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Immunodominance of the CD8+ T-Cell Response in Autoimmune Diabetes

by

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ABSTRACT

Insulin-dependent diabetes mellitus is a highly complex autoimmune disease. Through studies performed on the nonobese diabetic (NOD) mouse -- an excellent disease model -- it is well established that T-cells are essential for disease pathogenesis. CD8+ T-cells are responsible for initiating pancreatic β-cell destruction mediated by an unknown antigen. The majority of CD8+ T-cells isolated from pre- and acutely diabetic NOD mice possess identical or homologous T-cell receptor (TCR) α-chains, and are H-2K^d-restricted. This thesis defines an antigenic mimic that stimulates the proliferation, cytokine secretion, differentiation, and cytotoxicity of a CD8+ T-cell bearing a representative TCR α-chain utilized by islet-derived CD8+ T-cells. The elucidation of this mimic leads to the finding that CD8+ T-cells directed to the pancreatic islets are specific for an immunodominant antigen. Therefore, a single peptide/H-2K^d complex may trigger autoimmune diabetes.

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DEDICATION

Without question, I am dedicating this thesis to my parents, Ed & Hilda Anderson. Their devotion, encouragement, and assistance over my life have been indispensable in my achievements. With all my love. . .

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A/Ala	Alanine	FMOC	Nα-9-fluorenylmethyl-oxycarbonyl
APC	antigen presenting cell	G/Gly	glycine
BSA	bovine serum albumin	GAD	glutamic acid decarboxylase
C/Cys	cysteine	H/His	histidine
CD4/CD8	T-cell co-receptors	H-2	murine MHC
cDMEM	complete Dulbecco's Modified Eagle Media	I/Ile	isoleucine
cDNA	complementary deoxyribonucleic acid	ICA	islet cell antigen
CDR	complementarity determining region	IDDM	insulin-dependent diabetes mellitus
CM	complete media	IFN-γ	interferon-gamma
CTL	cytotoxic T-lymphocyte	IL	interleukin
D/Asp	aspartic acid	K/Lys	lysine
D/K	murine MHC class I loci	L/Leu	leucine
DMSO	dimethylsulfoxide	M/Met	methionine
E/Glu	glutamic acid	mAb	monoclonal antibody
EDTA	ethylenediamine tetraacetic acid	mfi	mean fluorescence intensity
F/Phe	phenylalanine	MHC	major histocompatibility complex
FACS	fluorescence activated cell sorter	mRNA	messenger ribonucleic acid
FITC	fluorescein isothiocyanate	N/Asn	asparagine

NCBI	National Center for Biotechnological Information
NOD	nonobese diabetic
NRP	NOD-relevant peptide
ori	origin of replication
P/Pro	proline
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PE	phycoerythrin
PSCPL	positional scanning combinatorial peptide library
Q/Gln	glutamine
R/Arg	arginine
RAG	recombination-activating gene
RBC	red blood cell
RIP	rat insulin promoter
rpHPLC	reverse-phase high-performance -liquid-chromatography
S/Ser	serine
scid	severe combined immunodeficiency
T/Thr	threonine
TAP	transporter associated with antigen processing
TCR	T-cell receptor
TE	Tris-EDTA buffer
TFA	trifluoroacetic acid
TNF-α	tumor necrosis factor-alpha
tum	tumor-associated peptide
V/Val	valine
W/Trp	tryptophan
Y/Tyr	tyrosine

EPIGRAPH

Miracles do not happen in contradiction of nature, but in contradiction of what we know about nature.

Saint Augustine (AD 354 - 430)

A. INTRODUCTION

A.1 Insulin-Dependent Diabetes Mellitus

"Diabetes is a dreadful affliction . . . being a melting down of the flesh and limbs into urine . . . Life is short, unpleasant, and painful . . . the patients are affected by nausea, restlessness and a burning thirst, and within a short time, they expire." This quotation, from the Greek physician Aretaeus of Cappadocia (2nd century AD), describes a disease first recorded almost two-thousand years earlier by the ancient Egyptians (1).

Today, we know that insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease resulting in the destruction of insulin-producing β-cells in the pancreatic islets of Langerhans (herein referred to as islets) (2). The prognosis is better for diabetic individuals than in 2nd century Greece. Victims, however, become dependent on daily insulin injections and may suffer severe complications, including retinopathy, nephropathy, neuropathy, and atheroma. A susceptibility to renal failure and coronary heart disease results in a mortality rate 4-7 times higher than healthy individuals (1).

The development of the nonobese diabetic (NOD) mouse, one of the best animal models for studying IDDM, has allowed for a dramatic expansion of our understanding of this illness. A spontaneous disease very similar to human diabetes occurs in these animals. Pathogenesis commences with infiltration of the islets by immune cells (a process called insulitis) at 3-5 weeks of age and culminates in β-cell destruction and subsequent hyperglycemia at about 12-15 weeks of age in these animals (3, 4). In humans and the NOD mouse, there is a strong genetic disposition to the affliction with multiple genes (linked and unlinked to the major histocompatibility complex [MHC]) associated with disease susceptibility (1, 5, 6). However, dependence on genetic factors is not absolute (there is only a 36% concordance rate between monozygotic twins [7]) and environmental elements such as diet (8) or viral infection (9) may play a crucial role in disease pathogenesis.

A.2 Autoimmune Diseases

The immune system is an exquisitely complex entity capable of selectively eliminating an inconceivably vast array of foreign pathogens. The price paid for the immense diversity of this response is the generation of immune cells specific for self-antigens. If unchecked, this subset of autoreactive effectors would wreak havoc on an individual. Fortunately, powerful regulatory systems, which remain poorly understood, exist to prevent these rogue cells from doing harm. In some individuals, though (~5-7% of the population), these regulatory systems fail and self-induced tissue damage ensues. This pathogenic process, often initiated by T-cells, is called autoimmunity, and it can be organ specific (e.g. IDDM) or systemic (e.g. systemic lupus erythematosus) (5, 7, 10).

There are two general mechanisms of eliminating or inactivating autoreactive immune cells -- central and peripheral tolerance (5). Central tolerance occurs in the thymus during T-cell maturation. T-cells are first selected for their ability to recognize an MHC molecule associated with peptide (positive selection). Then, elimination of autoreactive T-cells capable of high affinity/avidity recognition of self-peptides expressed in the thymus complexed with MHC occurs (negative selection).

Nonetheless, some autoreactive cells do mature and enter the blood stream, necessitating the need for peripheral tolerance. Common belief suggests circulating autoreactive B- and T-cells are restricted from attacking their target tissue either by lack of antigen expression by professional antigen presenting cells (APCs), sequestering of potentially autoantigenic peptides, anergy induction by down-regulation of antigen receptors or co-receptors, indifference of unprimed T-cells, or deletion (11).

The question remains, how does this breakdown in tolerance to self-tissue by autoreactive cells occur? There is definitely an inheritable predisposition to autoimmunity with disease susceptibility under polygenic control. Genes in the MHC locus play key roles, which is not surprising considering the importance of T-cells in autoreactivity.

However, genetic predisposition is not enough to develop autoimmunity -- poorly understood environmental factors play a role. Theories as to the events leading to the breakdown of tolerance abound. Initial tissue damage may release previously sequestered antigen resulting in a tissue-specific immune response. Perhaps foreign epitopes are similar enough to self-antigen to initiate a response initially aimed at a pathogen, but which will eventually redirect to the host's own tissue (a process called molecular mimicry). Inappropriate MHC class II expression, cytokine imbalances, dysfunctional idiotype networks, and poor T-cell mediated immune regulation may also lead to autoimmunity. In many diseases, the hormonal status of the patient is important, with disease-onset occurring at the commencement of sexual maturity, or the gender-skewing of some afflictions. The actual mechanisms of tolerance, and their subsequent breakdown in autoimmunity, still remain largely unknown (5, 7, 10).

A.3 Immunopathology of IDDM

The diabetogenic immune response is complex and encompasses all arms of the immune system (12). T-cells, however, are essential mediators of disease pathogenesis, as demonstrated by the inability of athymic NOD mice to develop IDDM (13), and the acceleration of disease in neonatal NOD mice by transfer of T-cell enriched splenocytes from a diabetic donor (14). The importance of both the CD4+ and CD8+ T-cell subsets has been demonstrated by adoptive transfer experiments where CD4+ and/or CD8+ T-cell-enriched populations were injected into irradiated adult (15) or immunodeficient NOD mice (16). In both studies, CD4+ and CD8+ T-cells were required for the efficient transfer of IDDM to donor animals. The exact role of the CD4+ and CD8+ T-cell subsets in disease progression has, however, remained a subject of debate.

Experiments aimed at resolving this controversy have shown that the age of IDDM onset in young NOD animals can be accelerated by the transfer of CD4+ islet-specific T-cell clones (17) and in NOD mice with CD4+ T-cells expressing a transgenic, pathogenic

T-cell receptor (TCR) (18). A differential requirement for CD8+ T-cell help in disease progression has been observed between different pathogenic CD4+ T-cell clones, though (19). Furthermore, the CD4+ dependence and CD8+ independence of syngeneic islet graft destruction (20, 21), and abrogation of disease by anti-CD4 monoclonal antibody treatment (22) has been observed. Likewise, CD4+ T-cells from NOD mice transgenic for a highly pathogenic TCR in a recombination-activating gene-2-(RAG-2)-deficient background are able to accelerate IDDM onset without CD8+ T-cell help (23). Taken together, these studies suggest that CD4+ T-cells are responsible for β-cell destruction, and the role CD8+ T-cells fill in this process is secondary. In addition, along with their role in disease pathogenesis, there is extensive evidence suggesting that a subset of CD4+ T-cells play a regulatory/suppressive role in IDDM pathogenesis (24-29).

However, there is strong experimental evidence suggesting that CD8+ T cells are extremely important in disease pathogenesis. For example, human clinical trials reveal aberrantly activated peripheral CD8+ T-cell subsets in patients with pre- (30) and recent onset IDDM (31). Adoptive transfer experiments of CD4+ and CD8+ T-cell subsets to athymic NOD mice, and studies on MHC class $I^{-/-}$ NOD mice demonstrated that CD8+ T-cells were required for insulitis initiation and terminal β-cell destruction. CD4+ T-cells, in these experiments, were needed for the effective recruitment of immune cells to the pancreas (13, 32-36). However, once insulin-dependence has developed in donor NOD mice, their CD8+ T-cells are no longer essential to transfer disease to immunodeficient, MHC class $I^{-/-}$ NOD recipients (36, 37). Moreover, the majority of CD8+ T-cell clones isolated from the islets of pre- and acutely diabetic NOD mice were, despite the lack of skewed TCR usage, H-$2K^d$-restricted and possessed TCRα-chains bearing homologous complementarity-determining region (CDR) 3 sequences (responsible for interaction with antigen [38]). This suggests the existence of an immunodominant MHC class I-restricted antigen (or a restricted set of antigens) in the NOD mouse that, when displayed by H-$2K^d$,

are recognized by the CD8+ T-cells which accumulate in the islets of Langerhans during disease pathogenesis. In addition, these clones elicit MHC class I-restricted β-cell destruction *in vivo* with the assistance of CD4+ T-cells, and recruit naive CD4+ cells to the islets (37, 39-41). As well, NOD mice transgenic for a representative TCR isolated from islet-derived CD8+ cytotoxic T-lymphocytes (CTLs) show a drastic acceleration of spontaneous disease (42), and initiate IDDM in RAG-2-deficient NOD mice, which are completely devoid of CD4+ T-cells (albeit with a delayed onset and lower incidence of disease due to defects in insulitis progression) (23).

In summary, both CD4+ and CD8+ T-cells are essential in disease pathogenesis. Present consensus suggests that CD8+ T-cells initiate the disease, thereby mobilizing CD4+ T-cells to the islets where they become the major recruiter of immune cells. Both may act as final mediators of β-cell death through the following theoretical mechanisms. Cytotoxicity may be the product of direct recognition of a β-cell antigen in the context of MHC class I by CD8+ CTLs resulting in perforin or FasL-Fas mediated destruction. Since β-cells do not express MHC class II molecules (43), antigen shed from β-cells may be displayed by the MHC class II of APCs resulting in the activation of CD4+ T-cells. The cytotoxic CD4+ T-cells now assist in eliciting β-cell destruction either through FasL-Fas interactions, the release of soluble cytolytic cytokines, or the activation of cytocidal macrophages (4).

A.4 Rationale & Hypothesis

Given the importance of T-cell involvement in IDDM, it becomes essential to elucidate the nature of their activity. Since very little is known about the response of CD8+ T-cells in IDDM, other than their extreme importance in disease initiation and as mediators of β-cell destruction, this study concentrates on the nature of this T-cell subset. It is the hypothesis of this thesis that CD8+ T-cells recruited to the islets of Langerhans in

spontaneous autoimmune diabetes recognize a single (or highly restricted set of) immunodominant antigen/H-2K^d complex(es) on pancreatic β-cells.

There are two lines of evidence that support this hypothesis. i) Most CD8+ T-cell clones isolated from the islets of pre- and acutely diabetic NOD mice use similar, if not identical, TCRα-chain CDR3 motifs ([hydrophobic]-[Arg]-[Asp/Glu or Asn/Gly]-[Tyr/Ser]) (39). Clones bearing this motif are H-2K^d-restricted (40), and they are cytotoxic towards β-cells *in vitro*, and *in vivo* (39, 41). Importantly, CD8+ T-cell clones isolated from the earliest insulitic lesions show similar TCRα-chain CDR3 usage ([Met]-[Arg]-[Asp/Glu]) (37). This suggests that a single (or restricted set of) peptide/MHC class I complex(es) may initiate disease pathogenesis as well as mediate final β-cell damage. ii) NOD mice transgenic for the TCRβ-chain of a clone (NY8.3, see ref. 40) bearing this islet associated TCRα-chain CDR3 motif found in the majority of islet-derived CD8+ T-cells have an accelerated age of IDDM onset. Furthermore, CD8+ T-cells isolated from the islets of these animals bear endogenous TCRα-chains identical to the TCRα-chain of the clonotype donating the TCRβ-chain transgene (42).

If the hypothesis holds true, then the suggested immunodominant CD8+ T-cell response differs drastically from the CD4+ T-cell response observed in IDDM. Diabetogenic CD4+ T-cells target a large number of autoantigens (see Section A.4.1) and are subject to the phenomenon of epitope (determinant) spreading, whereby an immune response originally directed against an antigen spreads intramolecularly (to other determinants of that antigen) and intermolecularly (to epitopes from different proteins) (44).

To address such a hypothesis, knowledge about the peptide recognized by CD8+ T-cells bearing this common CDR3 motif must be possessed. At the outset of this study, there was no information regarding the target antigen. To resolve this, the antigen either had to be identified, or a mimic of the target peptide had to be synthesized.

There are two approaches to identifying the natural target of a CD8+ T-cell. The first is expression cloning. In this method, cDNA generated from an immunogenic cell is transfected into a non-immunogenic target. A cDNA encoding a peptide that, when displayed by the appropriate MHC class I, sensitizes the non-immunogenic target to the desired CD8+ T-cell recognition is identified. A second option is peptide purification. Here, MHC-bound peptides are eluted from MHC molecules and separated by reverse-phase high-performance-liquid-chromatography (rpHPLC). The ability of each fraction eluted from the rpHPLC column to be recognized by the desired CD8+ T-cell in an MHC class I-restricted manner is tested. Subsequent identification of peptides in active fractions follows (45).

Through the use of random peptide libraries, it is also possible to generate antigenic mimics. In this system, each position of a randomly generated peptide library is fixed with a particular amino acid to determine which amino acids are antigenic at which positions. From this information, an antigen-mimic (a mimetic or mimotope) is generated (46).

To apply any of the aforementioned techniques, large populations of CD8+ T-cells bearing identical TCRs are essential. Generating islet-derived clones from acutely diabetic NOD mice is not feasible since CD8+ T-cell populations are very short-lived *in vitro*, and there is a heterogeneous mixture of TCRs. Therefore, it would be impossible to perform long-range experiments with CD8+ T-cells bearing the same TCR.

To address this issue, our research team made an NOD strain transgenic for the TCRα/β-chains of the islet-derived clone NY8.3 (40) bearing the following TCRα-chain CDR3: [Met]-[Arg]-[Asp]-[Ser] (39). With this transgenic CD8+ T-cell population (herein called 8.3-CD8+ T-cells), it was possible to attempt to identity this β-cell-associated antigen/mimotope.

A.4.1 Potential Autoantigens

Knowledge of an array of antigens possibly involved in the pathogenesis of diabetes exists through the detection of circulating autoantibodies and CD4+ T-cell reactivity profiles (47-58), and they are summarized in Appendix I. In fact, certain circulating autoantibodies and autoreactive T-cell, such as those specific for insulin or glutamic acid decarboxylase (GAD), for example, are commonly used as predictive markers for disease onset (58, 59). Interestingly, some of these autoantigens are intracellularly localized along the insulin secretory pathway (60). The most heavily studied IDDM-related autoantigens are insulin and GAD.

Proinsulin/insulin is a favoured autoantigen in IDDM due to its unique expression in β-cells. Circulating anti-insulin antibodies and peripheral CD4+ T-cells have been detected in pre- and recent-onset diabetic patients and healthy first-degree relatives (47, 48), as well as in the islet-infiltrates of pre-diabetic NOD mice (61). P815 cells transfected with preproinsulin, however, were not able to elicit the activation of NOD islet-derived CD8+ T-cell clones (unpublished observations).

Another important autoantigen, GAD, has two isoforms -- 65 kD and 67 kD. This enzyme is expressed on β-cells (with the exception of GAD67 in humans) and neuroendocrine cells (48). GAD specific CD4+ and CD8+ T-cells have been detected in the peripheral blood of pre/recent onset diabetics (62, 63). However, GAD-specific cytotoxic CD8+ T-cells have yet to be isolated from the pancreas of acutely diabetic subjects. To date, the predominant islet-specific antigen stimulating diabetogenic CD8+ T-cell activity remains elusive.

B. METHODS

B.1 Reagents

B.1.1 Generation of TCR Transgenic Mice

The CD8+, H-2K^d-restricted, β-cell cytotoxic T-cell clone NY8.3 (40), which possesses a CDR3 motif homologous or identical to many NOD islet-derived CD8+ clones ([Met]-[Arg]-[Asp]-[Ser]) donated the TCR-α (Vα17.1-Jα42) and TCR-β (Vβ8.1-Dβ2.1-Jβ2.4) transgenes to generate the 8.3-NOD mice. These TCR rearrangements were subcloned into TCRα and TCRβ shuttle vectors containing upstream TCR enhancers and regulatory sequences. The transgenic constructs were microinjected into fertilized (SJL/J x C57BL/6)F2 eggs (DNX, Princeton, NJ) and implanted into pseudopregnant female mice. Offspring were screened for inheritance of the transgenes by Southern blotting using VJα or VDJβ probes. Two founder mice expressing the transgene were backcrossed with NOD/Lt mice for 3-6 generations to generate the 8.3-NOD mouse (23)

B.1.2 Mice

NOD/Lt (H-2I-A^{g7}, -K^d, -D^b) and C57BL/6 mice (H-2I-A^b, -K^b, -D^b) were produced from breeding stocks purchased from The Jackson Laboratory (Bar Harbor, Maine). 8.3-NOD and 8.3-TCRβ-NOD mice, expressing the TCRα/β or TCRβ rearrangements, respectively, of the H-2K^d restricted β-cell-reactive CD8+ T-cell clone NY8.3 (40) have been described (23, 42). Severe combined immunodeficient NOD (NOD.*scid*) transgenic for B7.1 expression under control of the rat-insulin promoter (RIP) (herein called NOD.*scid*-RIP-B7) were donated from D. Serreze (Albert Einstein College of Medicine, NY). These animals possess no endogenous B- or T-cells, and express B7.1 exclusively on the pancreatic β-cells. B7.1 is an important co-stimulatory molecule for CD8+ T-cell activation and reduces the effector's susceptibility to activation-induced apoptosis, resulting in a longer life span of the clonal population. These islets act as professional APCs to β-cell specific CD8+ T-cells, resulting in a more potent

activation/proliferation than normal NOD islets (64, 65). All mice were housed under specific pathogen-free conditions.

B.1.3 Media

Complete Media (CM) -- RPMI 1640 media (Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Grand Island, NY), 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin (Flow Laboratories, McLean, VA), and 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO).

rIL-2 Media (rIL-2) -- CM containing 0.5 U/mL rIL-2 (Takeda, Osaka, Japan).

Complete DMEM (cDMEM) -- Dulbecco's Modified Eagle Media (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 μg/mL streptomycin.

MIN Media -- High glucose DMEM (Life Technologies, Grand Island, NY) with 20% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, 50 μg/mL streptomycin, and 25 μM 2-mercaptoethanol.

B.1.4 Cell Lines, Antibodies, and Flow Cytometry

NOD islet-derived NIT-1 insulinoma cells (66) were provided by E. H. Leiter (The Jackson Laboratory), and maintained in MIN media. WEHI-164 clone 13 cells, maintained in CM were provided by D. Remick (University of Michigan, Ann Arbor, MI). H-2K^d transfected RMA-S cells (RMA-S-K^d, H-2K^{d,b}/D^b), maintained in CM supplemented with 600 μg/mL geneticin (G418 sulfate) (Life Technologies, Grand Island, NY), were provided by B. Wipke and M. Bevan (University of Washington, Seattle, WA). COS-7 cells maintained in cDMEM were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). WOP 32-4 & 3027-3 cells were maintained in CM, and provided by C. Basilico (New York School of Medicine, New York, NY). H-2K^d-

transfected L929 cells (L929-K^d) were obtained from J. Yewdell (National Institutes of Health, Bethesda, MD).

A hybridoma secreting the Vβ8.1/8.2 specific monoclonal antibody (mAb) KJ16 was a gift from P. Marrack (National Jewish Center for Immunology, Denver, CO). The hybridoma secreting the mAb GK1.5 (rat anti-CD4) was obtained from the ATCC. Anti-Lyt-2 (CD8α)-phycoerythrin (PE) (53-6.7), anti-L3T4 (CD4)-biotin (H129.19), anti-Vβ 8.1/8.2-fluorescein isothiocyanate (FITC) (MR5-3), anti-H-$2K^d$-FITC (SF1-1.1), and anti-H-$2D^b$-FITC (KH95) were purchased from PharMingen (San Diego, CA). Streptavidin-PerCP was obtained from Becton-Dickinson (Mississauga, ON).

Cell lines or single cell suspensions of splenocytes were obtained for flow cytometry. Red blood cells (RBCs) were lysed with 0.87% ammonium chloride if necessary. Cells were washed twice with fluorescence activated cell sorting (FACS) buffer (phosphate-buffered saline [PBS], 1% fetal bovine serum, 0.1% sodium azide). Cells were stained either with anti-H-$2K^d$-FITC or anti-H-$2D^b$-FITC [single stain], or with anti-CD8α-PE, anti-L3T4 (CD4)-biotin and anti-Vβ8.1/8.2-FITC [triple stain] (1 μg mAb/10^6 cells) for 30 minutes at 4°C. Cells were washed, resuspended in FACS buffer [single stain] or incubated for 30 minutes at 4°C with Streptavidin-PerCP to reveal biotin stained cells [triple stain], washed, resuspended in FACS buffer, and analyzed with a FACscan (Becton-Dickinson).

