diabetic	SDAQNTL	12 (100%)	36 (100%)	23 (100%)
		Total	12	36	23
#5	Diabetic	SDPAYEQ	8 (57.1%)	11 (55%)	7 (33.3%)
		SGDDYEQ	3 (21.4%)	7 (35%)	7 (33.3%)
		SDPGNTL	3 (21.4%)	2 (10%)	2 (9.5%)
		SSGTDYEQ	0	0	1 (4.8%)
		SDGLYTL	0	0	3 (14.3%)
		SSDWGYEQ	0	0	1 (4.8%)
		Total	14	20	21
#6	Nondiabetic	SSDTYEQ	34 (100%)	29 (90.6%)	ND
		SSLEYKYEQ	0	2 (6.3%)	
		SNDTYEQ	0	1 (3.1%)	
		Total	34	32	
#7	Nondiabetic	SSDTYEQ	30 (96.8%)	32 (97%)	ND
		SNDTYEQ	1 (3.2%)	0	
		SSWDSSYEQ	0	1 (3%)	
		Total	31	33	
#8	Nondiabetic	SSLDRVEQ	5 (35.9%)	0	ND
		SSGPGQNSDYT	3 (21.4%)	0	
		SSRATGGDTGQLY	3 (21.4%)	0	
		SSRDWGGADEQ	1 (7.1%)	0	
		SSGDNYEQ	1 (7.1%)	9 (36%)	

SSWTGTNERLF	1 (7.1%)	0
PARDTKNTL	0	1 (4%)
SSDPGMNTL	0	1 (4%)
SSDTKNTL	0	4 (16%)
SSDPGNTL	0	2 (8%)
SSDDTYEQ	0	1 (4%)
TGDNSYEQ	0	2 (8%)
SSDNLYEQ	0	3 (12%)
SSDDTYEQ	0	1 (4%)
SSGDNYKQ	0	1 (4%)
Total	14	25

[a] Represents the number of TCR sequences identified using the respective CDR3β in single-cell-sorted K^d-$V7^+$ $CD8^+$ T cells in five individual mice.

[b] $p < 0.05$, PBL vs islet;

[c] $p < 0.01$, PBL vs PLN;

[d] $p < 0.02$, islets vs PLN; Fisher's exact test. Percentage of $CD8^+$ T cells staining with K^d-V7 for PBL, islets, and PLN, respectively, were as follows: mouse #1, 0.43, 0.9, and 0.2%; mouse #2, 0.82, 1.61, and 0.4%; mouse #3, 0.6, 1.2, and 0.5%; mouse #4, 1.5, 2.2, and 0.5%; mouse #5, 4.4, 5.94, and 1.6%; mouse #6, 1.3 and 4.9%, ND; mouse #7, 0.9 and 3.7%, ND; mouse #8, 1.1 and 5.4%, ND.

Table III. *TCR CDR3β repertoire in single-cell-sorted K^d-$V7^+$ $CD8^+$ T cells in female NOD mice pre- and post- VRP-IGRP immunization*

	CDR3β	PBL Prevaccination[a]	PBL Postvaccination[a]	Islets Postvaccination[a]	PLN Postvaccination[a]
#1	SDWGTNTGQL	22 (100%)	31 (96.9%)	19 (90.5%)	7 (100%)
	SDGTYEQ	0	1 (3.1%)	1 (4.8%)	0
	SDAQNTL	0	0	1 (4.8%)	0
	Total	22	32	21	7
#2	SDAQNTL	40 (100%)[c]	12 (100%)[d]	8 (61.5%)	17 (70.8%)
	SDPQNTL	0	0	1 (7.7%)	1 (4.2%)
	SDSQNTL	0	0	3 (23.1%)	6 (25%)
	SDEQNTL	0	0	1 (7.7%)	0
	Total	40	12	13	24
#3	SDSQNTL	16 (76.2%)	10 (58.8%)	12 (48%)	7 (50%)
	SDEKNTL	4 (19%)	4 (23.5%)	4 (16%)	1 (7.1%)
	SDDNYEQ	1 (4.8%)	2 (11.8%)	0	4 (28.6%)
	SGDSSYEQ	0	1 (5.9%)	0	0
	SDPENTL	0	0	2 (8%)	0
	SDGSYEQ	0	0	0	1 (7.1%)
	SDAQNTL	0	0	7 (28%)[cd]	1 (7.1%)
	Total	21	17	25	14
#4	SDSQNTL	9 (52.9%)[c]	10 (45.5%)[d]	20 (80%)	5 (41.7%)
	SSDIYEQ	5 (29.4%)	12 (54.5%)[d]	5 (20%)	7 (58.3%)
	SDEKNTL	2 (11.8%)	0	0	0
	SLGSAETL	1 (5.9%)	0	0	0
	Total	17	22	25	12
#5	SDPQNTL	25 (92.6%)[b]	15 (68.8%)	19 (73.1%)	16 (80%)
	SDEQNTL	2 (7.4%)[b]	6 (27.3%)[d]	2 (7.7%)	4 (20%)
	SSDRYEQ	0	1 (4.5%)	0	0

SDSQNTL	0	0	2 (7.7%)	0
SDPGNTL	0	0	2 (7.7%)	0
SDGTQAPL	0	0	1 (3.8%)	0
Total	27	22	26	20

[a] Represents the number of TCR sequences identified using the respective CDR3β in single-cell-sorted K^d-$V7^+$ $CD8^+$ T cells in five individual mice pre- and postimmunization with VRP-IGRP.

[b] $p < 0.03$, PBL preimmunization vs postimmunization;

[c] $p < 0.05$, preimmunization PBL vs islets;

[d] $p < 0.04$, postimmunization PBL vs islets; Fisher's exact test. Percentage of $CD8^+$ T cells staining with K^d-V7 for PBL prevaccination, PBL-postvaccination, islets, and PLN, respectively, were as follows: mouse #1, 0.3, 5.04, 4.8, and 0.61%; mouse #2, 0.15, 6.1, 5.3, and 0.63%; mouse #3, 0.7, 3.44, 8.6, and 0.73%; mouse #4, 0.37 and 4.5%, ND,ND; mouse #5, 0.28 and 6.3%, ND,ND.

References

1. Anderson, M. S., and J. A. Bluestone. 2005. The NOD mouse: a model of immune dysregulation. *Annu. Rev. Immunol.* 23: 447–485.

2. Delovitch, T. L., and B. Singh. 1997. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity* 7:727–738.

3. Tisch, R., and H. O. McDevitt. 1996. Insulin-dependent diabetes mellitus. *Cell* 85: 291–297.

4. DiLorenzo, T. P., and D. V. Serreze. 2005. The good turned ugly: immunopathogenic basis for diabetogenic CD8_ T cells in NOD mice. *Immunol. Rev.* 204:250–263.

5. Lieberman, S. M., A. M. Evans, B. Han, T. Takaki, Y. Vinnitskaya, J. A. Caldwell, D. V. Serreze, J. Shabanowitz, D. F. Hunt, S. G. Nathenson, et al. 2003. Identification of the _ cell antigen targeted by a prevalent population of pathogenic CD8_ T cells in autoimmune diabetes. *Proc. Natl. Acad. Sci. USA* 100: 8384–8388.

6. Anderson, B., B. J. Park, J. Verdaguer, A. Amrani, and P. Santamaria. 1999. Prevalent CD8_ T cell response against one peptide/MHC complex in autoimmune diabetes. *Proc. Natl. Acad. Sci. USA* 96: 9311–9316.

7. Han, B., P. Serra, J. Yamanouchi, A. Amrani, J. F. Elliott, P. Dickie, T. P. Dilorenzo, and P. Santamaria. 2005. Developmental control of CD8 T cell-avidity maturation in autoimmune diabetes. *J. Clin. Invest.* 115: 1879–1887.

8. Amrani, A., J. Verdaguer, P. Serra, S. Tafuro, R. Tan, and P. Santamaria. 2000. Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. *Nature* 406: 739–742.

9. Verdaguer, J., D. Schmidt, A. Amrani, B. Anderson, N. Averill, and P. Santamaria. 1997. Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. *J. Exp. Med.* 186: 1663–1676.

10. Han, B., P. Serra, A. Amrani, J. Yamanouchi, A. F. Maree, L. Edelstein-Keshet, and P. Santamaria. 2005. Prevention of diabetes by manipulation of anti-IGRP autoimmunity: high efficiency of a low-affinity peptide. *Nat. Med.* 11: 645–652.

11. Trudeau, J. D., C. Kelly-Smith, C. B. Verchere, J. F. Elliott, J. P. Dutz, D. T. Finegood, P. Santamaria, and R. Tan. 2003. Prediction of spontaneous autoimmune diabetes in NOD mice by quantification of autoreactive T cells in peripheral blood. *J. Clin. Invest.* 111: 217–223.

12. Toma, A., S. Haddouk, J. P. Briand, L. Camoin, H. Gahery, F. Connan, D. Dubois-Laforgue, S. Caillat-Zucman, J. G. Guillet, J. C. Carel, et al. 2005. Recognition of a

subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients. *Proc. Natl. Acad. Sci. USA* 102: 10581–10586.