B.2 CD8+ T-Cell Isolation

Studies of islet-associated CD8+ T-cells in IDDM are notoriously difficult to undertake. These T-cell populations are highly susceptible to activation induced apoptosis, to down-regulate TCR and co-receptors, and to lose cytotoxic activity within days to weeks. The spleen or islets of acutely diabetic 8.3-NOD were used as a source of CTL's unless otherwise mentioned. The T-cells of these mice all express the same TCR, making it easy to isolate multiple CD8+ T-cell populations with the same specificity.

B.2.1 Generation of Splenic 8.3-CD8+ T-Cell Lines

Twelve wells of a 96-well flat bottom tissue culture plate were coated with the anti-Vβ8.1/8.2 mAb KJ16 in 50 mM Tris/150 mM HCl (pH 9.5) for 90 minutes at 37°C, then overnight at 4°C. The next day, a spleen was removed from an acutely diabetic 8.3-NOD mouse to prepare single cell suspensions. After lysing RBCs, CD4+ T-cell depletion proceeded by GK1.5 incubation with the splenocytes at 4°C for half an hour. The cells were washed and incubated with pre-washed magnetic beads coated with goat-anti-rat IgG. Again, the mixture was incubated for 30 min. at 4°C. CD4+ T-cell/magnetic bead complexes were depleted by placing the mixture in a magnetic particle concentrator (Dynal, MPC-6, Great Neck, NY) for 5 minutes. The CD4+ T-cell-depleted suspension was plated (2×10^5 cells/well) in the KJ16-coated wells for three days. After this time, three wells were pooled into one well of a 24-well flat bottom tissue culture plate with rIL-2 media and allowed to proliferate for a week. Expansion of proliferating cells and addition of fresh rIL-2 media was performed as required. CD4+ depletion was confirmed with three-colour flow cytometry.

B.2.2 Generation of Islet-Derived 8.3-CD8+ T-Cell Lines

Islets were isolated from acutely diabetic 8.3-NOD mice by collagenase (Collagenase Type 4, Worthington Biochemical Corp., Freehold, NJ) digestion and ficoll (Sigma, St. Louis, MO) density-gradient centrifugation as described previously (67). Incubation in rIL-2 media for approximately 3-5 days followed. The remaining cells were CD4+ T-cell depleted and either cloned or further expanded.

B.2.3 Generation of Islet-Derived 8.3-CD8+ T-Cell Clones

Islet derived CD8+ T-cell lines were used to generate clones through limiting dilution. Approximately 5-12 days after generation of an islet-derived line, the CD8+ T-cells were plated in a 96-well round bottom plate at a concentration of 1-100

cells/well/200μL of rIL-2 media (cloning efficiency of 8.3% ± 1.9%). These cells were stimulated with 7-10 γ-irradiated (3 000 rad) NOD islets/well.

After 1 week, fresh rIL-2 media was added. Proliferating colonies were tested for serine esterase content (described below). Serine esterase positive wells were expanded in 24-well plates.

Clones expanded in 24-well plates were restimulated once every 1-2 weeks as required with 40 γ-irradiated (3 000 rad) NOD.*scid*-RIP-B7 islets/well. Wells were split and fresh media given as required.

B.2.3.1 Serine Esterase Assay. Approximately 5 x 10^4 T-cells in 50 μL CM were lysed with an equal volume of 1% Triton X-100 (Sigma, St. Louis, MO) for 30 minutes. To 50 μL of lysate, 950 μL Nα-CBZ-L-lysine thiobenzyl ester hydrochloride (BLT; Sigma, St. Louis, MO) was added, and the mixture incubated at 37°C for 20 minutes at which time the reaction was stopped by lowering the temperature by ice bath and adding 1 mL cold PBS. Then, 200 μL was transferred to a 96-well flat bottom tissue culture plate, and absorbance at 405 nm was read. A_{405} readings >0.106 OD (2 standard deviations above the mean absorbance of negative control samples) were considered positive.

B.2.4 NOD Relevant Peptide (NRP)-Stimulated Generation of CD8+ T-cells

Approximately 2 x 10^5 γ-irradiated APCs derived from the spleen of a non-diabetic NOD mouse were pulsed for 1 hour at 37°C with 1μg/mL of the 8.3-CD8+ T-cell agonist NRP (described in detail in Section C.3) in 100 μL CM/well of a 96-well round bottom plate. Splenocytes from an acutely diabetic 8.3-NOD mouse were depleted of CD4+ T-cells and 1 x 10^4 CD8+ T-cells in 100 μL CM were added to the NRP-pulsed APCs and allowed to incubated for 3 days in 37°C, 5% CO_2. At this time, rIL-2 media was provided and cells were transferred to a 24-well tissue culture plate. Wells were split and fresh media given as required.

B.3 Expression Cloning

B.3.1 Transient Transfection of COS-7 Cells

As delineated in figure 1, NIT-1 (an NOD-derived β-cell insulinoma line [66]) mRNA was isolated as outlined in Dynal's Dynabeads mRNA Direct Kit (Lake Success, NY). Briefly, 4 x 10^6 NIT-1 cells were disrupted with lysis buffer (supplied by Dynal; 100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM ethylenediamine tetraacetic acid [EDTA], pH 8.0, 1% SDS, and 5 mM dithiothreitol). Lysate was mixed with Dynabeads Oligo$(dT)_{23}$, which anneals to the polyA-tail of mRNA. The Dynabeads Oligo$(dT)_{23}$-mRNA complex was isolated from the remainder of the lysate with a Dynal Magnetic Particle Concentrator. To elute the purified mRNA, 2 mM EDTA (pH 8.0) was added.

From this mRNA, NIT-1 cDNA was generated using a cDNA synthesis kit (Invitrogen, San Diego, CA). First strand cDNA synthesis was initiated with Oligo-dT primer and reverse transcriptase. Second strand synthesis commences with the addition of DNA polymerase I. The cDNA termini are blunted with T4 DNA polymerase, and EcoRI adapters ligated to the ends with T4 DNA ligase. The cDNA is then cut with XhoI, and ligated into the pcDNA I/Amp (Invitrogen, San Diego, CA) vector pre-cut with EcoRI and XhoI, ensuring unidirectional ligation of inserts.

Three libraries were made according to the average size of their cDNA inserts -- <400 bp, 400-800 bp, and >800 bp, as determined by agarose gel electrophoresis. Competent XL-1 blue *E. coli* were transformed by cDNA from each library and plated. Plasmid DNA from 200 successfully transformed colonies were pooled. A total of 962 pools were generated in this manner, representing ~2 x 10^5 total cDNA (isolation of mRNA and cDNA generation was performed by another individual within the lab).

COS-7 cells (a cell line transfected with the SV-40 large T antigen [68]) were transfected using the DEAE-dextran/chloroquine method of transfection (69) with some modifications (68, unpublished data). Vectors possessing the SV-40 origin of replication

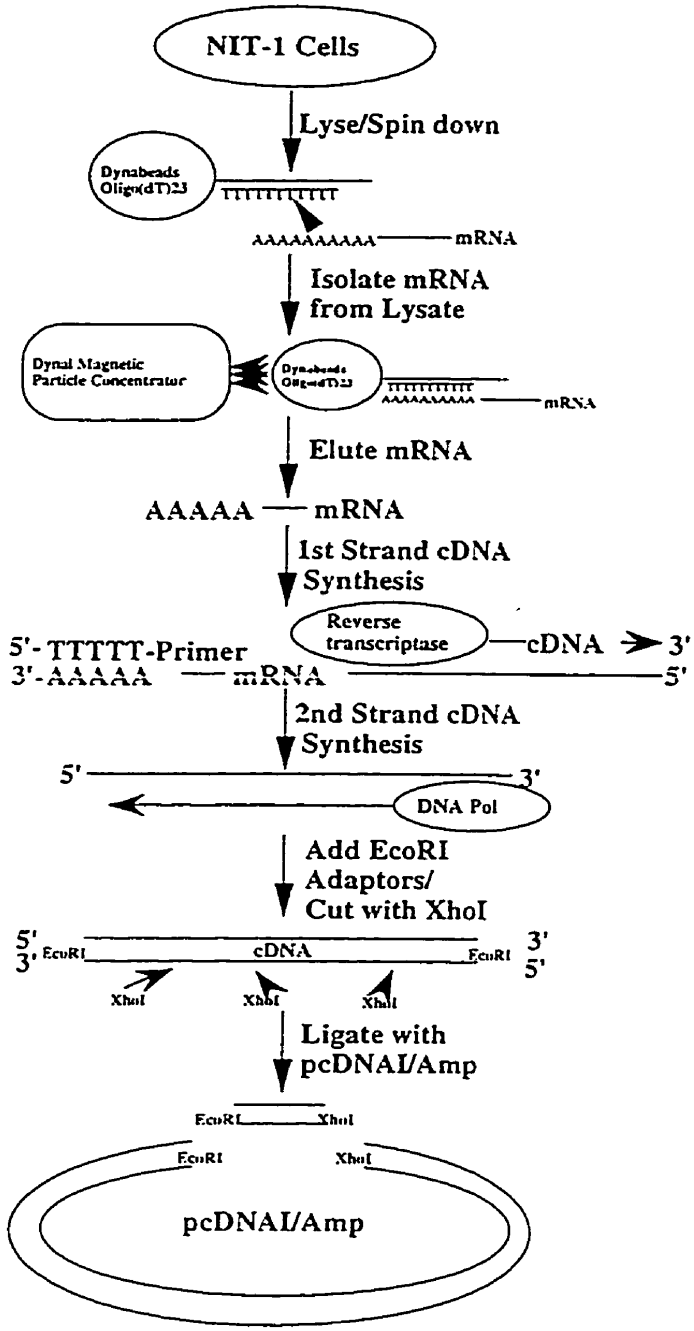

Figure 1. Generation of NIT-1 cDNA. NIT-1 cells were lysed and mRNA was isolated using Dynabeads Oligo$(dT)_{23}$ in conjunction with Dynal's magnetic particle concentrator. Using Invitrogen's cDNA synthesis kit, NIT-1 cDNA was generated from mRNA, ligated to EcoRI adapters, cleaved with XhoI, then ligated to pcDNA I/Amp pre-cut with EcoRI and XhoI.

(ori) will undergo massive amplification when transfected into this cell line (68). Since COS-7 do not express H-2K^d (the restricting MHC class I molecule of the 8.3-CD8+ T-cells being tested), H-2K^d cDNA cloned into the pcDNA I/Amp vector was co-transfected with the NIT-1 cDNA pools and probed with KJ16-stimulated 8.3-CD8+ T-cell lines (see Section B.2.1) as follows.

As summarized in figure 2, 2 x 10^4 COS-7 cells were plated per well of a 96-well, flat bottom tissue culture plate in cDMEM and then expanded overnight. On the day of transfection, in each well of a separate 96-well V-bottom plate, 13.3 ng/μL of NIT-1 cDNA and 3.3 ng/μL of H-2K^d cDNA was plated with 40 μL of DMEM-10% DNase free NuSerum IV (Collaborative Biomedical Products, Chicago, Il) containing 400 μg/mL DEAE-dextran and 200 μM chloroquine. The volume was brought up to 70 μL with Tris-EDTA (TE) buffer. This comprised the transfection solution.

The COS-7 cells were washed twice with DMEM, then, 60 μL of the transfection solution was added to each well, and the cells incubated for three hours at 37°C, 6.5% CO_2. After this period, the transfection solution was removed, and the cells were incubated for 2 min. at room temperature with PBS-10% dimethylsulfoxide (DMSO; Fisher, Fair Lawn, NJ), followed by two washings with DMEM-10% NuSerum IV. cDMEM was added, and the cultures were incubated for 48 hours at 37°C, 6.5% CO_2 (68, unpublished observations).

Forty-eight hours after transfection, cDMEM was removed from the transfected COS-7 cells and 1 x 10^4 KJ16-stimulated splenic 8.3-CD8+ T-cells were added to each well in 100 μL of CM, and the co-cultures incubated overnight. T-cell activation was monitored by measuring the secretion of tumor necrosis factor (TNF)-α into supernatant. Briefly, TNF-α-sensitive WEHI 164 clone 13 cells (10^6 cells/mL) in CM were incubated for 3 hours with 1 μg/mL actinomycin D (Boehringer Mannheim, Laval, Quebec) to stop DNA transcription and replication. Then, 50 μL of this solution was added to wells of a

96-well flat bottom plate. To these wells, 50 µL of the COS-7/8.3-CD8+ T-cell co-culture supernatant was added. After 24 hours, WEHI 164 clone 13 cell death was measured by the dye sodium 3'-[1-(phenylaminocarbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) from Boehringer Mannheim's Cellular Proliferation Kit II (Laval, Quebec). Live cells metabolize this dye resulting in the formation of a characteristic colour monitored at 490 nm (reference wavelength of 630 nm) using a spectrophotometer. Thus, a low absorbance reading is indicative of WEHI 164 clone 13 cell death, and, therefore, TNF-α secretion. A positive result was considered to be two standard deviations below the mean for any group of data.

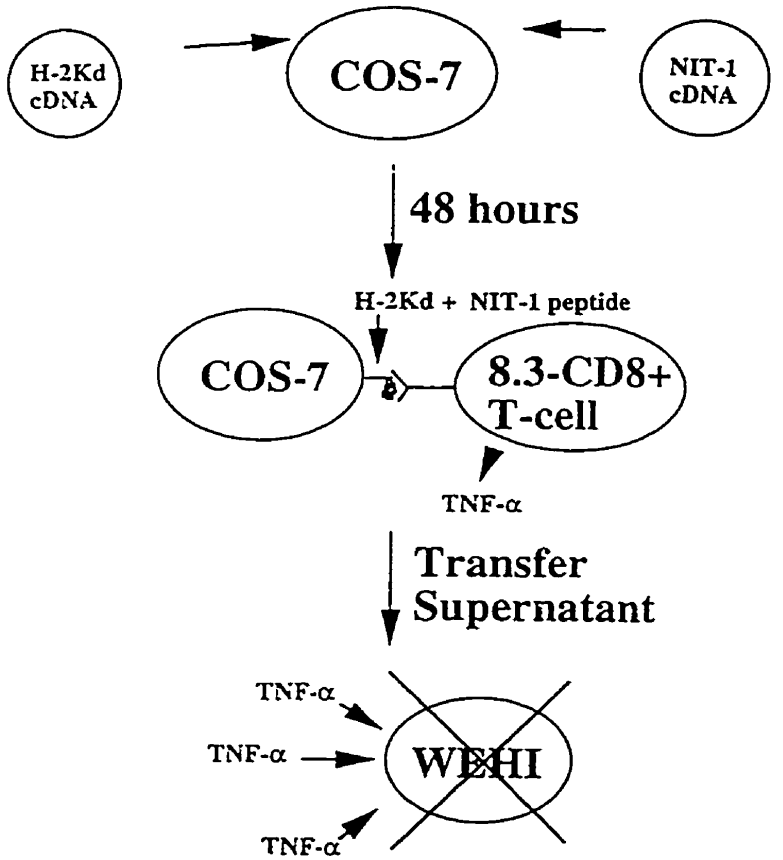

Figure 2. Transfection and Screening of COS-7 Cells. COS-7 cells are co-transfected with H-2K^d cDNA and the NIT-1 cDNA libraries. After 48 hours, the transfected COS-7 cells are tested for their ability to stimulate TNF-α secretion from KJ16-stimulated 8.3-CD8+ T-cells using TNF-α-sensitive WEHI 164 clone 13 cells as described in Methods.

Variants of this method have been useful in identifying several tumor antigens recognized by tumor-specific CD8+ T-cells in humans, including BAGE (70), MART-1

(71), and a variety of others (72-79). It has never been used for the isolation of autoantigens displayed by non-transformed cell types. It is important to note, however, that in rare cases this protocol has resulted in the identification of incorrect antigens (80).

B.3.2 Transient Transfection of WOP cells

A murine cell line analogous to COS-7 was also utilized. The murine cells, derived from NIH 3T3 mice, are named WOP, and they have been transfected with the polyoma large T antigen (68, 81). Analogous to the COS-7 transfection system, although not as effective, the polyoma large T antigen results in large scale amplification of vectors possessing the polyoma ori. There are two strains of these cells, WOP 3027-3 and WOP 32-4. Through our own observations, it has been noted that the WOP 32-4 constitutively express H-2K^d whereas the WOP 3027-3 do not.

The transfection protocol developed for these lines is identical to that described above for the COS-7 with the following exceptions. First, TE was toxic to WOP cells (unpublished observations), so PBS was used as a buffer. Second, WOP cells were quite sensitive to the DEAE-dextran/chloroquine solution used during the transfection, thus, the incubation with the transfection solution was reduced from 3 hours to 1 hour.

B.4 Peptide Purification

This technique utilizes transporter associated with antigen processing (TAP) II$^{-/-}$ RMA-S-K^d cells. These cells, transfected to express H-2K^d, are unable to load endogenous peptide onto their MHC class I molecules. Empty MHC molecules are not stable on the cell surface at 37^{o}C, but are at 26^{o}C. Therefore, exogenous peptides can be loaded onto the expressed empty MHC class I molecules at 26^{o}C, resulting in a peptide/MHC class I structure that remains stable when the temperature is raised to 37^{o}C (82-86).

B.4.1 Acid Extraction

Peptides were isolated from 1 x 10^{10} NIT-1 cells or 10 spleens of 6-8 week old NOD mice as follows. Samples were suspended in 20 mL 0.1% (v/v) trifluoroacetic acid (TFA; Fisher, Fair Lawn, NJ), and homogenization of samples was achieved by douncing (10 strokes; borosilicate glass dounce-homogenizer) and ultrasonification (20 pulses of 1s, sonifer model B15; Branson, Danbury, CT). The homogenates were stirred for 30 minutes at 4°C (pH kept at 2.0 by adding 1% TFA as required). Supernatants were taken after centrifugation (150,000g for 30 min. at 4°C). The remaining pellets were extracted again using 10 mL of 0.1% TFA. Combined supernatants of both extractions were pooled and passed through Centricon 10 membranes (87). Materials of Mr <10 000 were collected, lyophilized, and stored at -70°C (88).

B.4.2 First rpHPLC Fractionation

Acid extracted fractions were separated by reverse-phase high-performance-liquid-chromatography (rpHPLC) as follows. Acid extracts were solubilized in 2 mL of 0.1% TFA and subjected to rpHPLC (uBondpack C_{18} column, 3.9 x 300 mm; Waters, Milford, MA). Peptides were eluted off with the following acetonitrile gradients. 0-5 min., 0% acetonitrile, 5-40 min., linear increase to 60% acetonitrile (~1.7% acetonitrile/min.), 45-50 min., maintain 60% acetonitrile, followed by linear decrease to 0% acetonitrile. A flow rate of 1 mL/min. and fraction size of 1 mL were used. Absorbance of fractions was monitored at 214 nm and 280 nm using a Waters multiwavelength detector. Individual fractions were dried by vacuum centrifugation (Speedvac; Savant, Farmingdale, NY), and stored at -70°C until used (88).

Each fraction was resuspended in 200-700 µL of RPMI 1640 and loaded onto RMA-S-K^d to determine their ability to elicit TNF-α secretion from 8.3-CD8+ T-cells as described previously. Fractions eliciting TNF-α were pooled, dried by vacuum centrifugation, and stored at -70°C until used.

B.4.3 Second rpHPLC Fractionation

The pool of active fractions was resuspended in 0.1% TFA, again subjected to rpHPLC, and eluted off with the following acetonitrile profiles. 0-5 min., 0% acetonitrile, 5-15 min., 0-25% acetonitrile, 25-133 min., 25-50% acetonitrile (0.25%/minute), 133-135 min., 50-80% acetonitrile, and 135-150 min., linear decrease to 0% acetonitrile. Again, a flow rate of 1 mL/min and fraction size of 1 mL were used. Absorbance of fractions were monitored at 214 nm and 280 nm. Individual fractions were dried by vacuum centrifugation, and stored at -70°C until used.

Each fraction from 15-133 minutes was resuspended in 100 µL RPMI 1640-0.25% bovine serum albumin (BSA; Sigma, St. Louis, MO), loaded onto RMA-S-K^d cells as previously described, and used as targets in a cytotoxicity assay (chromium release, described in Section B.5.3) with NRP-stimulated 8.3-CD8+ T-cells (Section B.2.4). BSA was used rather than heat-inactivated fetal calf serum to reduce peptide degradation by serum proteases (89). This procedure has been used successfully by others to identify CD8+ T-cell antigens (90-92).

B.5 Generation of an 8.3-CD8+ T-Cell-Specific Mimotope Using Positional Scanning Combinatorial Peptide Libraries

B.5.1 Peptide Synthesis

Peptide libraries and single peptide sets with free amino and carboxy termini were synthesized by multipin technology using standard Nα-9-fluorenylmethoxycarbonyl (FMOC) chemistry (Chiron Technologies, San Diego, CA). Following assembly, side-chain protecting groups were removed by acidolysis. The pin-bound peptides were then subjected to a rigorous sonication/washing procedure to remove organic contaminants, cleaved from the pins, and dried down. Single custom peptides were purified through rpHPLC to ≥90% purity, and confirmed as containing the target peptide by ion spray mass

spectrometry. In this manner, 1 μmol of peptide libraries and 1 mg of custom peptide sets were obtained.

Peptide libraries were resuspended in 0.1 M Hepes (Sigma, St. Louis, MO) in 40% acetonitrile (Fisher Scientific, Fair Lawn, NJ) at pH 7.4, and custom peptides were resuspended in 0.1% acetic acid (Fisher Scientific, Fair Lawn, NJ), as per the manufacturer's directions, to a concentration of 1 mg/mL. Dilutions were made with RPMI 1640-0.25% BSA.

B.5.2 Chromium Release Assay

RMA-S-K^d (display empty H-$2K^d$ molecules at 26°C; described in Section B.4) cells were incubated overnight at 26°C and then labeled with [^{51}Cr]-sodium chromate (DuPont-NEN, Boston, MA) for 2 hours at the same temperature. For experiments where NOD islet cells were used, targets were kept and labeled at 37°C. Approximately 1 x 10^4 radio-labeled RMA-S-K^d cells were pulsed with peptide in 100 μL RPMI-0.25% BSA in 96-well round bottom plates for 1 hour at 26°C. Then, 100 μL of 1 x 10^5 CD8+ T-cells (10:1 effector/target ratio) in CM-20% heat inactivated fetal bovine serum were added. After 8 hours at 37°C in 5% CO_2, 100 μL of supernatant was collected and tested in a γ-counter. Plain media or 5% Triton X-100 were added to sets of target cells for examination of spontaneous and total cell lysis, respectively. %^{51}Cr Release was determined with the formula [(experimental lysis) - (spontaneous lysis)]/[(total lysis) - (spontaneous lysis)] x 100%.

B.6 Immunological Properties of NRP, NRP Analogs, and NRP Homologues

B.6.1 H-$2K^d$ Binding Assay

RMA-S-K^d cells were grown overnight at 26°C, incubated with 1μg/mL peptide for 1 hour, and placed at 37°C for 3 hours to allow for down-regulation of empty MHC molecules. Cells were then stained with either anti-H-$2K^d$-FITC or anti-H-$2D^b$-FITC mAb, and analyzed by flow cytometry. Data was calculated by subtracting the mean

fluorescence intensity (mfi) for K^d or D^b on non-peptide-pulsed cells from the mfi for the same MHC molecules on peptide-pulsed cells.

B.6.2 Proliferation Assay

Pancreatic islet cells were prepared from 4-8 week-old NOD mice as described (39), and used as a source of antigen. Splenocytes from 8.3-NOD mice were depleted of CD4+ T-cells by using rat anti-CD4 mAb (GK1.5) and goat anti-rat IgG coated magnetic beads as described in Section B.2.1. CD4+ T-cell depletion was analyzed by flow cytometry, and the cell population adjusted to 1 x 10^4 CD8+ T-cells/100 µL of CM , and incubated, in triplicate, with γ-irradiated (3 000 rad) islet cells (1-100 x 10^3/well) in 96-well round bottom plates for 3 days at 37°C in 5% CO_2. For wells where peptide-pulsed APCs were used to stimulate proliferation, 1 x 10^5 γ-irradiated splenocytes from a non-transgenic, healthy NOD mouse incubated with the desired concentration of peptide were used in place of islet cells. Cultures were pulsed with 1 µCi of (^{3}H)-thymidine during the last 18 hours of culture and harvested. Thymidine incorporation was measured by scintillation counting, and specific proliferation calculated following subtraction of background proliferation from target-cell induced proliferation.

B.6.3 *In Vitro* Cytokine Secretion

Splenic CD8+ T-cells isolated from an acutely diabetic 8.3-NOD mouse (2 x 10^4/well) were incubated with γ-irradiated NOD islet cells or splenocytes (1 x 10^5/well) pulsed with 1 µg/mL of the desired peptide in 96-well plates for 48 hours at 37°C in 5% CO_2. The supernatants (100 µL/well) were then harvested and assayed for interleukin (IL)-2, IL-4, and interferon (IFN)-γ content by ELISA using commercially available kits (Genzyme Diagnostics, Cambridge, MA).

B.6.4 CTLp Differentiation into CTL

Irradiated APCs (1 x 10^5) derived from the spleen of a non-diabetic NOD mouse (pulsed for 1 hour at 37°C with 1µg/mL of either NRP or tum [both are described in

Section C.3]) or NOD islet cells were resuspended in 100 μL final volume/well of a 96-well round bottom plate. Splenocytes from an acutely diabetic 8.3-NOD mouse were depleted of CD4+ T-cells and 1 x 10^4 CD8+ T-cells in 100 μL CM were added and incubated for 7 days in culture media. Fresh media was provided and wells were split as required. At this time, these cells were used in a ^{51}Cr-release assay using NOD islet cells or peptide-pulsed RMA-S-K^d cells as targets.

B.7 Database Searches for Protein Homologues to NRP

The National Center of Biotechnological Information's (NCBI) non-redundant GenBank CDS translations, PDB, SwissProt, PIR, and PRF databases were searched for ungapped alignments of proteins with query sequences using the BLAST search program. An Expect value of 1000 was used, resulting in the lowest stringency homology searches available. Lack of sequence homology between NRP and GAD65, GAD67, and preproinsulin type I/II were confirmed using sequence homology programs of the Genetics Computer Group's (GCG; Wisconsin package version 9.1, Madison, WN) database.

B.8 Determination of the Immunodominance of the CD8+ T-Cell Response in IDDM

B.8.1 TCR Repertoire Studies

TCRα-specific cDNA libraries were generated by anchor-polymerase chain reaction (PCR) by another individual within the lab as previously described (39) . Briefly, total cellular RNA was isolated from islet-derived CD8+ T-cells of 5 diabetic 8.3-TCRβ-transgenic mice, from NOD-islet stimulated (3 times at 1 week intervals) or NRP-stimulated (2 times at 1 week intervals) CD8+ T-cells from 3 non-diabetic 8.3-TCRβ-transgenic NOD mice, and from naive, CD4+ T-cell-depleted splenocytes from 3 non-diabetic 8.3-TCRβ-transgenic NOD mice was isolated. This RNA was reverse transcribed with Moloney leukemia virus reverse transcriptase (GIBCO BRL) by using a synthetic antisense oligonucleotide annealing to Cα (Z743). An oligo(dG) tail was added onto the

3' end of the cDNA with terminal deoxynucleotidyl transferase (GIBCO BRL) and amplified by PCR using a nested TCR constant region-specific antisense primer containing a restriction site for SstI, a primer with an oligo(dC) tail, and an anchor primer (JB4). The PCR products underwent digestion with SstI and NotI, and were electrophoresed in 1% NuSieve low melting agarose gel (FMC Corporation, Marine Colloids Division, Rockland, ME) in Tris-acetate buffer. The appropriate size fraction (400-800 bp) was cut from the gel, and 5 μL of the band was ligated into pBluescript and transformed into XL-1 blue (Stratagene, La Jolla, CA). Recombinant plasmids from white colonies were sequenced using a Sequenase DNA Sequencing Kit (USB, Cleveland, OH) (all sequencing reactions were performed by myself).

B.8.2 Immunodominance of Non-Transgenic CD8+ T-Cell Specificity

Islet derived lines were generated from acutely diabetic non-transgenic NOD mice as described in Section B.2.2. Cells were depleted of CD4+ T-cells after 3-5 days as described in Section B.2.1. Once ~8 x 10^5 proliferating cells were present (~7 days after depletion of CD4+ T-cells), the CD8+ T-cells were tested in a ^{51}Cr-release assay (Section B.5.2) against RMA-S-K^d targets pulsed with 1 μg/mL of either NRP or tum in duplicate as described in Section B.5.

These lines were also cloned as described in Section B.2.3, except that γ-irradiated NOD.*scid*-RIP-B7 islets were used as a source of antigenic stimulation. To test targets pulsed with NRP and tum in duplicate at an effector/target ratio of 10:1 (Section B.5.3), a minimum of 4 x 10^5 effectors are required. These clones were not restimulated in order to minimize the potential effects *in vitro* manipulations may have had on their functional capabilities. However, non-transgenic CD8+ T-cell clones rarely proliferate quickly enough, or survive long enough without restimulation to achieve the desired cell number.

Therefore, the following modifications were made. Proliferating clones were evenly divided into four wells (2 of which were destined to test RMA-S-K^d with NRP, the

other 2 wells against tum). When an estimated 1 x 10^4 cloned CD8+ T-cells/well was achieved, they were tested in a ^{51}Cr Release assay against 1 x 10^4 RMA-S-K^d targets/well pulsed with either NRP or tum (effector/target ratio of ~1:1).

B.9 Statistical Analysis

Statistical analyses were performed using Mann-Whitney U, Student's *t*, and χ^2 tests.

C. RESULTS

C.1 Expression Cloning

To address the hypothesis that CD8+ T-cells recruited to the pancreas during the development of IDDM in the NOD mouse recognize an immunodominant antigen, the peptide specificity of the 8.3-TCR, which is representative of a large fraction of islet-associated CD8+ T-cells, must be known. Initially, an expression cloning system was utilized in hopes of identifying one or more cDNAs encoding peptides that were recognized by 8.3-CD8+ T-cells when displayed by H-2K^d (the restricting MHC class I molecule).

First, a source of mRNA encoding antigenic peptide(s) to be used for the generation of the cDNA library was required. The NOD-derived insulinoma cell line NIT-1 (66) was capable of activating KJ16-stimulated 8.3-CD8+ T-cells, as measured by TNF-α secretion (a cytokine typically secreted by diabetogenic CD8+ T-cell clones in response to β-cells [93]), when compared to the negative control cell line L929-K^d ($p < 0.008$, figure 3). Therefore, it was concluded that NIT-1 expressed mRNA encoding a peptide recognized by 8.3-CD8+ T-cells in an H-2K^d-restricted manner. NIT-1 was, thus, chosen as the source of mRNA used as a template for the cDNA library generation.

A cell line susceptible to transfection was next required. COS-7 cells are African green monkey kidney cells (CV-1) transfected with an SV-40 virus bearing a defective origin of replication (SV-40 ori). The SV-40 large T antigen, however, is fully functional

and will initiate the large scale amplification of transfected cDNA ligated to a vector bearing an intact SV-40 ori. Observation of a 1000-fold amplification of cDNA under the influence of this origin within 48 hours of transfection can result (68). This amplification eventually overloads the COS-7's cellular machinery and cell death ensues, hence, the name transient transfection. The COS-7 transient transfection system was chosen due to the great amount of vector amplification attainable, the presence of simple, well established transfection protocols, and an availability of vectors that possess the SV-40 ori (68).

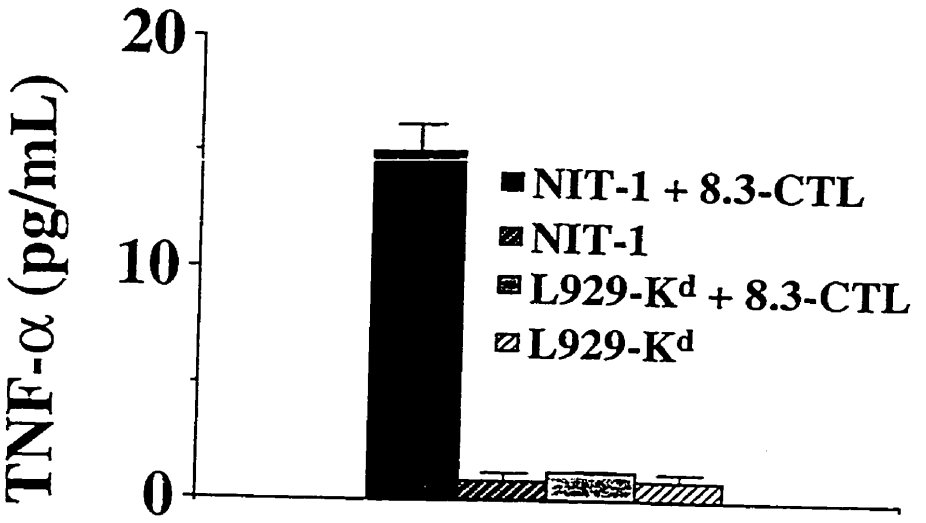

Figure 3. Ability of NIT-1 to stimulate TNF-α secretion from 8.3-CD8+ T-cells. NIT-1 cells were cultured in 96-well flat bottom tissue culture plates over night, then KJ16-stimulated 8.3-CD8+ T-cells were added at a effector/target ratio of 1:1. After 24 hours, TNF-α secretion was measured by adding supernatant to TNF-α-sensitive WEHI 164 clone 13 cells. WEHI 164 clone 13 cell death was measured with Boehringer Mannheim's Cell Proliferation Kit II. NIT-1 cells were capable of stimulating TNF-α secretion from 8.3-CD8+ T-cells when compared to the negative control cell line L929 transfected with H-2K^d cDNA (L929-K^d) ($p < 0.008$). Bars show the standard error of the mean. Data was compared by the Mann-Whitney U test.

The DEAE-dextran/chloroquine method of transfection was employed (see Section B.3.1). Since COS-7 cells do not naturally express H-2K^d (figure 4A), the NIT-1 cDNA

library was co-transfected with cDNA encoding this MHC class I molecule. Thus, the gene products encoded by the NIT-1 cDNA could be displayed by H-2K^d to the 8.3-CD8+ T-cells. Both NIT-1 and H-2K^d cDNA were cloned into the vector pcDNA I/Amp which contains the SV-40 ori as described in Section B.3.1.

To increase the chances of successfully displaying a peptide/H-2K^d complex recognized by 8.3-CD8+ T-cells on COS-7 cells, the transfection protocol first had to be optimized to achieve the highest possible levels of cDNA expression. As mentioned, COS-7 cells do not synthesize their own H-2K^d (figure 4A). Therefore, transfection efficiency was determined by monitoring H-2K^d expression on the surface of transfected cells by FACS analysis.

Initially, the ideal concentration of cDNA used to transfect COS-7 cells was determined (figure 4). Equal amounts of NIT-1 and H-2K^d cDNA (figure 4B; 6.7 ng/μL of each), an increased quantity of NIT-1 cDNA (figure 4C; 6.7 ng/μL of H-2K^d and 20 ng/μL of NIT-1 cDNA), or a decreased amount of H-2K^d cDNA (figure 4D; 3.3 ng/μL of H-2K^d and 20 ng/μL of NIT-1 cDNA) were tested to observe the effect on the transfection efficiency. Regardless of the amount of cDNA transfected, approximately 60% H-2K^d expression resulted.

Since the amount of cDNA transfected did not affect the transfection efficiency, it was decided that future experiments would be performed with 3.3 ng/μL of H-2K^d and 13.3 ng/μL of NIT-1 cDNA. This ratio of NIT-1 to H-2K^d cDNA was employed in hopes of maximizing the amount of NIT-1 cDNA amplified while maintaining high levels of H-2K^d expression.

Until this point, 30 μL of the transfection solution (containing the cDNA to be transfected and the DEAE-dextran/chloroquine solution) per well of a 96-well flat bottom plate was applied to the COS-7 cells being transfected. It was observed that, through capillary action, the majority of the transfection solution clung to the walls of the 96-well

plate. The H-2K^d and NIT-1 cDNAs, therefore, may not have had an opportunity to transfect the entire COS-7 cell population efficiently. To address this situation, the volume of the transfection solution was doubled while the concentration of cDNA and DEAE-dextran/chloroquine was kept the same. From figure 5, it can be seen that this increased the transfection efficiency from 60% to 70%, as determined by H-2K^d expression.

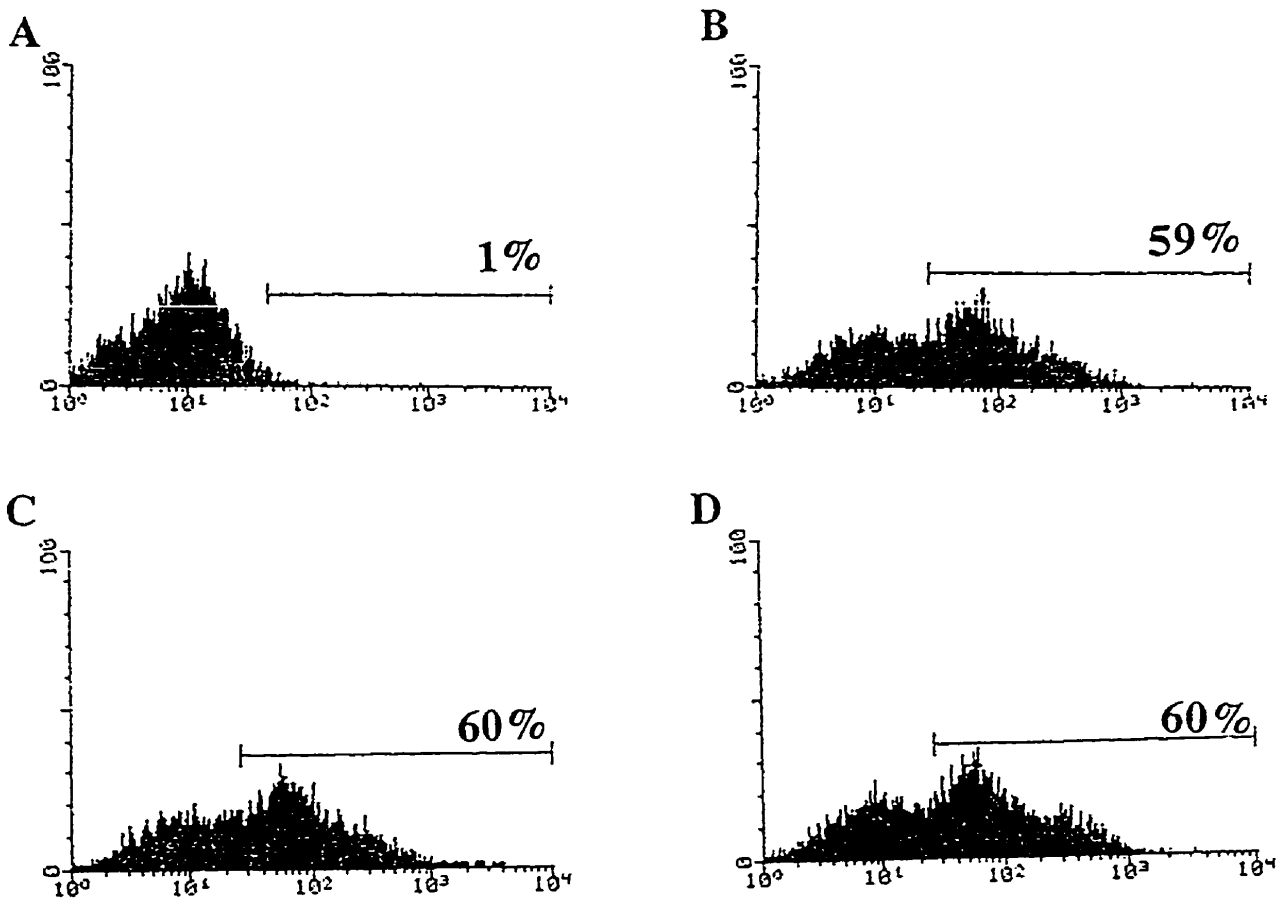

Figure 4. Optimization of cDNA Concentrations for the COS-7 Transfections. The transfection efficiency was measured by H-2K^d expression. COS-7 cells were transfected with the indicated amount of cDNA and incubated at 37°C, 6.5% CO_2 for 48 hours. The cells were then stained with anti-H-2K^d-FITC mAb and observed by flow cytometry. (A) No H-2K^d cDNA or NIT-1 cDNA – no H-2K^d expression. (B) 6.7 ng/μL of H-2K^d cDNA and 6.7 ng/μL of NIT-1 cDNA – 59% H-2K^d expression. (C) 6.7 ng/μL H-2K^d cDNA and 20 ng/μL of NIT-1 cDNA – 60% H-2K^d expression. (D) 3.3 ng/μL H-2K^d cDNA and 20 ng/μL of NIT-1 cDNA – 60% expression of H-2K^d.

Now that an efficient COS-7 transfection protocol had been developed, the 962 pools of NIT-1 cDNA libraries generated (a total of 2 x 10^5 cDNAs) were each co-transfected with H-2K^d cDNA using DEAE-dextran/chloroquine as described in Section B.3.1. The transfected COS-7 cells were then screened for their ability to elicit TNF-α secretion from KJ16-stimulated 8.3-CD8+ T-cells. This process was repeated 3 times, but no NIT-1 cDNA pools, when transfected into COS-7 cells, were able to stimulate TNF-α secretion from 8.3-CD8+ T-cells (data not shown).

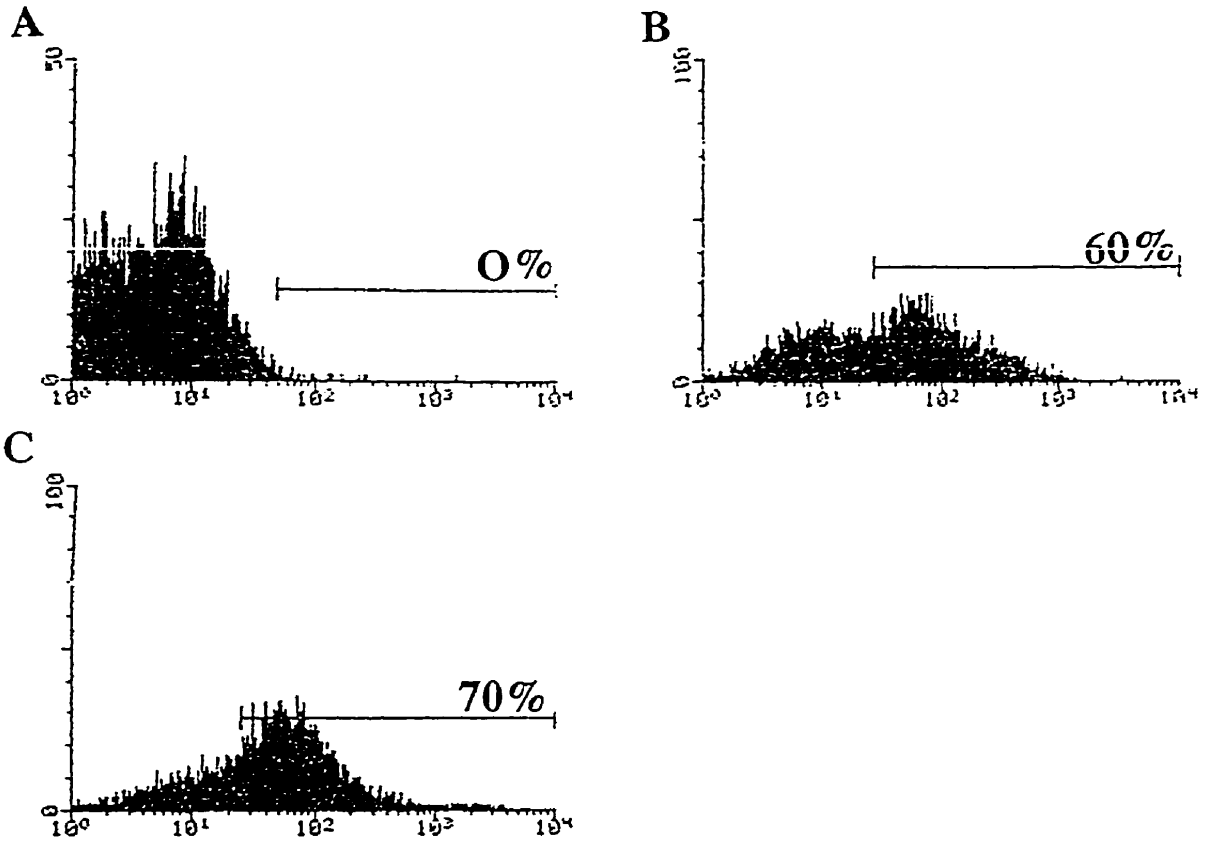

Figure 5. Optimization of Transfection Solution Volume Incubated with the Transfectants. (A) COS-7 cells were transfected with no cDNA as a control. (B) and (C) COS-7 cells were co-transfected with 3.3 ng/μL of H-2K^d cDNA and 13.3 ng/μL NIT-1 cDNA. The cells were incubated for 3 hours at 37°C, 6.5% CO_2 with the indicated volumes of transfection solution, washed, then allowed to incubate at the same conditions for 48 hours in cDMEM. Transfection efficiencies were then determined by staining with anti-H-2K^d-FITC mAb and observing H-2K^d expression. (B) 30 μl of transfection solution was added – 60% H-2K^d expression. (C) 60 μl of transfection solution added – 70% expression of H-2K^d.