13. Arif, S., T. I. Tree, T. P. Astill, J. M. Tremble, A. J. Bishop, C. M. Dayan, B. O. Roep, and M. Peakman. 2004. Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J. Clin. Invest.* 113: 346–349.

14. Reijonen, H., W. W. Kwok, and G. T. Nepom. 2003. Detection of CD4_ autoreactive T cells in T1D using HLA class II tetramers. *Ann. NY Acad. Sci.* 1005: 82–87.

15. Kent, S. C., Y. Chen, L. Bregoli, S. M. Clemmings, N. S. Kenyon, C. Ricordi, B. J. Hering, and D. A. Hafler. 2005. Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope. *Nature* 435: 224–228.

16. Baker, F. J., M. Lee, Y. H. Chien, and M. M. Davis. 2002. Restricted islet-cell reactive T cell repertoire of early pancreatic islet infiltrates in NOD mice. *Proc. Natl. Acad. Sci. USA* 99: 9374–9379.

17. Wong, C. P., L. Li, J. A. Frelinger, and R. Tisch. 2006. Early Autoimmune destruction of islet grafts is associated with a restricted repertoire of IGRP-specific CD8_ T cells in diabetic nonobese diabetic mice. *J. Immunol.* 176: 1637–1644.

18. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigenspecific T lymphocytes. *Science* 274: 94–96.

19. Santamaria, P., T. Utsugi, B. J. Park, N. Averill, S. Kawazu, and J. W. Yoon. 1995. _-Cell-cytotoxic CD8_ T cells from nonobese diabetic mice use highly homologous T cell receptor _-chain CDR3 sequences. *J. Immunol.* 154:2494–2503.

20. DiLorenzo, T. P., R. T. Graser, T. Ono, G. J. Christianson, H. D. Chapman, D. C. Roopenian, S. G. Nathenson, and D. V. Serreze. 1998. Major histocompatibility complex class I-restricted T cells are required for all but the end stages of diabetes development in nonobese diabetic mice and use a prevalent T cell receptor _ chain gene rearrangement. *Proc. Natl. Acad. Sci. USA* 95:12538–12543.

21. Pushko, P., M. Parker, G. V. Ludwig, N. L. Davis, R. E. Johnston, and J. F. Smith. 1997. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. *Virology* 239: 389–401.

22. MacDonald, G. H., and R. E. Johnston. 2000. Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. *J. Virol.* 74: 914–922.

23. Davis, N. L., I. J. Caley, K. W. Brown, M. R. Betts, D. M. Irlbeck, K. M. McGrath, M. J. Connell, D. C. Montefiori, J. A. Frelinger, R. Swanstrom, et al. 2000. Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. *J. Virol.* 74: 371–378.

24. van Halteren, A. G. S., M. J. Kardol, A. Mulder, and B. O. Roep. 2005. Homing of human autoreactive T cells into pancreatic tissue of NOD-scid mice. *Diabetologia* 48: 75–82.

25. Sollid, L. M. 2002. Coeliac disease: dissecting a complex inflammatory disorder. *Nature Rev.* 2: 647–655.

26. Molberg, O., S. N. McAdam, R. Korner, H. Quarsten, C. Kristiansen, L. Madsen, L. Fugger, H. Scott, O. Noren, P. Roepstorff, et al. 1998. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat. Med.* 4: 713–717.

27. Anderson, R. P., P. Degano, A. J. Godkin, D. P. Jewell, and A. V. S. Hill. 2000. In vivo antigen challenge in celiac disease identifies a single transglutaminasemodified peptide as the dominant A-gliadin T cell epitope. *Nat. Med.* 6: 337–342.

28. Ben-Horin, S., P. H. R. Green, I. Bank, L. Chess, and I. Goldstein. 2006. Characterizing the circulating, gliadin-specific CD4_ memory T cells in patients with celiac disease: linkage between memory function, gut homing and Th1 polarization. *J. Leukocyte Biol.* 79: 676–685.

29. Goronzy, J. J., and C. M. Weyand. 2003. Aging, autoimmunity and arthritis: T cell senescence and contraction of T cell repertoire diversity-catalysts of autoimmunity and chronic inflammation. *Arthritis Res. Ther.* 5: 225–234.

30. Hafler, D. A., M. G. Saadeh, V. K. Kuchroo, E. Milford, and L. Steinman. 1996. TCR usage in human and experimental demyelinating disease. *Immunol. Today* 17: 152–159.

31. Sarukhan, A., P. Bedossa, H. J. Garchon, J. F. Bach, and C. Carnaud. 1995. Molecular analysis of TCR junctional variability in individual infiltrated islets of non-obese diabetic mice: evidence for the constitution of largely autonomous T cell foci within the same pancreas. *Int. Immunol.* 7: 139–146.