A potential confounding factor of the above protocol stems from the fact that COS-7 cells are derived from the African green monkey. The T-cells being stimulated and the antigens being expressed are derived from the NOD mouse. It is possible that a monkey cell will not process a peptide as a mouse cell will due to improper glycosylation patterns or amino acid substitutions resulting from the absence or presence of additional modifying enzymes (94, 95). This results in an inability to display a peptide/H-2K^d complex on the surface of COS-7 cells capable of being recognized by the 8.3-CD8+ T-cell. It is also possible that the co-receptors present on monkey cells will not bind with high affinity to their ligands on a murine T-cell, making it difficult, if not impossible, to activate that T-cell (68).

To deal with these possibilities, a murine cell line analogous to COS-7 was employed. The murine cells, derived from NIH 3T3 mice, are named WOP, and they have been transfected to express the polyoma large T antigen (68, 81). Analogous to the COS-7 transfection system, although not as effective, the polyoma large T antigen results in large scale amplification of vectors possessing the polyoma ori.

As described in Section B.3.2, there are 2 strains of WOP. WOP 3027-3 cells do not naturally express H-2K^d, whereas WOP 32-4 cells do display this MHC class I molecule at high levels (figure 6). Since transfection efficiency was determined by monitoring H-2K^d expression, optimization of the transfection protocol was performed on WOP 3027-3 cells, then both cell lines were used to screen the NIT-1 cDNA libraries.

Original attempts at transfecting WOP 3027-3 cells using the protocol developed for COS-7 cells demonstrated that TE buffer (used in the transfection solution) was highly toxic, and, therefore, had to be replaced with PBS (data not shown). Furthermore, this cell line was highly sensitive to the DEAE-dextran/chloroquine used to transfect the cells. Since DEAE-dextran/chloroquine was essential for performing transfections, their concentrations could not be lowered (68). It was felt, though, that reducing the time

WOP 3027-3 cells were exposed to DEAE-dextran/chloroquine may increase the viability of transfectants. Thus, WOP 3027-3 cells were incubated with 60 μL of transfection solution (containing 3.3 ng/μL and 13.3 ng/μL of H-2K^d and NIT-1 cDNA, respectively, 400 μg/mL DEAE-dextran, and 200 μM chloroquine) for 1 hour (figure 7A & B), 2 hours (figure 7C & D), and 3 hours (figure 7E & F). Viability of transfectants was determined 48 hours after transfection via propridium iodide, which exclusively stains dead cells (figure 7A, C, & E), as well as H-2K^d expression to determine transfection efficiency (figure 7B, D, & F). After a 1 hour incubation, 60% of transfectants were still viable with a transfection efficiency of 23%. Longer incubation times did not increase the transfection efficiency, and resulted in the death of the majority of transfected cells. Therefore, it was decided that WOP cells would only be incubated for 1 hour with the transfection solution for future transfection experiments.

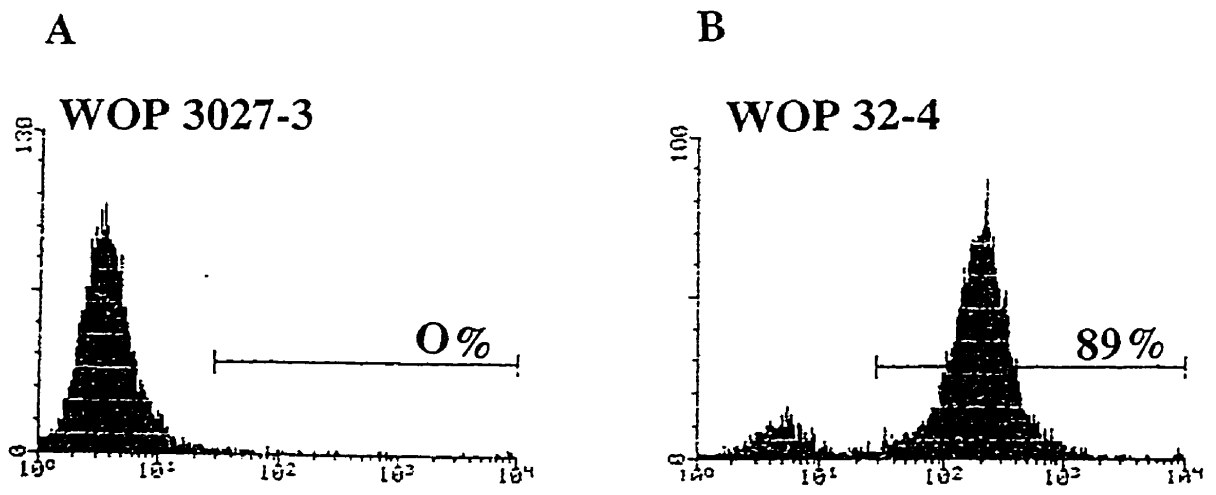

Figure 6. H-2K^d Expression on WOP cells. WOP 3027-3 (A), and WOP 32-4 (B) were stained with anti-H-2K^d-FITC mAb, then observed under FACscan to determine the levels of H-2K^d expression.

Using these optimized transfection conditions, co-transfection with each of the 962 pools of NIT-1 cDNA with H-2K^d cDNA in WOP 3027-3 and WOP 32-4 cells over three trials using DEAE-dextran/chloroquine (as described in Section B.3.2) were performed.

Unfortunately, no NIT-1 cDNA pools capable of stimulating TNF-α secretion from KJ16-stimulated 8.3-CD8+ T-cells were identified (data not shown).

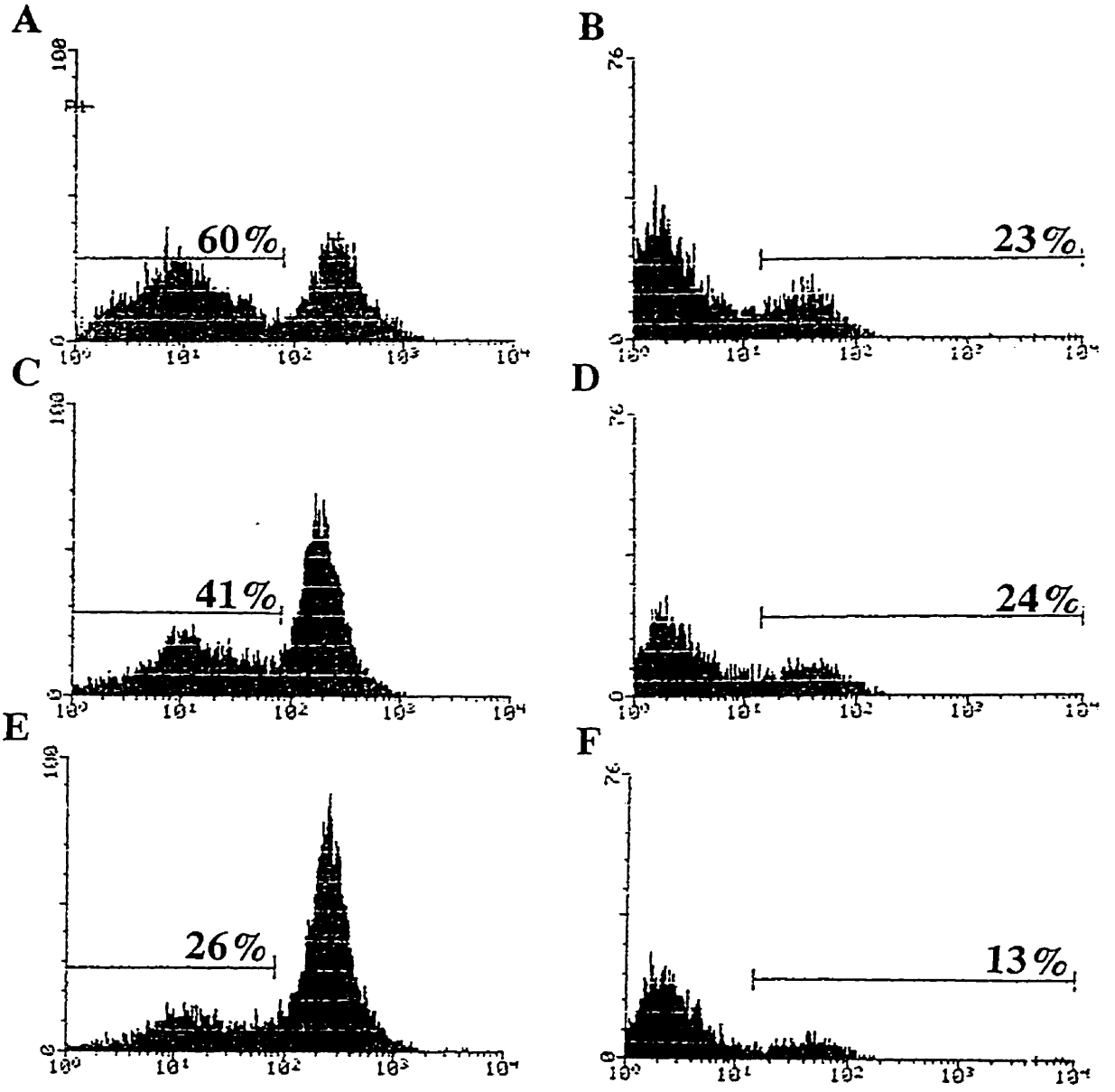

Figure 7. Optimization of the Incubation Time with Transfection Solution for WOP 3027-3 Transfectants. WOP 3027-3 were incubated for the indicated times with the transfection solution at 37°C, 6.5% CO_2, washed, and resuspended in cDMEM. The cells were incubated for 48 hours at which point the transfection efficiency was monitored by H-2K^d expression as previously described. **(A) and (B)** were incubated for 1 hour, **(C) and (D)** for 2 hours, and **(E) and (F)** for 3 hours. **(A), (C), and (E)** show the viability of the WOP transfectants via propridium iodide staining. **(B), (D), and (F)** show the expression of H-2K^d. One hour incubation results in a viability of 60% and 23% H-2K^d expression. Two hours incubation gives 41% viability and 24% H-2K^d expression. Three hours gives 26% viability and 13% H-2K^d expression.

In conclusion, expression cloning could not be utilized to identify a cDNA encoding a peptide that, when displayed in the context of H-2K^d, was recognized by 8.3-CD8+ T-cells. This suggests that either the peptide-encoding cDNA was rare in the NIT-1 cDNA libraries generated, or this method was not sensitive enough to detect antigens targeted by 8.3-CD8+ T-cells. For further discussion of the complications which may hinder this study, see Section D.1.

C.2 Peptide Purification

Since expression cloning had failed to yield a NIT-1-derived cDNA encoding a peptide recognized by 8.3-CD8+ T-cells in an H-2K^d-restricted manner, it was next attempted to isolate the antigenic peptide from the surface of NIT-1 cells, and then purify it so that sequencing could be performed. As described in Section B.4, peptides (Mr < 10,000) were acid extracted from the surface of 1×10^{10} NIT-1 cells and from the splenocytes derived from 10 NOD spleens as a negative control. These peptides were then subjected to separation by rpHPLC (figure 8). Peptides bound to the rpHPLC column were eluted off over an acetonitrile gradient of 0-60% over 35 minutes (~ 1.7%/min.). Elution of splenocyte-derived peptides (figure 8A, top panel) and NIT-1-derived peptides (figure 8A, bottom panel) was monitored by A_{214} readings. TAP II$^{-/-}$ RMA-S-K^d cells at 26^{o}C expressing empty H-2K^d molecules on the cell surface (see Section B.4) were incubated for 1 hour with each rpHPLC fraction collected. This allowed peptides present within the rpHPLC fractions to bind the empty H-2K^d molecules on the RMA-S-K^d cell's surface. Once exogenous peptide had bound to H-2K^d at 26^{o}C, the peptide/MHC class I complex is stable at higher temperatures. These peptide-pulsed RMA-S-K^d cells were then assayed at 37^{o}C for their ability to stimulate TNF-α secretion from KJ16-stimulated 8.3-CD8+ T-cells (figure 8B).

From figure 8B, it was observed that fractions 26-32 (corresponding to an acetonitrile gradient of 36%-45% with the major peak at 44%) clearly contained a peptide

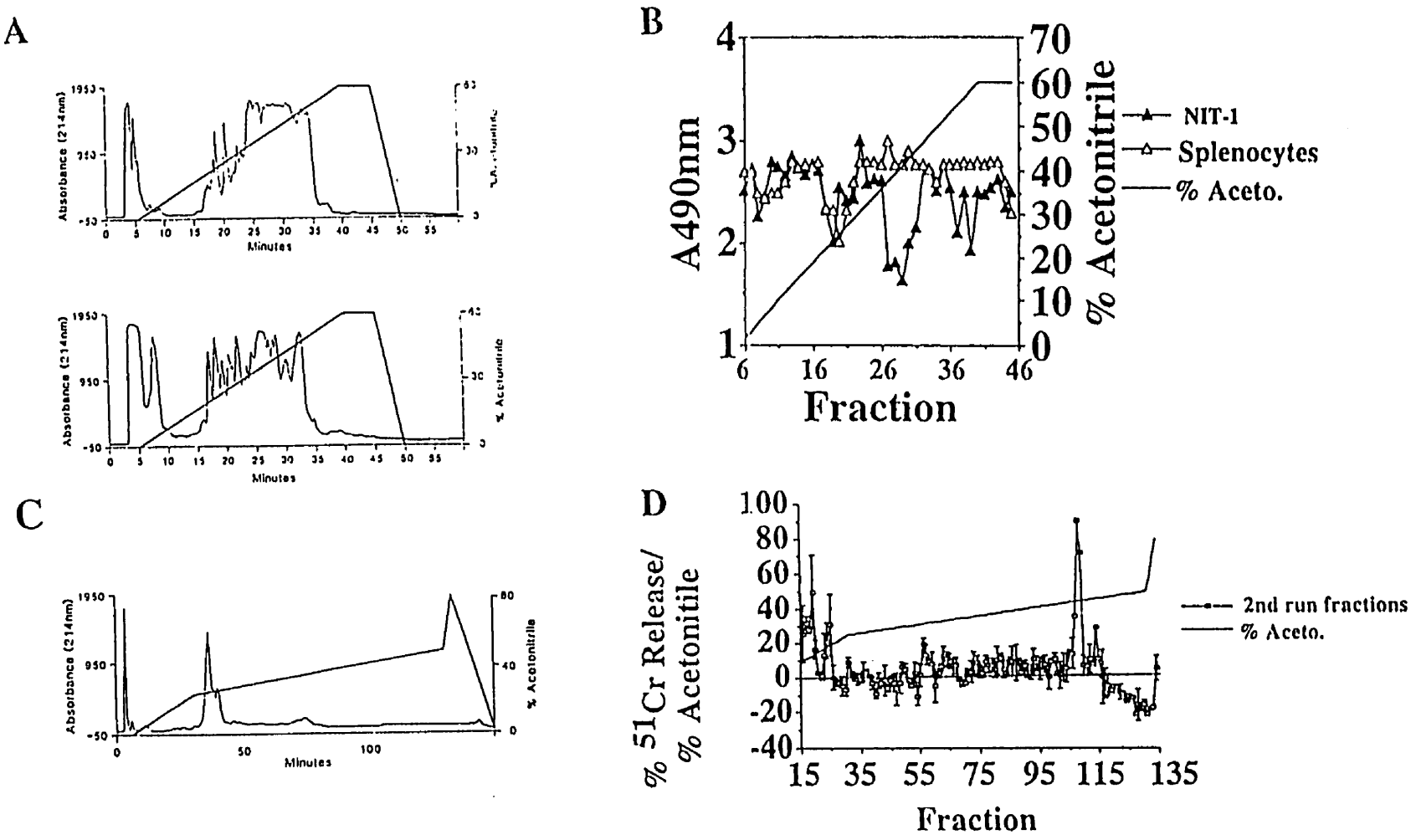

Figure 8. Purification of NIT-1 Peptides Through rpHPLC. (A) and (B) First run of NIT-1 peptides through the rpHPLC. Peptides were acid extracted from NOD splenocytes (A, top panel) or NIT-1 cells (A, bottom panel) and underwent rpHPLC. Bound peptides were eluted off with an acetonitrile gradient increasing from 0-60% over 35 minutes (~ 1.7%/minute). A flow rate of 1 mL/min. was used, and 1 mL fractions were collected. Elution of peptides was measured by absorbance at 214 nm. (B) Peptide fractions from NIT-1 (closed triangles) and splenocytes (open triangles) collected from the rpHPLC were incubated with RMA-S-K^d cells at 26°C for 1 hour. At this time, KJ16-stimulated 8.3-CD8+ T-cells were added at an effector/target ratio of 1:1 and incubated overnight at 37°C. At this time, 8.3-CD8+ T-cell activation was monitored via TNF-α secretion as previously described. (C) and (D) Second Run of NIT-1 Peptides Through the rpHPLC. (C) Previously purified immunogenic peptide fractions were pooled and re-run through the rpHPLC. Bound peptides were eluted off with an increasing acetonitrile concentration of 0-25% from 5-30 minutes (1%/minute), at which point the acetonitrile gradient increased from 25-50% from 30-130 minutes (0.25%/minute). A flow rate of 1 mL/min. was used, and 1 mL fractions were collected. Elution of peptide was monitored by absorbance at 214 nm. (D) One-fifth of each fraction was loaded onto 1 x 10^4 [^{51}Cr]-labeled RMA-S-K^d cells at 26°C for 1 hour. NRP-stimulated 8.3-CD8+ T-cell lines were added at an effector/target ratio of 10:1, incubated at 37°C for 8 hours, and %^{51}Cr-release determined as described in Methods. Bars show the standard error of the means.

that, when displayed by H-2K^d molecules on the RMA-S-K^d cell surface, initiated TNF-α secretion from KJ16-stimulated 8.3-CD8+ T-cells. From figure 8A, lower panel, it can be observed that there is a large amount of NIT-1-derived peptide that elutes off through the acetonitrile range of 36%-45%. This suggested a large amount of contaminating peptides were present in these fractions. Since a relatively purified peptide capable of being recognized by 8.3-CD8+ T-cells for sequencing was desired, it was decided to pool fractions 26-32 and re-run them through the rpHPLC to obtain better peptide separation.

Peptides bound to the rpHPLC column during this second run were again eluted off with acetonitrile and monitored by A_{214} readings (figure 8C). Whereas previously the acetonitrile gradient increased at a rate of ~1.7%/minute, now 0.25%/minute was used through the acetonitrile range in which the antigenic peptide was expected to elute off the rpHPLC column. This shallow acetonitrile gradient was employed in an effort to better separate the pooled peptides. Originally, RMA-S-K^d cells were pulsed with each fraction and tested for their ability to stimulate TNF-α secretion from 8.3-CD8+ T-cells, as was done with the fractions from the first rpHPLC separation. However, the sensitivity of the TNF-α assay was too low to detect any stimulation resulting from these fractions (data not shown).

Only recently, utilizing the powerful agonist NRP to stimulate 8.3-CD8+ T-cell lines (see Section B.2.4), has the highly sensitive chromium release assay (see Section B.5.3) been employed to test these fractions (figure 8D). RMA-S-K^d cells pulsed with each fraction eluted off the rpHPLC column during the second run were now tested for their ability to elicit a cytotoxic response from NRP-stimulated 8.3-CD8+ T-cell lines. Fractions corresponding to an acetonitrile gradient of 44.00% to 44.50%, with a major peak at 44.25%, appeared to contain a peptide that, when displayed by H-2K^d on the RMA-S-K^d cell surface, initiated a cytotoxic response from NRP-stimulated 8.3-CD8+ T-cells. Since the major fraction containing peptide(s) recognized by 8.3-CD8+ T-cells

eluted off at 44% during the first rpHPLC separation, it can be concluded that the antigenic peptide(s) obtained from the second rpHPLC separation are the same as those obtained from the first.

From the elution profile of the second rpHPLC separation (figure 8C), it appears that very little peptide eluted off at 44.00-44.50% acetonitrile. This suggests that the peptide responsible for initiating a cytotoxic response in NRP-stimulated 8.3-CD8+ T-cells had been significantly purified. However, a large number of contaminating peptides may still be present. Collaboration is presently being sought out with another laboratory to separate the peptides eluted off at 44.25% acetonitrile and subsequently sequence them using a tandem capillary rpHPLC/mass spectrometer. RMA-S-K^d cells will be pulsed with peptides identified in this manner and tested for their ability to elicit a cytotoxic response from NRP-stimulated 8.3-CD8+ T-cells. If the amount of sequenced peptide obtained is below the detection abilities of the chromium release assay, synthetic peptides of these sequenced fractions will be synthesized (assuming the number of different peptides identified is reasonable). These synthetic peptides will then be tested for their ability to sensitize RMA-S-K^d cells to 8.3-CD8+ T-cell-mediated cytotoxicity. Although this procedure could not be implemented in a timely manner allowing us to address this thesis's hypothesis, there is hope it may yet yield the naturally occurring β-cell antigen recognized by 8.3-CD8+ T-cells.

C.3 Generation of an 8.3-CD8+ T-Cell-Specific Mimotope Using Positional Scanning Combinatorial Peptide Libraries

Since an antigen-encoding cDNA or the naturally expressed target peptide that was recognized by 8.3-CD8+ T-cells could not be identified, it was decided to design a mimotope of this β-cell-associated antigen. Positional scanning combinatorial peptide libraries (PSCPL) were employed to design this mimetic that would eventually be used to test the hypothesis of this thesis.

To elucidate an antigenic mimotope through PSCPL, some preliminary information about the target peptide must be known. For example, it has been shown that the 8.3-CD8+ T-cells are H-2K^d restricted (40). H-2K^d binding peptides are nonamers with a tyrosine (or rarely phenylalanine) at position 2 and leucine, isoleucine, or valine at position 9 as anchor residues (96-98). All peptide libraries generated had the anchor residue at position 2 fixed with tyrosine and position 9 with a leucine. Leucine was chosen rather than isoleucine or valine for position 9 to increase the peptide's polarity for synthesis purposes. To generate the PSCPL, amino acids were divided into groups of 1-2 amino acids sharing similar chemical characteristics as follows. Asp & Glu are acidic, Lys/Arg (basic), Gln/Asn (polar), Gly/Ser (neutral, small), Phe/Tyr (contain a benzene ring), Ala/Leu (hydrophobic), Val/Thr (neutral), Trp/Ile (neutral and large), Pro, and His. Sulfur containing amino acids (Cys and Met) were excluded to prevent the cyclization of peptide through the formation of disulfide bonds with other thiol groups. Then, 72 first generation libraries in which all peptides per library had one of two amino acids/group fixed at individual non-anchor positions, and a random assortment of 19 amino acids (excluding Cys) at the remaining positions were synthesized. For example, library 1 was: (Asp/Glu)-(Tyr)-X-X-X-X-X-X-(Leu), and library 2 was: X-(Tyr)-(Asp/Glu)-X-X-X-X-X-(Leu), etc.

RMA-S-K^d cells (expressing empty H-2K^d molecules at 26^{o}C; described in Section B.4) were pulsed with each library at a concentration of 60 μg/mL at 26^{o}C. At this concentration, there were a total of 3.0×10^{16} peptides composed of 9.4×10^7 different peptide sequences. Therefore, a total of 3.2×10^8 peptides (6.3 pg/mL) bearing an antigenic sequence were present within this mixture. Since CD8+ T-cell mediated lysis only requires recognition of <100 peptide/MHC class I complexes (99) as a result of the TCR's ability to serially engage its target (100), it was expected that 60 μg/mL of an

antigen-containing library contained sufficient target to initiate 8.3-CD8+ T-cell-mediated cytotoxicity.

The contribution of each amino acid group at each position of the peptide towards stimulating 8.3-CD8+ T-cell-mediated lysis of peptide-pulsed RMA-S-K^d cells could then be tested through the highly sensitive chromium release assay (see Section B.5.2). The amino acid group at each position of the target peptide that elicits the most cytotoxicity from a clonal population of islet-derived 8.3-CD8+ T-cells towards peptide-pulsed RMA-S-K^d targets was regarded as occupying that position in the target antigen. This process was repeated up to five times for each position, and figure 9A displays a representative trial. Six out of 7 non-anchor positions gave a clear cytotoxic response with an individual amino acid group. In one experiment, 8.3-CD8+ T-cells recognized libraries containing Asn/Gln or His at position 3, and Asn/Gln, Gly/Ser, and Phe/Tyr at position 6. However, only libraries containing Asn/Gln at both positions 3 and 6 could reproducibly elicit 8.3-CD8+ T-cell-mediated lysis of peptide-pulsed RMA-S-K^d targets over 5 trials (data not shown). Only position 5 did not yield any reproducible results over five trials, suggesting that either a Cys or Met occupied this position, or there was no interaction between the TCR and peptide at this residue. The following putative target peptide recognized by islet-derived, clonal populations of 8.3-CD8+ T-cells was deduced: (Lys or Arg)-Tyr-(Asn or Gln)-(Lys or Arg)-X-(Asn or Gln)-(Trp or Ile)-(Phe or Tyr)-Leu.

To identify a single amino acid sequence of the peptide recognized by clonal populations of islet-derived 8.3-CD8+ T-cells, 12 second generation PSCPL were generated. Now, each position of the target peptide was tested with 1 of the 2 amino acids defined for each of the non-anchor residues in the first generation library, and the remaining non-anchor residues occupied by a random assortment of 19 amino acids (excluding Cys). For example, to test position 1 (either a Lys or Arg, as determined in the first generation PSCPL), library 1 was: (Lys)-(Tyr)-X-X-X-X-X-X-(Leu), and library 2

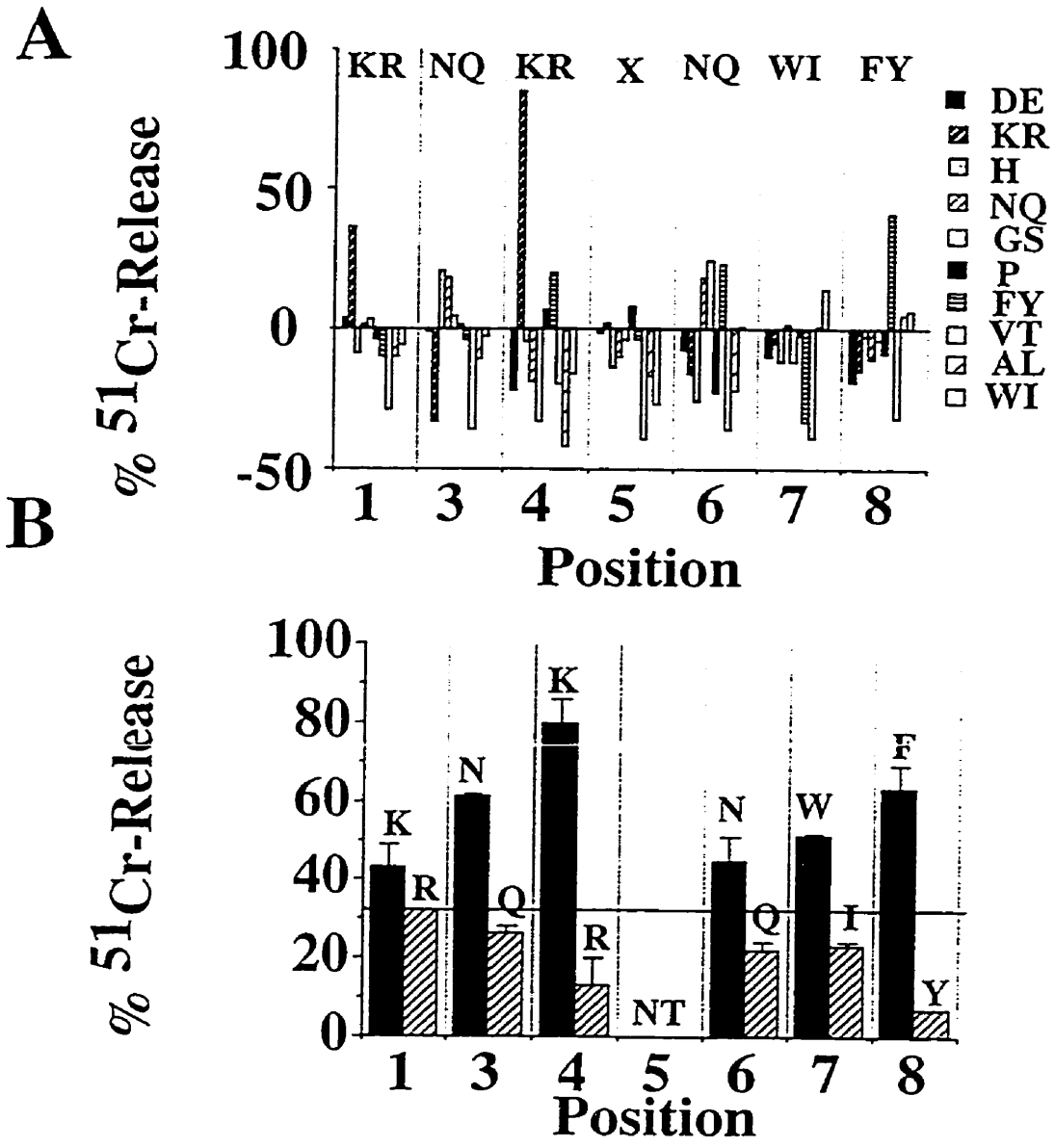

Figure 9. Generation of an 8.3-CD8+ T-Cell Mimotope by PSCPL. (A) Screen of the first generation libraries' ability to elicit 8.3-CD8+ T-cell-mediated cytotoxicity. Amino acids were placed into groups sharing similar chemical characteristics. The groups were tested individually at each position in a PSCPL where H-2K^d anchor residues Y2 and L9 were fixed. Then, 60 μg/mL of each library was loaded onto 1 x 10^4 [^{51}Cr]-labeled RMA-S-K^d cells at 26°C for 1 hour. Islet-derived 8.3-CD8+ T-cell clones were added at an effector/target ratio of 10:1, incubated at 37°C for 8 hours, and %^{51}Cr-release determined as described in Methods. The figure shows one representative trial. Amino acid groups capable of eliciting the greatest lysis of peptide-loaded targets over 5 trials were defined as antigenic, and are labeled at the top of each position column. (B) Screen of the second generation libraries' ability to elicit 8.3-CD8+ T-cell-mediated cytotoxicity. The individual amino acids of the antigenic amino acid groups defined for each position in the first generation library are tested as above to determine the most antigenic amino acid for each position. The solid line represents 2 standard deviations above the mean lysis incurred by the negative control peptide, tum, at 60 μg/mL.

was (Arg)-(Tyr)-X-X-X-X-X-X-(Leu). The ability of these second generation libraries at 60 μg/mL (each containing 12.6 pg/mL of an individual target peptide) to initiate 8.3-CD8+ T-cell-mediated lysis of peptide-pulsed RMA-S-K^d cells was compared to the negative control peptide, tum (see Table 1; ref. 101). Amino acids yielding $\%^{51}Cr$-release results 2 standard deviations above tum were considered antigenic (figure 9B).

From this data, a potential mimotope for the 8.3-transgenic TCR was inferred to be (Lys)-(Tyr)-(Gln)-(Lys)-X-(Gln)-(Trp)-(Phe)-(Leu) and was named the NOD Relevant Peptide (NRP) (table 1). It must be understood that PSCPL may not yield the exact antigen found in nature. Many studies, however, indicate that the mimetic deduced has similar characteristics to the natural epitope (46, 102-104). It was assumed that position 5 was not important for TCR recognition, and thus it was decided to place an alanine there for the synthesis of the antigenic mimotope.

Table 1: Peptides Synthesized

Peptide	Sequence
NRP	KYNKANWFL
tum	KYQAVTTTL
Aminopeptidase C (*S. therm.*)	KYEKSNWFL
Aminopeptidase C (*L. lactis*)	KYEKSNWFM
Hypothetical protein (*P. horikoshii*)	KYSKEVWFV
C23d3.15 (*S. pombe*)	RFNKSNFFI
A1	AYNKANWFL
A3	KYAKANWFL
A4	KYNAANWFL
A6	KYNKAAWFL
A7	KYNKANAFL
A8	KYNKANWAL

Amino acids differing from NRP are underlined

C.4 Immunological Properties of NRP

C.4.1 Agonistic Properties of NRP

Next, it was wished to determine whether NRP possessed the same agonistic qualities as NOD islet cells, the natural target of 8.3-CD8+ T-cells (39, 41). To study this,

four 8.3-CD8+ T-cell responses were studied: 1) proliferation, 2) cytokine secretion, 3) differentiation, and 4) cytotoxic response.

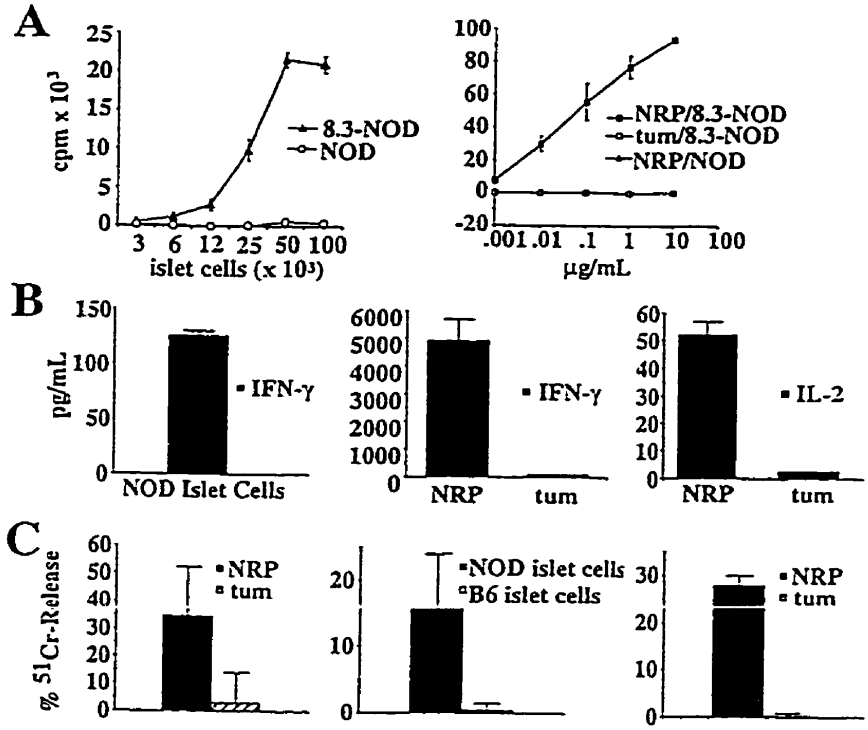

Figure 10. Immunological Properties of NRP. (A) Proliferation of splenic 8.3-CD8+ T-cells to NOD islet-cells (**left panel**) or APCs pulsed with varying concentrations of NRP or tum (**right panel**). Splenic 8.3-CD8+ T-cells (2×10^4) were incubated with γ-irradiated NOD islet-cells or peptide-loaded APCs for three days, pulsed with (^{3}H)-thymidine, harvested, and counted. (B) Cytokine profile of splenic 8.3-CD8+ T-cells to NOD islet cells (**left panel**), or APCs pulsed with NRP or tum (**centre & right panels**). Splenic 8.3-CD8+ T-cells (2×10^4) were incubated with 1×10^5 γ-irradiated NOD islet cells or splenocytes pulsed with 1 µg/mL NRP or tum for 48 hours. The supernatants were then harvested and assayed for IFN-γ (**left & centre panels**), IL-2 (**right panel**), or IL-4 (data not shown). NOD islet cells stimulate 8.3-CD8+ T-cells to secrete IFN-γ and IL-2 when compared to wells with no T-cells added ($p < 0.006$ and data not shown, respectively), as do NRP-pulsed APCs when compared to tum-pulsed APCs ($p < 0.006$ and $p < 0.009$, respectively). IL-4 secretion was not stimulated in any instance (data not shown). (C) The differentiation of naive, splenic 8.3-CD8+ T-cells into CTLs. Splenic 8.3-CD8+ T-cells (1×10^4) were incubated with 1×10^5 γ-irradiated NOD islet cells (**left panel**) or APCs pulsed with 1µg/mL NRP (**central & right panels**) or tum (data not shown) for 7 days. At this time, cells were used in a ^{51}Cr-release assay against NOD or C57BL/6 (B6) islet cells (**central panel**), or RMA-S-K^d cells pulsed with 1 µg/mL NRP or tum (**left & right panels**). The data demonstrate that NOD islet cells are capable of differentiating naive 8.3-CD8+ T-cells into cytotoxic effectors capable of lysing NRP-pulsed targets, but not tum-pulsed targets ($p < 0.05$). Furthermore, NRP-pulsed APCs differentiated naive 8.3-CD8+ T-cells into cytotoxic effectors capable of lysing NOD, but not B6 islet cells (due to the great variability observed in 8.3-CD8+ T-cell mediated cytotoxicity towards NOD islet cells over different experiments, no statistical analysis was performed – the data presented is representative of four separate trials), as well as targets pulsed with NRP, but not tum ($p < 0.05$). Bars show the standard error of the means. Data was compared by the Mann-Whitney U test.

In figure 10 (left panels in A, B, and C), *in vitro* stimulation of naive, splenic 8.3-CD8+ T-cells (isolated from RAG-2$^{-/-}$, non-diabetic 8.3-NOD animals) with NOD islet

cells results in their proliferation, IFN-γ secretion (when compared to wells with no 8.3-CD8+ T-cells [$p < 0.006$]), low levels of IL-2 secretion (when compared to wells with no 8.3-CD8+ T-cells [$p < 0.009$]), no IL-4 secretion (data not shown), and differentiation into cytotoxic effectors capable of lysing RMA-S-K^d targets pulsed with NRP (but not tum; $p < 0.05$). *In vitro* stimulation of naive, splenic 8.3-CD8+ T-cells with NRP-pulsed APCs (derived from NOD splenocytes) resulted in qualitatively similar results (figure 10, middle and/or right panels in A, B, and C).

Figures 10A and 10B (central and/or right panels) show naive, splenic 8.3-CD8+ T-cells, in response to NRP-pulsed APCs, proliferate in a manner dependent on antigen concentration and secrete high amounts of IFN-γ ($p < 0.006$), low amounts of IL-2 ($p < 0.009$), and no IL-4 (data not shown) when compared to tum-pulsed APCs to perform the same function. These results were NRP-specific (no responses observed with tum-pulsed APCs, APCs alone [data not shown], or T-cells alone [data not shown]) and 8.3-TCR-driven (no observed response from non-transgenic, splenic CD8+ T-cells). The quantitative differences observed between the agonistic effects of NOD islet cells and NRP-pulsed APCs are not surprising. NRP was used at concentrations most likely orders of magnitude greater then the amount of endogenous antigen expressed on the surface of NOD islet cells.

Finally, figure 10C shows naive, splenic 8.3-CD8+ T-cells incubated with NRP-pulsed APCs for 1 week without exogenous IL-2 differentiated into cytotoxic effectors capable of killing NOD islet cells, but not C57BL/6 (B6) islet cells (figure 10C, central panel; due to the great variability observed in 8.3-CD8+ T-cell mediated cytotoxicity towards NOD islet cells over different experiments, no statistical analysis was performed -- the data presented is representative of four separate trials) and RMA-S-K^d cells pulsed with NRP, but not tum (figure 10C, right panel; $p < 0.05$). Furthermore, naive, splenic 8.3-CD8+ T-cells incubated with tum-pulsed APC's or in the absence of APC's were not

cytotoxic towards NOD or B6 islet cells, or RMA-S-K^d cells pulsed with NRP or tum (data not shown).

To summarize NRP's immunological properties, it can induce the proliferation, cytokine secretion, differentiation, and cytotoxic response from naive 8.3-CD8+ T-cells in a manner qualitatively identical to NOD islets. It is concluded that NRP is an excellent mimetic of an antigen expressed on NOD islet cells recognized by 8.3-CD8+ T-cells.

C.4.1.1 Immunological Properties of Alanine-Substituted NRP Analogs. It was next desired to identify which residues of NRP were essential for its observed immunological properties. To do this, NRP analogs were generated by sequentially replacing each residue with an Ala, except the anchor residues at positions 2 and 9, and position 5 (already an Ala) (table 1). These analogs were tested for their MHC class I binding and agonistic properties.

To demonstrate that all NRP analogs, NRP, and tum bound to H-$2K^d$ equally well, the ability of these peptides to stabilize H-$2K^d$ molecules on the surface of RMA-S-K^d cells (H-$2K^{d,b}/D^b$) was compared to the peptide ESP (a non-H-$2K^d$-binding peptide with the sequence DKGSNKGFE). This was determined by flow cytometry using anti-H-$2K^d$-FITC or anti-H-$2D^b$-FITC (control) mAb. Figure 11A, left panel, shows all the peptides tested could stabilize H-$2K^d$ ($p < 0.02$) when compared to ESP (at baseline, data not shown). Since RMA-S-K^d cells also express H-$2D^b$, there was a concern that some peptides may preferentially associate with this MHC class I molecule resulting in the observed responses. Figure 11A, right panel, demonstrated that there was no significant peptide binding to H-$2D^b$ when compared to ESP ($p < 0.2$).

Next, it was observed whether NRP analogs could stimulate proliferation and cytokine secretion from naive, splenic 8.3-CD8+ T-cells. Figures 11B, 11C, and 11D demonstrate that of the 6 Ala substitutions in NRP, 5 resulted in variable reductions in proliferation, IFN-γ secretion, and IL-2 secretion from 8.3-CD8+ T-cells (A4 and A8

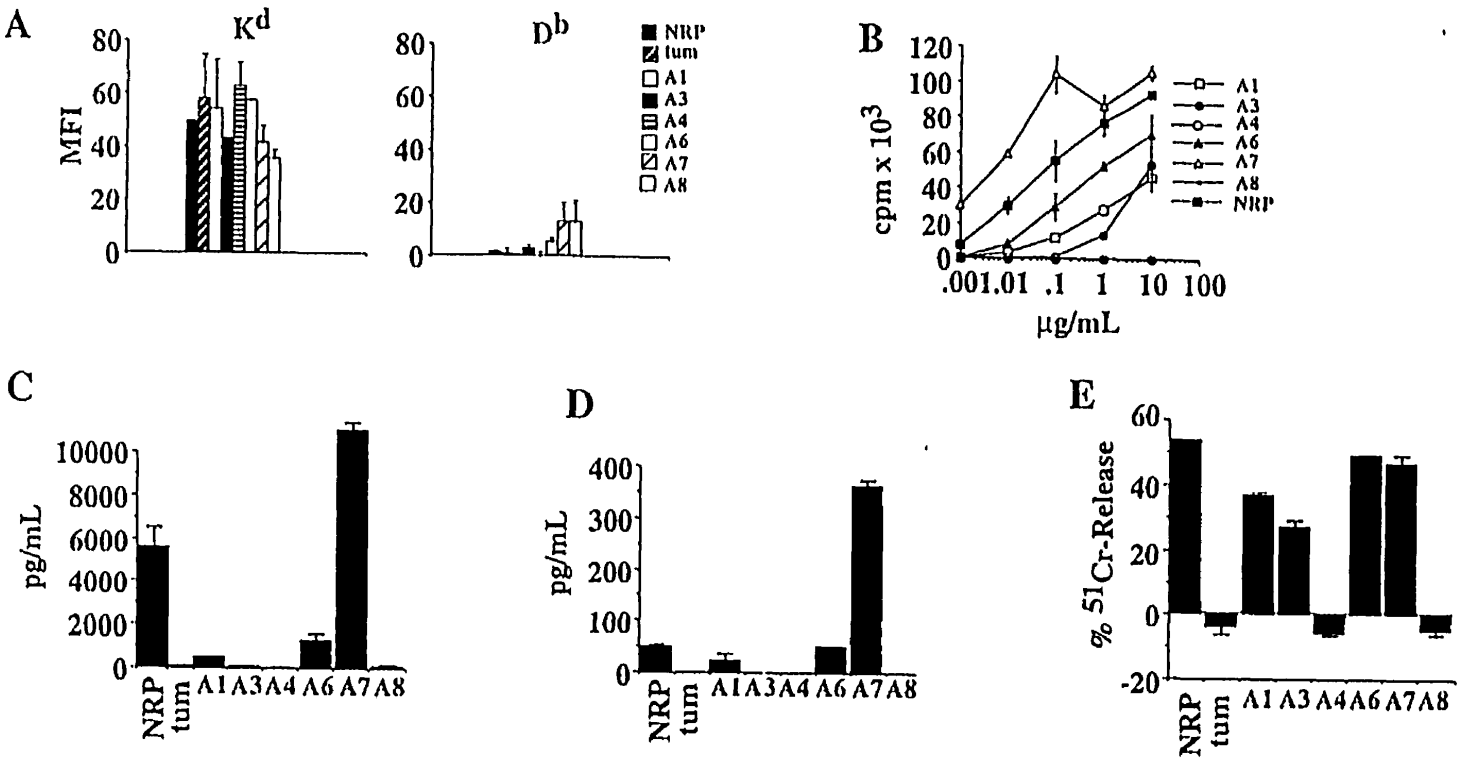

Figure 11. Alanine Scans of NRP. (A) Ability of peptides to selectively bind H-2K^d. RMA-S-K^d cells at 26°C were incubated with 1µg/mL peptide for 1 hour, then incubated at 37°C for 3 hours to allow for down-regulation of empty MHC molecules. Cells were stained with anti-H-2K^d-FITC or anti-H-2D^b-FITC mAb, then analyzed by flow cytometry. Data was calculated by subtracting the mean fluorescence intensity (mfi) for K^d or D^b on non-peptide-pulsed cells from the mfi for the same MHC molecules on peptide-pulsed cells. (B) Proliferation of splenic 8.3-CD8+ T-cells in response to APCs pulsed with 1 µg/mL peptide. Splenic 8.3-CD8+ T-cells (2 x 10^4) were incubated with γ-irradiated peptide-loaded APCs for three days, pulsed with (^{3}H)-thymidine, harvested, and counted. (C & D) Cytokine profile of splenic 8.3-CD8+ T-cells in response to APCs pulsed with peptide. Splenic 8.3-CD8+ T-cells (2 x 10^4) were incubated with 1 x 10^5 γ-irradiated splenocytes pulsed with 1 µg/mL peptide for 48 hours. The supernatants were then harvested and assayed for IFN-γ (C), or IL-2 (D). (E) Cytotoxic response of NRP-stimulated splenic 8.3-CD8+ T-cells in response to peptide-pulsed APCs. Splenic 8.3-CD8+ T-cells (1 x 10^4) were incubated with 1 x 10^5 γ-irradiated APCs pulsed with 1µg/mL NRP for 7 days. At this time, cells were used in a ^{51}Cr-release assay against RMA-S-K^d cells pulsed with 1 µg/mL peptide. Bars show the standard error of the means.

analogs induced no responses, whereas A1, A3, and A6 analogs stimulated partial responses). None of the peptides tested stimulated 8.3-CD8+ T-cells to secrete IL-4 (data not shown).

The ability of NRP analogs to elicit a cytotoxic response from NRP-stimulated 8.3-CD8+ T-cells towards peptide-pulsed RMA-S-K^d cells was next determined. Figure 11D demonstrates that A1, A6, and A7 were as effective as NRP at stimulating a cytotoxic response from 8.3-CD8+ T-cells. A3, however, was only partially capable, and A4 & A8 were completely incapable of stimulating cytotoxicity from NRP-stimulated 8.3-CD8+ T-cells.

Table 2: Immunological Properties of Peptides

Peptide	Proliferation	IFN-γ/IL-2 Secretion	Cytotoxicity
NRP	+++	+++	+++
tum	-	-	-
A1	+	+	++
A3	+	-	++
A4	-	-	-
A6	++	++	+++
A7	+++	+++	+++
A8	-	-	-

+++ > 90 000 cpm (proliferation, 10 μg/mL peptide); > 4000 pg/mL (IFN-γ)/> 50 pg/mL (IL-2); > 45% (^{51}Cr Release)

++ 60 000 - 90 000 cpm (proliferation, 10 μg/mL peptide); 1000 - 4000 pg/mL (IFN-γ)/30 - 50 pg/mL (IL-2); 25-45% (^{51}Cr Release)

\+ 1 000 - 60 000 cpm (proliferation, 10 μg/mL peptide); 100 - 1000 pg/mL (IFN-γ)/ 10 - 30 pg/mL (IL-2); 5 - 25% (^{51}Cr Release)

\- 0 - 1 000 cpm (proliferation, 10 μg/mL peptide); 0 - 100 pg/mL (IFN-γ)/ 0 - 10 pg/mL (IL-2); < 5% (^{51}Cr Release)

Therefore, NRP's agonistic abilities appear to be susceptible to amino acid substitution at several of its non-anchor residues (table 2). Positions 3, 4, and 8 were particularly sensitive to substitutions. Thus, it was concluded that Asn at position 3, Lys at position 4, and Phe at position 8 were primary TCR contact residues. Lys at position 1 and Asn at position 6 were secondary TCR contact residues. Finally, Trp at position 7 did not appear to be TCR contact residues, and neither did Ala at position 5 (figure 12). The conclusion that position 5 does not contact the TCR (rather than being occupied by a Cys or Met) followed from the following observations: i) no amino acid groups stimulated

8.3-CD8+ T-cell-mediated cytotoxicity in the first generation PSCPL and ii) an Ala occupying position 5 in no way hinders NRP agonistic abilities towards the 8.3-CD8+ T-cell.

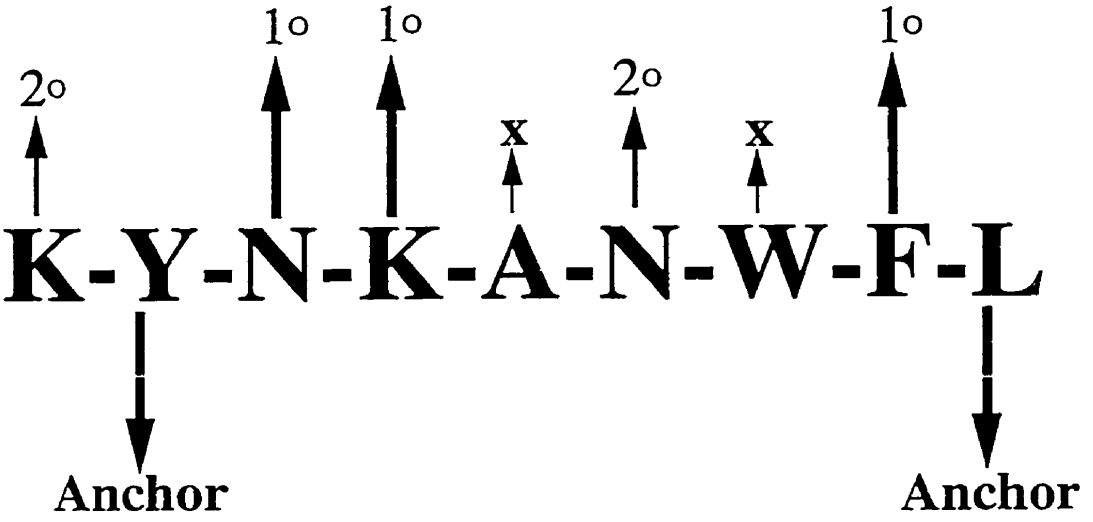

Figure 12. Contribution of Each Residue Towards NRP's Antigenicity. The primary (1°), secondary (2°), and non- (x) TCR contact residues of NRP are depicted in this figure as determined by observing the immunological properties of Ala-substituted NRP analogs.

C.5 Database Searches

It was next determined whether NRP was homologous to a known peptide in an effort to gain some insight into the natural β-cell-associated agonist of the 8.3-CD8+ T-cell. To accomplish this, a search of the NCBI database for proteins containing the following NRP or NRP-like sequences was undertaken as described in Section B.7

(underlined residues are those which differ from NRP): K(Y/F)NKANWF(L/I/V), K(Y/F)NKXNWF(L/I/V), K(Y/F)NKXNXF(L/I/V), K(Y/F)NKXXWF(L/I/V), K(Y/F)NKXXXF(L/I/V), X(Y/F)NKXNWF(L/I/V), K(Y/F)XKXNWF(L/I/V), X(Y/F)XKXNWF(L/I/V), and X(Y/F)XKXXXF(L/I/V).

Appendix II lists the results for all queries. Table 3 highlights the results that most closely resembled the query sequence. The following were synthesized for further experimentation (see table 1). They included aminopeptidase C from *Streptococcus thermophilus* (KYEKSNWFL), and *Lactobacillus lactis* (KYEKSNWFM), a 254 amino acid long hypothetical protein from *Pyrococcus horikoshii* (KYSKEVWFV), and the hypothetical protein C23d3.15 in chromosome 1 of *Schizosaccharomyces pombe* (RFNKSNFFI).

Table 3: NRP Sequence Homology

Organism	Protein Name	NRP Homologous Sequence
--	NRP	KYNKANWFL
Streptococcus thermophilus	Aminopeptidase C	KYEKSNWFL
Lactobacillus lactis	Aminopeptidase C	KYEKSNWFM
Pyrococcus horikoshii	Hypothetical Protein	KYSKEVWFV
Schizosaccharomyces pombe	Hypothetical Protein (C23d3.15)	RFNKSNFFI

Amino acids differing from NRP are underlined

Since aminopeptidase C derived from *S. thermophilus* contained a sequence most closely related to NRP, the NCBI's database was searched for a murine counterpart to this bacterial enzyme using the Entrez search program. The protein bleomycin hydrolase was discovered to be the mammalian homologue to aminopeptidase C. However, only partial cDNA sequence information was available on murine bleomycin hydrolase that did not extend to the region possessing the NRP homologous sequence in *S. thermophilus*-derived aminopeptidase C. Therefore, bleomycin hydrolase-specific cDNA libraries were prepared by anchor-PCR using total cellular RNA isolated from NOD islet cells, cloned into the vector pBluescript (Stratagene, La Jolla, CA), and transformed into competent XL-1 *E. coli* (Stratagene, La Jolla, CA). Recombinant plasmids from white colonies were

sequenced with a Sequenase DNA Sequencing Kit (USB, Cleveland, OH). The sequence results obtained were read up to the region possessing the NRP homologues in bacterial aminopeptidase C proteins. Unfortunately, this region contained the sequence KVERCYFFL. With only 3/9 residues matching NRP, and the lack of an anchor residue at position 2, no tests were performed with this peptide.

The NRP homologues synthesized (see Table 3) were recently tested for their ability to elicit a cytotoxic response from NRP-stimulated 8.3-CD8+ T-cells towards peptide-pulsed RMA-S-K^d cells. Of the 4 NRP homologues tested, only C23d3.15 from *S. pombe* (RFNKSNFFI) was recognized by 8.3-CD8+ T-cells (23.5% ± 2.5% specific lysis, data not shown). This was not surprising, since all 3 primary TCR contact residues (positions 3, 4, and 8) remain unchanged from NRP and a secondary TCR contact residue (position 1) underwent a conserved substitution (from a Lys in NRP to an Arg in C23d3.15 [both amino acids are basic]). In the non-TCR contact residues, one conserved substitution occurred (from Trp in NRP to a Phe in C23d3.15 at position 7 [both amino acids are aromatic]), and one non-conserved substitution occurred (from an Ala [aliphatic side chain] in NRP to a Ser [aliphatic hydroxyl side chain] in C23d3.15 at position 5). The anchor residues at positions 2 and 9 were substituted from Tyr and Leu, respectively, in NRP to Phe and Ile, respectively, in C23d3.15. However, these substitutions should not affect the ability of the C23d3.15 peptide to bind to H-$2K^d$ molecules (96, 97, 98). All of the peptides that did not elicit cytotoxicity from 8.3-CD8+ T-cells had an amino acid substitution in a primary TCR contact residue (From Asn in NRP to Asp at position 3 of both bacterial sources of aminopeptidase C, and from Asn in NRP to Ser in position 3 of *P. horikoshii*'s hypothetical protein).

Learning that 8.3-CD8+ T-cell recognition could still be maintained if secondary TCR contact residues (position 1 and 6) underwent conserved substitutions, a new search of the NCBI database was undertaken (see Section B.7) in hopes of finding a β-cell-

associated homologue. The following sequences were queried: RYNKANWFL and KYNKAQWFL. However, these searches did not yield any homologue matches that had not already been described with the previous NCBI database searches listed in Appendix II (data not shown)

Furthermore, NRP was compared against the sequences of the autoantigens GAD65, GAD67, and preproinsulin type I and II, but no areas of homology could be identified (data not shown). In summary, the database searches did not allow us to identify a β-cell-associated peptide recognized by 8.3-CD8+ T-cells when displayed by H-2K^d. However, the studies performed with NRP homologues demonstrated that peptides possessing H-2K^d binding residues (Tyr/Phe at position 2 and Leu/Ile/Val at position 9), primary 8.3-TCR contact residues (Asn at position 3, Lys at position 4, and Phe at position 8), conserved substitutions at secondary TCR contact residues (positions 1 and 6) and non-conserved substitutions at non-TCR contact residues (positions 5 and 7) may still elicit 8.3-CD8+ T-cell-mediated cytotoxicity towards peptide-pulsed RMA-S-K^d cells.

C.6 Establishment of the Immunodominance of the CD8+ T-Cell Response

C.6.1 TCR Repertoire Studies

Since NRP's agonistic effect on 8.3-CD8+ T-cells had been established (see Section C.4.1), this peptide was employed to address the hypothesis that the majority of CD8+ T-cells recruited to the islets during IDDM pathogenesis recognized an immunodominant antigen/MHC class I complex on the surface of β-cells. To demonstrate this, it was first determined whether a single peptide/H-2K^d complex could select for a splenic population of 8.3-TCRβ-chain transgenic CD8+ T-cells bearing an endogenous, highly restricted, TCRα-chain CDR3 motif commonly found in islet-associated CD8+ T-cells ([hydrophobic]-[Arg]-[Asp/Glu or Asn/Gly]-[Tyr/Ser]) (37, 39, 42).

This was accomplished by studying the endogenous TCRα-chain repertoire of unstimulated and NOD islet- or NRP-stimulated splenic TCRβ-transgenic CD8+ T-cells.

To generate TCRα-specific cDNA libraries, anchor-PCR was employed using total cellular RNA from islet-derived T-cells of 5 diabetic 8.3-TCRβ-transgenic mice (figure 13, upper panel) and from NOD-islet stimulated (3 times at 1 week intervals) or NRP-stimulated (twice) CD8+ T-cells from 3 non-diabetic 8.3-TCRβ-transgenic NOD mice (figure 13, second and third panels, respectively). Furthermore, TCRα-specific cDNA libraries generated by anchor-PCR of total cellular mRNA from naive, CD4+ T-cell-depleted splenocytes from 3 non-diabetic 8.3-TCRβ-transgenic NOD mice were also prepared (figure 13, lower panel). The TCRα cDNAs were cloned into pBluescript (Stratagene, La Jolla, CA), and sequenced using a Sequenase DNA Sequencing Kit (USB, Cleveland, OH).

As shown in figure 13, 14 out of 21 endogenous TCRα sequences employed by NRP-stimulated TCRβ-transgenic CD8+ T-cells were identical to the 8.3-TCRα sequence. This pattern is also observed in NOD islet-stimulated TCRβ-transgenic CD8+ T-cells, where 46/60 clones used the 8.3-TCRα-chain. This skewing was not observed in unstimulated cells (0/21), and is, thus, specific for NRP and NOD β-cells.

It has been well established that the majority of islet-derived CD8+ T-cell clones from pre- and acutely diabetic NOD mice express an endogenous TCRα-chain with the CDR3 motif ([hydrophobic]-[Arg]-[Asp/Glu or Asn/Gly]-[Tyr/Ser]) (37, 39, 42). Significantly, the majority (17/21) of NRP-stimulated splenic 8.3-TCRβ-chain transgenic CD8+ T-cells possessed this TCRα-CDR3 motif. It was, therefore, concluded that a single peptide/H-2K^{d} complex could stimulate the outgrowth of a CD8+ T-cell population possessing this motif in the same manner that β-cells do *in vitro* and *in vivo*.

C.6.2 Immunodominance of Islet-Derived Non-Transgenic CD8+ T-Cell Specificity

At this point, it had been discovered that NRP, a proven agonist of 8.3-CD8+ T-cells, could select for a population of CD8+ T-cells bearing endogenous TCRα-chain CDR3 motifs homologous to the majority of islet-derived CD8+ T-cells. To prove the

CELLS/Vα-Jα	N*	Vα	Nα (CDR3)	Jα
NY8.3				
Vα17.1-Jα42	-	CA	MRD	S...GGSNAKLTFGKGTKLSVKSN
8.3β-NOD ISLET-ASSOCIATED CD8+ T-CELLS				
Vα17.1-Jα42	57/78	CA	MRD	S...GGSNAKLTFGKGTKLSVKSN
Vα4-Jα47	14/78	CA	LRRMD	Y.....ANKMIFGLGTILRVRPH
Vα17.1-Jα42	1/78	CA	LRD	S...GGSNAKLTFGKGTKLSVKSN
Vα10-Jα57	1/78	CA	LY	QGGSRKLIFGEGTKLTVSSY
Vα1-Jα13	1/78	CA	ASS	NS.....GTYQRFGTGTKLQVVPN
Vα1-Jα13	1/78	CA	ASE	S....NSGTSQRFGTGTKLQVVPN
Vα3-Jα42	1/78	CA	VSG	S..SGGSNAKLTFGKGTKLSVKSN
Vα8-Jα9	1/78	CA	STVG	NMGYKLTFGTGTSLLVDPN
VαnP3-Jα31	1/78	CA	PGN	NRIFFGDGTQLVVKPN
8.3β-NOD CD8+ T-CELLS GROWN ON NOD ISLETS				
Vα17.1-Jα42	46/60	CA	MRD	S...GGSNAKLTFGKGTKLSVKSN
Vα1-Jα13	7/60	CA	AS S	NS.....GTYQRFGTGTKLQVVPN
VαnP4-Jα53	2/60	CA	A	S...GGSNYKLTFGKGTLLTVTPN
Vαn3-Jα18	2/60	CA	VRG	GSALGRLHFGAGTQLIVIP
Vα17.1-Jα40	1/60	CA	MRED	VNTGNYKYVFGAGTRLKVIAH
Vα10-Jα27	1/60	CA	ASTTMP	TFGAGTKLTVKPN
Vα8-Jα22	1/60	CA	LSER	S.....GSWQLIFGSGTQLTVMP
8.3β-NOD CD8+ T-CELLS GROWN ON NRP-PULSED APCs				
Vα17.1-Jα42	10/21	CA	MRD	T...GGSNAKLTFGKGTKLSVKSN
Vα17.1-Jα42	4/21	CA	MRD	S...GGSNAKLTFGKGTKLSVKSN
Vα4-Jαx	3/21	CA	LPHERD.....Y	FGLGTRVSVFPY
Vα10-Jαx	1/21	CA	AGPARN.....NY.....AQGLTFGLGTRVSVFPY	
Vα9n-Jα52	1/21	CA	MER.....SA	GANTGRLTFGAGTILRVAPN
Vα4-Jα32	1/21	CA	ALAMNYGG	SGNKLIFGTGTLLSVKPN
Vα9n-Jα42	1/21	C	GGI	NSGGSNAKLTFGKGTKLSVKSN
NAIVE 8.3β-NOD CD8+ T-CELLS				
Vαx-Jα18	2/17	CV	VARD.....GSALGRLHFGAGTQLIVI	
Vα2-Jα47	1/17	CA	ARGVD	YANKMIFGNGTIWRVRPH
Vα10-Jα52	1/17	CA	MRA	GANTGKLTFGHGTILRVH
Vα3-Jα24	1/17	CA	TVIDK	LASLGKLGFGTGTQVVVT
Vα8-Jαx	1/17	CA	WN	LSGSFNKWTFGAGNRLAVC
Vα1-Jα42	1/17	CA		SGGSNAKLTFGKGTKLSVK
Vα4-Jα23	1/17	CA	TEGP	LIFGQGTKLSIKP
Vα3-Jα18	1/17	CA	VRG	GSALGRLHFGAGTQLIVI
Vα2-Jα30	1/17	CA	AAYD	TNAYKVIFGKGTHLHVL
Vαx-Jα53	1/17	CA	AS	SGGSNYKLTFGKGTLLTVT
Vα5-Jα17	1/17	CA	VSG	NSAGNKLTFGIGTRVLVR
Vα3-Jα23	1/17	CA	TE	GKLIFGGGTKLSIK
Vα7-Jα42	1/17	CA	S	SGGSNAKLTFGKGTKLSVK
Vα8-Jα31	1/17	CA	L	NNNNRIFFGDGTQLVVK
Vα5-Jα22	1/17	CA	VR	SSGSWQLIFGSGTQLTVM
Vα1-Jα53	1/17	CA	V	NSGGSNYKLTFGKGTLLTVT

Figure 13. Endogenous VJα repertoire of islet-associated, NOD-stimulated, NRP-stimulated, and naive CD8+ T-cells from 8.3-TCRβ-transgenic NOD mice. Anchor-PCR was used to generate TCRα-specific cDNA from total, islet-derived, cellular RNA from T-cells of 5 diabetic 8.3-TCRβ-transgenic mice **(upper panel)** and NOD-islet-stimulated or NRP-stimulated CD8+ T-cells of 3 non-diabetic 8.3-TCRβ-transgenic mice **(second and third panels, respectively)**. TCRα-specific cDNA libraries were also generated by anchor-PCR of total cellular RNA from naive, CD4+ T-cell-depleted splenocytes from 3 non-diabetic 8.3-TCRβ-transgenic NOD mice **(lower panel)**. The TCRα cDNAs were cloned and sequenced. Underlined amino acids are N-terminal residues homologous to those of the 8.3-TCRα-chain **(top sequence)**. N* = the total number of copies of each sequence/total number of cDNAs sequenced. Vα and Jα genes were numbered according to refs. 105 and 106.

hypothesis that islet-derived CD8+ T-cells recognized an immunodominant antigen/MHC class I complex, it was next determined whether the majority of CD8+ T-cells, derived from the islets of non-transgenic NOD mice, could recognize NRP/H-2K^d complexes.

To that end, the cytotoxic response of short-term expanded, islet-derived, non-transgenic lines generated from 7 acutely diabetic NOD mice against NRP-pulsed RMA-S-K^d cells was determined. As shown in figure 14A, a representative non-transgenic line (#39/40) lyses RMA-S-K^d targets pulsed with NRP about half as well as an islet-derived TCRα/β-chain transgenic 8.3-CD8+ T-cell line. This suggests that approximately half of CD8+ T-cell clones within an islet-derived non-transgenic line recognized NRP, but not tum, complexed with H-2K^d. Figure 14C demonstrates that 87.5% (7/8) of islet-derived CD8+ T-cell lines isolated from non-transgenic, acutely diabetic NOD mice contained clonotypes that were cytotoxic towards targets displaying NRP (when compared to tum)/H-2K^d complexes ($p < 0.01$).

Next, the percentage of clones generated from islet-derived non-transgenic lines isolated from 9 acutely diabetic NOD mice able to specifically recognize NRP was determined. Due to the short-lived nature of non-transgenic clones, it was essential to perform the chromium release assay as soon as possible. Therefore, experiments were performed before large CD8+ T-cell populations could be generated. Thus, the results seen are at a target/effector ratio of ~1:1. Figure 14B shows the data obtained for a representative clone (clone #20), the average lysis of clones that specifically responded to NRP-pulsed targets (when compared to tum-pulsed targets, $p < 0.003$), and the average lysis of clones that did not recognize NRP. Of 31 clones, 14 clearly recognized NRP (but not tum) complexed with H-2K^d on the surface of RMA-S-K^d cells. The percentage of NRP-reactive clones/mouse ranged from 14% to 100%, with an average of 45%. No tum-reactive clones were observed (figure 14C). Overall, these results strongly suggested that, as the hypothesis states, the CD8+ T-cell response in autoimmune diabetes is

predominantly directed towards a single (or highly restricted set of) peptide/MHC class I complex(es).

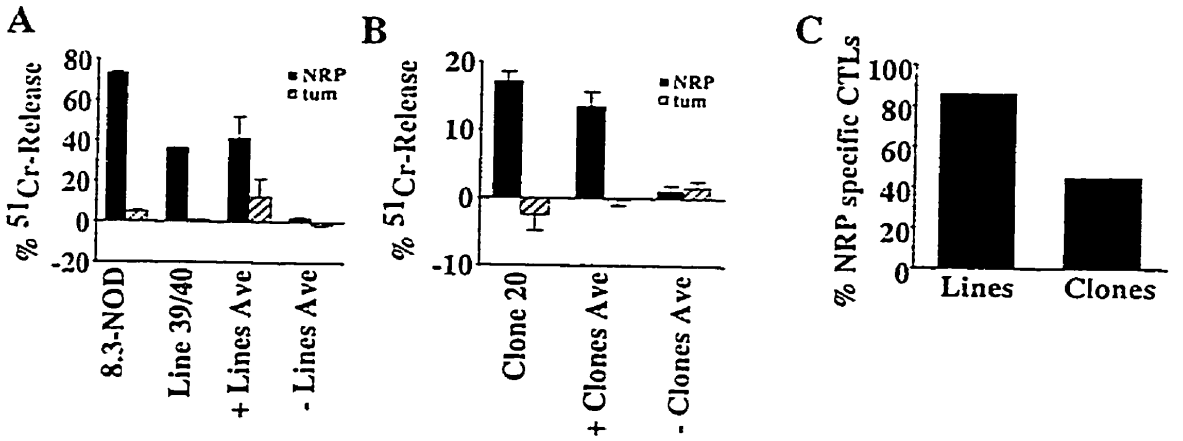

Figure 14. Functional Immunodominance of the CD8+ T-Cell Response in Non-Transgenic NOD Mice. (A) Ability of islet-derived, CD8+ T-cell lines to specifically recognize NRP. Islet-derived CD8+ T-cells (1 x 10^5) isolated from 8.3- and non-transgenic NOD mice were incubated with 1 x 10^4 [^{51}Cr]-labeled RMA-S-K^d targets pulsed with either NRP or tum for 8 hours. %^{51}Cr-release was determined as specified in Methods. Average of NRP specific (capable of lysing NRP-pulsed targets when compared to tum-pulsed targets; + lines ave; $p < 0.03$) and non-specific (- lines ave) lines was determined. Data was compared by the Mann-Whitney U test. (B) Ability of islet-derived CD8+ T-cell clones to specifically recognize NRP vs tum. Approximately 1 x 10^4 CD8+ T-cells cloned from islet-derived lines isolated from acutely diabetic, non-transgenic NOD mice were incubated with 1 x 10^4 [^{51}Cr]-labeled RMA-S-K^d targets pulsed with either NRP or tum for 8 hours. %^{51}Cr release determined as above. Average of NRP specific (+ clones ave; $p < 0.004$) and non-specific (- clones ave) clones determined. Bars show the standard error of the means. Data was compared by the Student's *t* test. (C) %NRP specific CTLs. The average percentage of NRP-specific islet-derived, non-transgenic lines or clones was calculated. The number of NRP reactive lines or clones was compared against the number of tum reactive lines or clones (of which there were none). The number or NRP reactive lines and clones was determined to be significant ($p < 0.0001$). Data was compared by the χ^2 test.

D. DISCUSSION

D.1 Overview

IDDM, in humans and the NOD mouse, is a complex disease on many levels. Genetically, there is an ever-growing array of genes (linked and unlinked to the MHC locus) being discovered that are associated with disease susceptibility (1, 5, 6). However, genetic factors account for only a third of an individual's susceptibility to IDDM, and a synergy between a patient's genetic predisposition and environmental elements seem to be essential for disease development (7-9). Immunologically, the entire immune system is involved in disease pathogenesis (12). A number of studies, however, have demonstrated

that T-cells play a very important part in IDDM development (14-16). Although both CD4+ and CD8+ T-cells were shown to be essential for disease progression, the exact role each T-cell subset plays has remained controversial.

Nevertheless, the inability of MHC class $I^{-/-}$ and anti-CD8+ mAb treated NOD mice to develop insulitis, let alone IDDM, suggests that CD8+ T-cells may be critical for disease initiation (32-35, 107). This is confirmed by the restoration of insulitis when MHC class I expression is reconstituted (34), and the inability of CD4+ T-cells from pre-diabetic NOD donors to transfer disease into NOD.*scid* recipients without CD8+ T-cell help (16). However, when compared to RAG-$2^{+/+}$ 8.3-NOD and RAG-$2^{-/-}$ NOD mice transgenic for a highly pathogenic MHC class II-restricted TCR specificity, RAG-$2^{-/-}$ 8.3-NOD animals (no endogenous B- or T-cell help) show a reduced incidence and delayed onset of IDDM. The delayed onset of disease in these RAG-$2^{-/-}$ 8.3-NOD mice is attributable to a decreased ability to develop insulitis (23). This suggests that CD4+ T-cells are more effective recruiters of immune cells to the pancreas than CD8+ T-cells, and is confirmed by a previous study demonstrating that CD8+ T-cells from pre- and acutely diabetic NOD donors could not transfer IDDM to NOD.*scid* recipients without CD4+ T-cell help (16).

Taken together, a consensus is emerging whereby CD8+ T-cells infiltrate the islets first, then somehow recruit CD4+ T-cells which initiate the large-scale mobilization of immune cells to the pancreas, resulting in insulitis (4). There is now an extensive body of evidence demonstrating the ability of both, CD4+ and CD8+ T-cells, to effect terminal β-cell damage (17-21, 23, 37, 39-42).

Interestingly, the majority of CD8+ T-cells isolated from the islets of pre- and acutely diabetic NOD mice possess the TCRα-chain CDR3 motif [hydrophobic]-[Arg]-[Asp/Glu or Asn/Gly]-[Tyr/Ser] (39). Moreover, many CD8+ T-cells isolated from the earliest insulitic lesions of NOD mice possessed the homologous CDR3 motif [Met]-[Arg]-[Asp/Glu] (37). This suggested the hypothesis that CD8+ T-cells recruited to the

islets of Langerhans in spontaneous autoimmune diabetes recognize a highly restricted repertoire of antigen/MHC class I complexes on pancreatic β-cells. This presupposition was strengthened by the observation that NOD mice transgenic for the TCRβ-chain from a clone bearing this islet-associated TCRα-chain CDR3 motif (NY8.3, see ref. 40) had an accelerated age of onset of IDDM. In addition, islet-derived clones from these animals possessed the same endogenous TCRα-chain of the transgene-donating clone (42).

To enable the testing of this hypothesis, a knowledge about the ligand(s) of CD8+ T-cells bearing the islet-associated TCRα-chain CDR3 motif was required. To study the nature of this (these) β-cell-associated antigen(s), a clonotypic source of CD8+ T-cells possessing this islet associated TCRα-chain CDR3 motif was necessary. Thus, an NOD strain transgenic for the TCRα/β-chains of an islet derived CD8+ T-cell clone (NY8.3; see ref. 40) which possessed the IDDM-associated CDR3 motif [Met]-[Arg]-[Asp]-[Ser] was generated (the 8.3-NOD mouse; see ref. 23 & 39).

With the 8.3-NOD mouse in hand, a search for cDNAs encoding a peptide or peptides that, when in the context of H-2K^{d}, was recognized by 8.3-CD8+ T-cells using an expression cloning system was undertaken. Originally, this was expected to be the simplest, most effective method of identifying the naturally occurring agonist(s) for 8.3-CD8+ T-cells. Despite the best efforts to improve transfection efficiencies and address potential short-comings, this was not a viable manner in which to pursue the identity of the β-cell-associated antigen(s) recognized by 8.3-CD8+ T-cells. There are three factors unable to be accounted for which may have confounded these attempts.

I. If the putative antigen/H-2K^{d} complex was expressed at low levels in the COS-7 or WOP transfected cells, the TNF-α assay may not have been sensitive enough to detect the small number of 8.3-CD8+ T-cells stimulated. Since it was not feasible to label each individual target population (one for each of 962 cDNA pools tested) with [^{51}Cr]-

sodium chromate, the highly sensitive chromium release assay could not be employed to screen these libraries in place of the TNF-α assay.

An avenue to overcome this obstacle would be to fuse the 8.3-CD8+ T-cells to the T-cell fusion partner α-β-BW5147 previously transfected with a CD8α gene and a β-galactosidase (lacZ) gene fused to the nuclear factor in activated T-cells (NFAT). NFAT is a DNA element within the IL-2 gene enhancer, and is important in the transcriptional regulation of the IL-2 gene. Hybrids capable of antigen-mediated stimulation possessing the 8.3-TCR are selected. When the T-cell hybridoma created with this fusion partner is activated via antigenic stimulation (resulting in transcription of the IL-2 gene, and, therefore, lacZ in this system), β-galactosidase synthesis occurs, and this can be monitored by its chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). Activation of T-cell hybrids can be observed at the single cell level with a simple microscope using this system (45, 108-110). Unfortunately, the CD8α and NFAT-lacZ transfected fusion partner could not be obtained.

II. Different post-translational modifications of peptides may occur in cells from different animals or animal strains. Thus, although a WOP or COS cell may have been transfected with a cDNA encoding the correct amino acid sequence of the antigenic peptide, the CD8+ T-cell may not recognize the resultant gene product due to an improper glycosylation pattern or amino acid substitution (94, 95).

The differences in post-translational modification may result from the saturation of modifying enzymes due to the great amplification achieved in these cell lines, or it could stem from the absence or presence of additional modifying enzymes (94, 95).

III. Finally, the possibility exists that the 962 pools (2×10^5 total cDNA) of NIT-1 cDNA do not possess the gene encoding the antigenic peptide, or the cDNA was cleaved during digestion with EcoRI and XhoI during construction of the cDNA library.

To address this, more cDNA pools could be generated using different restriction enzyme digestion, but this would not guarantee success.

Therefore, expression cloning was laid aside, and the process of purifying the antigenic peptide(s) acid extracted off the surface of NOD-derived NIT-1 insulinoma cells was applied. Acid extracted peptides were loaded onto an rpHPLC column and separated by elution with a steeply increasing acetonitrile gradient. This resulted in two elution peaks containing peptides that, when displayed by H-2K^d on RMA-S-K^d cells, were capable of stimulating TNF-α secretion from splenic, KJ16-stimulated 8.3-CD8+ T-cells. To better purify the antigenic peptide(s), the larger elution peak underwent a second separation by rpHPLC. A shallower gradient of the peptide elution buffer (acetonitrile) was applied in an attempt to gain greater separation of the peptide mixture. A large number of fractions were collected, and another TNF-α assay was performed on them. However, the levels of antigenic peptide were too low in the collected fractions to be detected using this assay system. It was attempted to implement the more sensitive chromium release assay, but generating a clonal population of cytotoxic, islet-derived 8.3-CD8+ T-cells large enough to perform a proper experiment was problematic. It had to wait until the powerful 8.3-CD8+ T-cell agonist, NRP, was elucidated that a sizable population of NRP-stimulated 8.3-CD8+ T-cells could be employed to perform a chromium release assay.

This allowed the recent discovery of a single, antigenic peak in the second-run rpHPLC fractions. The antigen(s) eluted off the rpHPLC column at the same acetonitrile concentration as it did in the first run (44% acetonitrile in the first run; 44.25% acetonitrile in the second run), suggesting that it was the same peptide(s) in both rpHPLC purification steps that resulted in the observed 8.3-CD8+ T-cell reactivity. This opens new doors of opportunity for discovering the true identity of this antigen. Presently, collaboration is being sought with a laboratory containing a capillary-HPLC/tandem mass spectrometer

system that will be used for the fine separation of the peptides which eluted off at 44.25% acetonitrile during the second rpHPLC purification. It is hoped that these peptides can then be sequenced and tested for antigenicity. The result of these efforts would be the identification of the naturally occurring β-cell associated peptide(s) that, when complexed with H-2K^d, are recognized by 8.3-CD8+ T-cells.

However, since the purification and subsequent sequencing of antigenic peptides has not yet led to their identification, the breakthrough that allowed us to address this thesis's hypothesis was the generation of NRP by PSCPL. Combinatorial peptide libraries are a powerful tool that have previously been used to identify CD8+ T-cell epitopes, mimetics of those epitopes, as well as alloepitopic mimics (46, 103, 111). The versatility of this system has been demonstrated in epitope/MHC class I binding studies (112, 113) and analyses of individual amino acid contribution within an epitope for CTL activity (89). Even CD4+ T-cell epitopes have been identified (102, 104) and antibody/epitope binding studies have been undertaken (114).

Although this system generally does not result in the identification of the natural peptide, it usually results in a mimic highly homologous to the natural antigen. For example, PSCPL employed to test the antigenic specificity of a clone targeting a vesicular stomatitis virus nucleoprotein-derived epitope generated the mimotope RGY(V/A)WQYT. When compared to the natural epitope (RGYVYQGL) 5 out of 8 positions are seen to be identical (46). In a separate study, mimics of epitopes for EL-4-specific CTLs were generated using PSCPL. These mimics were recognized by EL-4 specific CTLs, had the same HPLC elution profile, and behaved biochemically and immunologically identical to the naturally expressed EL-4 antigens (103). However, one study using combinatorial peptide libraries to identify a mimotope of a CD4+ T-cell antigen demonstrated that a peptide capable of eliciting T-cell reactivity with no sequence homology to the natural epitope could be generated (115).

Regardless of whether it is homologous to the naturally occurring peptide or not, NRP is an effective mimotope of a β-cell antigen recognized by 8.3-CD8+ T-cells in a H-2K^d-restricted manner. This has been shown by its ability to elicit the proliferation, cytokine secretion, differentiation, and cytotoxicity of 8.3-CD8+ T-cells in a manner homologous to NOD islet-cells. The creation of this mimotope is a powerful tool for the study of autoimmune diabetes. For example, the difficulty inherent in the generation of 8.3-CD8+ T-cell populations large enough to employ in some of our experiments crippled our studies for many years. Now, considering the powerful agonistic properties of NRP, it is remarkably simple to generate vast populations of 8.3-CD8+ T-cells to employ in our endeavors. Furthermore, under the assumption that the natural β-cell-derived antigen recognized by 8.3-CD8+ T-cells is similar to NRP, polyclonal NRP-specific rabbit antibodies have been generated and are presently being used to screen a NIT-1 cDNA expression library. It is hoped that a cDNA will be identified which encodes a protein that binds to the anti-NRP antibodies, for such a cDNA may be responsible for the expression of the naturally occurring antigen. Moreover, information generated while studying the contribution of each amino acid within NRP to its antigenicity opens the door to altered peptide ligand studies and searches for an antagonist to the 8.3-CD8+ T-cell (116).

With NRP in hand, the hypothesis that the CD8+ T-cell response initiating disease and effecting β-cell destruction was directed towards an immunodominant peptide/H-2K^d complex could now be addressed. First, it was sought to test whether NRP could select for a population of naive, TCRβ-chain transgenic CD8+ T-cells possessing the TCRα-chain CDR3 motif commonly found among islet-derived CD8+ T-cell lines ([hydrophobic]-[Arg]-[Asp/Glu or Asn/Gly]-[Tyr/Ser]). It was discovered that, indeed, NRP was capable of selecting the outgrowth of such a population of cells. This indicated that a single peptide/H-2K^d complex was capable of selecting for a population of naive

CD8+ T-cells bearing the TCRα-chain CDR3 motif homologous to those observed at insulitis onset (37) and insulin-dependence (39).

It was next determined whether the majority of islet-derived, non-transgenic CD8+ T-cells from acutely diabetic NOD mice could recognize a single peptide/MHC class I complex during disease onset. The majority of lines tested contained CD8+ T-cell populations cytotoxic towards NRP-pulsed RMA-S-K^d cells. The average lysis caused by non-transgenic CD8+ T-cells was 55% that of a typical 8.3-CD8+ T-cell line. This suggested that approximately half of the CD8+ T-cells present in the islets of non-transgenic, acutely diabetic NOD mice recognized a single peptide/MHC class I complex. Studies on clonal CD8+ T-cell populations generated from islet-derived lines from acutely diabetic NOD mice confirmed that about half of the islet-derived CD8+ T-cell population at IDDM onset recognize a single antigen/H-$2K^d$ complex. Considering the potential for TCR heterogeneity (~10^{15}-10^{17} possible TCR recombinations [5, 10]), this is highly significant. The ability of 8.3-CD8+ T-cells to recognize the *S. pombe*-derived peptide C23d3.15 possessing conserved amino acid substitutions at secondary TCR-contact residues within NRP indicates that, in nature, it may be a highly restricted, NRP-homologous, set of peptides which the majority of islet-derived CD8+ T-cells are reactive towards.

This finding is the first characterization of the CD8+ T-cell response in IDDM. The majority of islet-derived CD8+ T-cells at the onset of insulitis bear the TCRα-chain CDR3 motif [Met]-[Arg]-[Asp/Glu] (37). This is homologous to the majority of islet-derived CD8+ T-cells' TCRα-chain CDR3 usage preceding and at the onset of insulin-dependence ([hydrophobic]-[Arg]-[Asp/Glu or Asn/Gly]-[Tyr/Ser]) (39). CD8+ T-cells sharing this TCRα-chain CDR3 motif isolated at the onset of IDDM are capable of recognizing a single peptide in the context of MHC class I, are H-$2K^d$-restricted (40) and are cytotoxic towards β-cells *in vitro* (41) and *in vivo* (39). Taken together, this certifies

the hypothesis that CD8+ T-cells recruited to the islets of Langerhans in spontaneous autoimmune diabetes recognize a highly restricted (perhaps single), immunodominant repertoire of antigen/H-2K^d complexes on pancreatic β-cells in NOD mice.

Furthermore, the homology between TCRα-chain CDR3 regions from islet-derived CD8+ T-cells isolated from the earliest insulitic lesions, and from pre- and acutely diabetic NOD mice (37, 39) suggests that the TCR-specificity of islet-associated CD8+ T-cells does not diversify significantly during disease progression. This is in contradiction to the CD4+ T-cell response observed in IDDM and a variety of other autoimmune diseases that undergo epitope (determinant) spreading. This is a process whereby an immune response, originally directed against a single (or restricted number of) epitope(s) spreads intramolecularly to other regions of the immunogenic protein, and intermolecularly to new proteins. It is postulated that determinant spreading occurs as endogenous peptide is released during the immune attack on infected cells and is subsequently recognized by the immune system. In this manner, the immune response to a particular pathogen is broadened, thereby ensuring its complete abrogation (117).

Studies on pre-diabetic NOD mice suggest that the CD4+ T-cell response originally focuses on a single determinant of GAD65 at 4 weeks of age. By 12 weeks of age, the CD4+ T-cell response has spread intramolecularly to various epitopes of GAD65, and intermolecularly to other autoantigens, including insulin and hsp60 (44, 117). Furthermore, intramolecular epitope spreading has been observed in the CD4+ T-cell response to insulin (56) and the IDDM-associated autoantibody response to tyrosine phosphatase IA2 and phogrin (118).

The reason why the nature of the CD8+ T-cell response may differ from the epitope spreading observed in the CD4+ T-cell response is a matter of conjecture, and the following supposition may account for it. Perhaps the differing natures of the CD4+ and CD8+ T-cell responses stem from the different roles they play in IDDM progression.

CD4+ T-cells have many diverse roles throughout disease pathogenesis. When initially recruited to the islets, CD4+ T-cells are primarily responsible for the conscription of other immune cells, and this process could be mediated by an (oligo)clonal CD4+ T-cell population specific for a restricted repertoire of antigens. As insulitis develops, the need for a regulatory/suppresser subset of (oligo)clonal CD4+ T-cells (specific for a different array of antigens) will arise to moderate and restrict the burgeoning immune response. Finally, β-cell destruction could be mediated by a new (oligo)clonal population of CD4+ T-cells, specific for a new repertoire of antigens.

CD8+ T-cells, on the other hand, have a much simpler task -- they primarily attack β-cells. Since the CD8+ T-cell's role is more singular than the CD4+ T-cell's, a less diverse set of antigenic repertoires is required. Furthermore, cytotoxic T-cells destroy their target with surgical precision. Perhaps restricting the antigenic repertoire of cytotoxic T-cell subsets is the immune system's way of maintaining this accuracy.

These views are apparently contradicted by a study performed on monoclonal T-cell RAG-2$^{-/-}$ NOD mice. These animals possess either CD4+ or CD8+, highly pathogenic, monoclonal T-cell populations. Both the monoclonal CD4+ and CD8+ T-cell populations are observed to initiate IDDM in the absence of other T- or B-cell help, although CD8+ T-cells do so at a much lower incidence and delayed onset attributable to defective insulitis initiation (23). However, this study is quite reductionist in that it only observes the capabilities of strongly pathogenic T-cells with a single TCR-specificity, negating the opportunity for epitope spreading to be observed. Although important in delineating the roles each T-cell subset is capable of performing in IDDM pathogenesis, this model does not reflect the significantly elaborate natural immune response.

D.2 Future Considerations

The discoveries made during this project result in a broad spectrum of new research opportunities. Since, as opposed to CD4+ T-cells in IDDM, the CD8+ T-cell

response is focused primarily on a highly restricted (perhaps single) repertoire of antigen/MHC class I complexes, perhaps therapeutic procedures targeting this pathogenic subset of effectors would be fruitful. The information gathered regarding the contribution of each position within NRP towards its antigenicity could pave the way for the design of an antagonist to achieve these ends (116).

The definition of NRP opens the door for studies attempting to identify the naturally occurring β-cell antigen recognized by the majority of islet-derived CD8+ T-cells. Knowledge of such an antigen could be applied to the treatment of IDDM, since it has been possible in animal models to stave off autoimmunity by immunization with a peptide targeted in a T-cell mediated autoimmune response (59, 119-121). In a greater scope, knowledge about a diabetogenic antigen yields a powerful tool, allowing researchers to address fundamental questions about the nature of the CD8+ T-cell response in the process of IDDM progression.

Overall, IDDM is a very complex autoimmune disease whose precise mechanisms still, largely, elude us. However, the boundaries of the unknown are slowly being pushed back, permitting an understanding of immune regulation gone awry. Such research is essential, since studying a system malfunctioning gives us knowledge of how the system should work in health. This wisdom will yield tools hopefully allowing us to one day cure IDDM, and most likely other autoimmune disorders as well.

E. BIBLIOGRAPHY

1. Pickup, J. & G. Williams. 1997. Textbook of Diabetes: Volume 1, 2nd Ed. Blackwell Science Ltd. Malden, MA.

2. Bottazzo, G. F., A. Florin-Christenses, D. Doniach. 1974. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiences. *Lancet*. 3: 133-135.

3. Makino, S., K. Kunimoto, Y. Muraoka, Y. Mizushima, K. Katagire, & Y. Tochino. 1980. Breeding of a non-obese, diabetic strain of mice. *Exp. Anim*. 29: 1-13.

4. Benoist, C. & D. Mathis. 1997. Cell death mediators in autoimmune diabetes -- no shortage of suspects. *Cell*. 89: 1-3.

5. Male, D., A. Cook, M. Owen, & J. Trowsdale. 1996. Advanced Immunology, 3rd Ed. Mosby. New York, NY.

6. Serreze, D. & E. H. Leiter. 1994. Genetic and pathogenic basis of autoimmune diabetes in NOD mice. *Curr. Op. Immunol*. 6: 900-906.

7. Janeway, Jr., C. A. & P. Travers. 1996. Immunobiology, 2nd Ed. Garland Publishing Inc. New York, NY.

8. Li, X-B., F. W. Scott, Y. H. Park, & J-W Yoon. 1995. Low incidence of autoimmune type I diabetes in BB rats fed a hydrolysed casein-based diet associated with early inhibition on non-macrophage-dependent hyperexpression of MHC class I molecules on beta cells. *Diabetologia*. 38: 1138-1147.

9. Yoon, J-W. 1995. A new look at viruses in type 1 diabetes. *Diabetes/Metabolism Reviews*. 11(2): 83-107.

10. Kuby, J. 1994. Immunology, 2nd Ed. W. H. Freeman and Company. New York, NY.

11. Miller, J. F. A. P. & A. Basten. 1996. Mechanisms of tolerance to self. *Curr. Op. Immunol*. 8: 815-821.

12. Signore, A., P. Pozzilli, E. A. M. Gale, D. Andreani, & P. C. L. Beverley. 1989. The natural history of lymphocyte subsets infiltrating the pancreas of NOD mice. *Diabetologia*. 32: 282-289.

13. Yagi, H., M. Matsumoto, K. Kunimoto, J. Kawaguchi, S. Makino, & M. Harada. 1992. Analysis of the roles of CD4+ and CD8+ T cells in autoimmune diabetes of NOD mice using transfer to NOD athymic nude mice. *Eur. J. Immunol.* 22: 2387-2393.

14. Bendelac, A., C. Carnaud, C. Boitard, & J. F. Bach. 1987. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. *J. Exp. Med.* 166: 823-832.

15. Miller, B. J., M. C. Appel, J. J. O'Neil, & L. S. Wicker. 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): 52-58.

16. Christianson, S. W., L. D. Shultz, & E. H. Leiter. 1993. Adoptive transfer of diabetes into immunodeficient NOD-*scid*/*scid* Mice: relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-*Thy*-1^a donors. *Diabetes.* 42: 44-55.

17. Haskins, K. & M. McDuffie. 1990. Acceleration of diabetes in young NOD mice with a CD4+ islet-specific T cell clone. *Science.* 249: 1433-1436.

18. Katz, J. D., B. Wang, K. Haskins, C. Benoist, & D. Mathis. 1993. Following a diabetogenic T cell from genesis through pathogenesis. *Cell.* 74: 1089-1100.

19. Peterson, J. D. & K. Haskins. 1996. Transfer of diabetes in the NOD.*scid* mouse by CD4 T-cell clones: differential requirement for CD8+ T-cells. *Diabetes.* 45: 328-336.

20. Wang, Y., O. Pontesilli, R. G. Gill, F. G. La Rosa, & K. J. Lafferty. 1991. The role of CD4+ and CD8+ T cells in the destruction of islet grafts by spontaneously diabetic mice. *Proc. Natl. Acad. Sci. USA.* 88: 527-531.

21. Bradley, B. J., K. Haskins, F. G. La Rosa, & K. J. Lafferty. 1992. CD8 T cells are not required for islet destruction induced by a CD4+ islet-specific T-cell clone. *Diabetes*: 41: 1603-1608.

22. Kurasawa, K., A. Sakamoto, T. Maeda, T. Sumida, I. Ito, H. Tomioka, S. Yoshida, & T. Koike. 1993. Short-term administration of anti-L3T4 MoAb prevents diabetes in NOD mice. *Clin. Exp. Immunol.* 91: 376-380.

23. Verdaguer, J., D. Schmidt, A. Amrani, B. Anderson, N. Averill, & P. Santamaria. 1997. Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. *J. Exp. Med.* 186(10): 1663-1676.

24. Chosich, N. & L. C. Harrison. 1993. Suppression of diabetes mellitus in the non-obese diabetic (NOD) mouse by an autoreactive (anti-I-A^{g7}) islet-derived CD4+ T-cell line. *Diabetologia*. 36: 716-723.

25. Sempe, P., M-F. Richard, J-F. Bach, & C. Boitard. 1994. Evidence of CD4+ regulatory T cells in the non-obese diabetic male mouse. *Diabetologia*. 37: 337-343.

26. Katz, J. D., C. Benoist, & D. Mathis. 1995. T helper cell subsets in insulin-dependent diabetes. *Science*. 268: 1185-1188.

27. Healey, D., P. Ozegbe, S. Arden, P. Chandler, J. Hutton, & A. Cooke. 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: 2979-2985.

28. Akhtar, I., J. P. Gold, L-Y. Pan, J. L. M. Ferrara, X-D. Yang, J. I. Kim, & K-N. Tan. 1995. CD4+ beta islet cell-reactive T cell clones that suppress autoimmune diabetes in nonobese diabetic mice. *J. Exp. Med*. 182: 87-97.

29. Utsugi, T., M. Nagata, T. Kawamura, & J-W. Yoon. 1994. Prevention of recurrent diabetes in syngenic islet-transplanted NOD mice by transfusion of autoreactive T lymphocytes. *Transplantation*. 57: 1799-1804.

30. Peakman, M., R. D. G. Leslie, L. Alviggi, M. Hawa, & D. Vergani. 1996. Persistent activation of CD8+ T-cells characterizes prediabetic twins. *Diabetes Care*. 19(11): 1177-1184.

31. Hehmke, B., D. Michaelis, E. Gens, F. Laube, & K-D. Kohnert. 1995. Aberrant activation of CD8+ T-cell and CD8+ T-cell subsets in patients with newly diagnosed IDDM. *Diabetes*. 44: 1414-1419.

32. Katz, J., C. Benoist, D. Mathis. 1993. Major histocompatibility complex class I molecules are required for the development of insulitis in non-obese diabetic mice. *Eur. J. Immunol*. 23: 3358-3360.

33. Wicker, L. S., E. H. Leiter, J. A. Todd, R. J. Renjilian, E. Peterson, P. A. Fischer, P. L. Podolin, M. Zijlstra, R. Jaenisch, & L. B. Peterson. 1994. β2-microglobulin-deficient NOD mice do not develop insulitis or diabetes. *Diabetes*. 43: 500-504.

34. Kay, T. W. H., J. L. Parker, L. A. Stephens, H. E. Thomas, & J. Allison. 1996. RIP-β2-microglobulin transgene expression restores insulitis, but not diabetes, in β 2-microglobulin^{null} nonobese diabetic mice. *J. Immunol*. 157: 3688-3693.

35. Wang. B., A. Gonzalez, C. Benoist, & D. Mathis. 1996. The role of CD8+ T-cells in the initiation of insulin-dependent diabetes mellitus. *Eur. J. Immunol.* 26: 1762-1769.

36. Serreze, D. V., H. D. Chapman, D. S. Varnum, I. Gerling, E. H. Leiter, & L. D. Shultz. 1997. Initiation of autoimmune diabetes in NOD/Lt mice is MHC class I-dependent. *J. Immunol.* 158: 3978-3986.

37. DiLorenzo, T. P., R. T. Graser, T. Ono, G. J. Christianson, H. D. Chapman, D. C. Roopenian, S. G. Nathenson, & D. Serreze. 1998. MHC class I-restricted T cells are required for all but the end stages of diabetes development in NOD mice and utilize a prevalent T cell receptor α chain gene rearrangement. *Proc. Natl. Acad. Sci. USA.* 95: 12538-12542.

38. Sorger, S. B., L. A. Matis, I. Engel, D. L. McElligot, P. J. Fink, & S. M. Hedrick. 1988. The influence of MHC gene products on the generation of an antigen-specific T-cell repertoire. *Ann. NY Acad. Sci.* 532: 18-32.

39. Santamaria, P., T. Utsugi, B-J. Park, N. Averill, S. Kawazu, & J-W. Yoon. 1995. Beta-cell-cytotoxic CD8+ T cells from nonobese diabetic mice use highly homologous T cell receptor α-chain CDR3 sequences. *J. Immunol.* 154: 2494-2503.

40. Nagata, M., P. Santamaria, T. Kawamura, T. Utsugi, & J-W. Yoon. 1994. Evidence for the role of CD8+ cytotoxic T cells in the destruction of pancreatic β-cells in nonobese diabetic mice. *J. Immunol.* 152: 2042-2050.

41. Utsugi, T., J-W. Yoon, B-J. Park, M. Imamura, N. Averill, S. Kowazu, & P. Santamaria. 1996. Major histocompatibility complex class I-restricted infiltration and destruction of pancreatic islets by NOD mouse-derived β-cell cytotoxic CD8+ T-cell clones in vivo. *Diabetes.* 45: 1121-1131.

42. Verdaguer, J., J-W. Yoon, B. Anderson, N. Averill, T. Utsugi, B-J. Park, & P. Santamaria. 1996. Acceleration of spontaneous diabetes in TCR-β-transgenic nonobese diabetic mice by β-cell cytotoxic CD8+ T cells expressing identical endogenous TCR-α chains. *J. Immunol.* 157: 4726-4735.

43. McInerney, M. F., S. Rath, & C. A. Janeway, Jr. 1991. Exclusive expression of MHC class II proteins on CD45+ cells in pancreatic islets of NOD mice. *Diabetes.* 40: 648-651.

44. Tian, J., P. V. Lehmann, & D. L. Kaufman. 1997. Determinant spreading of T helper cell 2 (Th2) responses to pancreatic islet autoantigens. *J. Exp. Med.* 186(12): 2039-2043.

45. Shastri, N. 1996. Needles in haystacks: identifying specific peptide antigens for T cells. *Curr. Opin. Immunol.* 8: 271-277.

46. Gundlach, B. R., K-H. Wiesmuller, T. Junt, S. Kienle, G. Jung, P. Walden. 1996. Determination of T cell epitopes with random peptide libraries. *J. Immunol. Meth.* 192: 149-155.

47. Harrison, L. C. 1992. Islet cell antigens in insulin-dependent diabetes: Pandora's box revisited. *Immunol. Today.* 13(9): 348-352.

48. Roep, B. O. 1996. T-cell responses to autoantigens in IDDM: the search for the holy grail. *Diabetes.* 45: 1147-1156.

49. Gelber, C., L. Paborsky, S. Singer, D. McAteer, R. Tisch, C. Jolicoeur, R. Buelow, H. McDevitt, & C. G. Fathman. 1994. Isolation of nonobese diabetic mouse T-cells that recognize novel autoantigens involved in the early events of diabetes. *Diabetes.* 43: 33-39.

50. Aguilar-Diosdado, M., D. Parkinson, J. A. Corbett, G. Kwon, C. A. Marshall, R. L. Gingerich, J. V. Santiago, & M. L. McDaniel. 1994. Potential Autoantigens in IDDM: expression of carboxypeptidase-H and insulin, but not glutamate decarboxylase on the β-cell surface. *Diabetes.* 43: 418-425.

51. Ko, I. Y., S. H. Ihm, & J-W. Yoon. 1991. Studies on autoimmunity for initiation of beta-cell destruction VIII. Pancreatic beta-cell dependent autoantibody to a 38 kilodalton protein precedes the clinical onset of diabetes in BB rats. *Diabetologia.* 34: 548-554.

52. Arden, S. D., B. O. Roep, P. I. Neophytou, E. F. Usac, G. Duinkerken, R. R. P. de Vries, & J. C. Hutton. 1996. Imogen 38: A novel 38-kD islet mitochondrial autoantigen recognized by T cells from a newly diagnosed type 1 diabetic patient. *J. Clin. Invest.* 97: 551-561.

53. Durinovic-Bello, I. 1998. Autoimmune diabetes: the role of T cells, MHC molecules, and autoantigens. *Autoimmunity.* 27: 159-177.

54. Nepom, G. T. 1995. Glutamic acid decarboxylase and other autoantigens in IDDM. *Curr. Op. Immunol.* 7: 825-830.

55. Pietropaolo, M., J. C. Hutton, & G. S. Eisenbarth. 1997. Protein tyrosine phosphatase-like proteins: link with IDDM. *Diabetes Care*. 20(2): 208-214.

56. Schloot, N. C., B. O. Roep, D. Wegmann, L. Yu, H. P. Chase, T. Wang, & G. S. Eisenbarth. 1997. Altered immune response to insulin in newly diagnosed compared to insulin-treated diabetic patients and healthy control subjects. *Diabetologia*. 40: 564-572.

57. Christie, M. R. 1996. Islet cell autoantigens in type 1 diabetes. *Eur. J. Clin. Inves*. 26: 827-838.

58. Bonifacio, E. & M. R. Christie. 1997. Islet cell antigens in the prediction and prevention of insulin-dependent diabetes mellitus. *Annals of Medicine*. 29: 405-412.

59. Harrison, L. C. 1995. Antigen-specific therapy for autoimmune disease: prospects for the prevention of insulin-dependent diabetes. *Molecular Medicine*. 1(7): 722-727.

60. Dotta, F. & U. di Mario. 1996. Antigenic determinants in type 1 diabetes mellitus. *APMIS*. 104: 769-774.

61. Wegmann, D. R., M. Norbury-Glaser, & D. Daniel. 1994. Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. *Eur. J. Immunol*. 24: 1853-1857.

62. Honeyman, M. C., D. S. Cram, & L. C. Harrison. 1993. Glutamic Acid Decarboxylase 67-reactive T cells: A marker of insulin-dependent diabetes. *J. Exp. Med*. 177: 535-540.

63. Panina-Bordignon, P., R. Lang, P. M. van Endert, E. Benazzi, A. M. Felix, R. M. Pastore, G. A. Spinas, & F. Sinigaglia. 1995. Cytotoxic T cells specific for glutamic acid decarboxylase in autoimmune diabetes. *J. Exp. Med*. 181: 1923-1927.

64. Wong, S., S. Guerder, I. Visintin, E-P. Reich, K. E. Swenson, R. A. Flavell, & C. A. Janeway, Jr.. 1995. Expression of the co-stimulator molecule B7-1 in pancreatic β-cells accelerates diabetes in the NOD mouse. *Diabetes*. 44: 326-329.

65. Daniel, P. T., A. Kroidl, S. Cayeux, R. Bargou, T. Blankenstein, & B. Dorken. 1997. Costimulatory signals through B7.1/CD28 prevent T cell apoptosis during target cell lysis. *J. Immunol*. 159: 3808-3815.

66. Hamaguchi, K., H. R. Gaskins, and E. H. Leiter. 1991. NIT-1, a pancreatic beta-cell line established from a transgenic NOD/Lt mouse. *Diabetes*. 40: 842.

67. Gotch, M., T. Maki, T. Kiyoizumi, S. Satomi, A. P. Monaco. 1985. An improved method for isolation of mouse pancreatic islets. *Transplantation*. 40: 437-438.

68. de Plaen, E., C. Lurquin, V. Brichard, P. van der Bruggen, J-C. Renauld, P. coulie, J-P. Szikora, T. Wolfel, A. van Pel, & T. Boon. 1996. Cloning of genes coding for antigens recognized by cytolytic T lymphocytes. Immunology Methods Manual. Academic Press.

69. Seed, B. & A. Aruffo. 1987. Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA*. 84: 3365-3369.

70. Boel, P., C. Wildmann, M. L. Sensi, R. Brasseur, J-C. Renauld, P. Coulie, T. Boon, & P. van der Bruggen. 1995. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. *Immunity*. 2: 167-175.

71. Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, L. Rivoltine, S. L. Topalian, T. Miki, & S. A. Rosenberg. 1994. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci.* USA. 91: 3515-3519.

72. Wolfel, T., M. Hauer, J. Schneider, M. Serrano, C. Wolfel, E. Klehmann-Hieb, E. de Plaen, T. Hankeln, K-H. M. zum Buschenfelde, & D. Beach. 1995. A $p16^{INK4a}$- insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science*. 269: 1281-1284.

73. van den Eynde, B., O. Peeters, O. de Backer, B. Gaugler, S. Lucas, & T. Boon. 1995. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. *J. Exp. Med.* 182: 689-698.

74. Coulie, P. G., F. Lehmann, B. Lethe, J. Herman, C. Lurouin, M. Andrawiss, & T. Boon. 1995. A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proc. Natl. Acad. Sci. USA*. 92: 7976-7980.

75. Robbins, P. F., M. El-Gamil, Y. F. Li, S. L. Topalian, L. Rivoltine, K. Sakaguchi, E. Appella, Y. Kawakami, & S. A. Rosenberg. 1995. Cloning of a new gene encoding an antigen recognized by melanoma-specific HLA-A24-restricted tumor-infiltrating lymphocytes. *J. Immunol.* 154: 5944-5950.

76. Wang, R-F., P. F. Robbins, Y. Kawakami, X-Q. Kang, & S. A. Rosenberg. 1995. Identification of a gene encoding a melanoma tumor antigen recognized by HLA-A31-restricted tumor-infiltrating lymphocytes. *J. Exp. Med.* 181: 799-804.

77. Wolfel, T., A. van Pel, V. Brichard, J. Schneider, B. Seliger, K-H. M. zum Buschenfelde, & T. Boon. 1994. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. *Eur. J. Immunol.* 24: 759-764.

78. Kawakami, Y., S. Eliyahu, K. Sakaguchi, P. F. Robbins, L. Rivoltini, J. R. Yannelli, E. Apella, & S. A. Rosenberg. 1994. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J. Exp. Med.* 180: 347-352.

79. Brichard, V., A. van Pel, T. Wolfel, C. Wolfel, E. de Plaen, B. Lethe, P. Coulie, & T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 178: 489-495.

80. Coulie, P. G., V. Brichard, A. van Pel, T. Wolfel, J. Schneider, C. Traversari, S. Mattei, E. de Plaen, C. Jurquin, J-P. Szikora, J-C. Renauld, & T. Boon. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 180: 35-42.

81. Dailey, L., and C. Basilico. 1985. Sequences in the polyomavirus DNA regulatory region involved in viral DNA replication and early gene expression. *J. Virol.* 54: 739-749.

82. Schumacher, T. N. M., M-T. Heemels, J. J. Neefjes, W. M. Kast, C. J. M. Melief, & H. L. Ploegh. 1990. Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. *Cell.* 62: 563-567.

83. Elliot, T. 1997. How does TAP associate with MHC class I molecules? *Immunol. Today.* 18(8): 375-379.

84. Ljunggren, H-G., N. J. Stam, C. Ohlen, J. J. Neefjes, P. Hoglund, M-T. Heemels, J. Bastin, T. N. M. Schumacher, A. Townsend, K, Karre, & H. L. Ploegh. 1990. Empty MHC class I molecules come out in the cold. *Nature.* 346: 476-480.

85. Attaya, M., S. Jameson, C. K. Martinez, E. Hermel, C. Aldrich. M. J. Bevan, & J. J. Monaco. 1992. Ham-2 corrects the class I antigen-processing defect in RMA-S cells. *Nature.* 355: 647-649.

86. Hosken, N. A. & M. J. Bevan. 1992. An endogenous antigenic peptide bypasses the class I antigen presentation defect in RMA-S. *J. Exp. Med.* 175: 719-729.

87. Tsomides, T. J. 1993. Sample preparation for HPLC by Centricon® ultrafiltration. *BioTechniques.* 14(4). 656-659.

88. Walker, J. M. 1994. Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols. Humana Press Inc. Totowa, NJ.

89. Udaka, K., K-H. Wiesmuller, S. Kienle, G. Jung, & P. Walden. 1995. Decrypting the structure of major histocompatibility complex class I-restricted cytotoxic T lymphocyte epitopes with complex peptide libraries. *J. Exp. Med.* 181: 2097-2108.

90. Tsai, S-L., M-H. Chen, C-T. Yeh, C-M. Chu, A-N. Lin, F-H. Chior, T-H. Chang, & Y-F. Liaw. 1996. Purification and characterization of a naturally processed hepatitis B virus peptide recognized by CD8+ cytotoxic T lymphocytes. *J. Clin. Invest.* 97: 577-584.

91. Uenaka, A., T. Ono, T. Akisawa, H. Wada, T Yasuda, & E. Nakayama. 1994. Identification of a unique antigen peptide pRL1 on BALB/c RL1 leukemia recognized by cytotoxic T lymphocytes and its relation to the Akt oncogene. *J. Exp. Med.* 180: 1599-1607.

92. Udaka, K., T. J. Tsomides, & H. N. Eisen. 1992. A naturally occurring peptide recognized by alloreactive CD8+ cytotoxic T lymphocytes in association with a class I MHC protein. *Cell.* 69: 989-998.

93. Schloot, N. C. & B. O. Roep. 1997. Islet antigen-specific T cell clones in autoimmune diabetes: from mice to men. *Diabetes/Metabolism Reviews.* 13(3): 127-138.

94. Meadows, L., W. Wang, J. M. M. den Haan, E. Blokland, C. Reinhardus, J. W. Drijfhout, J. Shabanowitz, R. Pierce, A. I. Agulnik, C. E. Bishop, D. F. Hunt, E. Goulmy, & V. H. Engelhard. 1997. The HLA-A*0201-restricted H-Y antigen contains a posttranslationally modified cysteine that significantly affects T cell recognition. *Immunity.* 6: 273-281.

95. Skipper, J. C. A., R. C. Hendrickson, P. H. Gulden, V. Brichard, A. van Pel, Y. Chen, J. Shabanowitz, T. Wolfel, C. L. Slingluff, Jr., T. Boon, D. F. Hunt, & V. H. Engelhard. 1996. An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. *J. Exp. Med.* 183: 527-534.

96. Falk, K., O. Rotzschke, S. Stevanovic, G. Jung, & H-G Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature*. 351: 290.

97. Gill, R. F., J-P. Abastado, & W-Z Wei. 1994. Systematic identification of H-2K^d binding peptides and induction of peptide specific CTL. *J. Immunol. Meth.* 176: 245-253.

98. Engelhard, V. H. 1994. Structure of peptides associated with MHC class I molecules. *Curr. Op. Immunol.* 6: 13-23.

99. Kageyama, S., T. J. Tsomides, Y. Sykulev, & H. N. Eisen. 1995. Variations in the number of peptide-MHC class I complexes required to activate cytotoxic T cell responses. *J. Immunol.* 154: 567-576.

100. Viola, A. & A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science*. 273: 104-106

101. Wallny, H. J., K. Deres, S. Faath, G. Jung, A. van Pel, T. Boon, & H. G. Rammensee. 1992. Identification and quantification of a naturally presented peptide as recognized by cytotoxic T lymphocytes specific for an immunogenic tumor variant. *Int. Immunol.* 4(10): 1085-1090.

102. Hiemstra, H. S., P. A. van Veelen, N. C. Schloot, A. Geluk, K. E. van Meijgaarden, S. J. M. Willemen, J. A. M. Leunissen, W. E. Benckhuijsen, R. Amons, R. R. P. de Vries, B. O. Roep, T. H. M. Ottenhoff, & J. W. Drijfhout. 1998. Definition of natural T cell antigens with mimicry epitopes obtained from dedicated synthetic peptide libraries. *J. Immunol.* 161: 4078-4082.

103. Blake, J., J. V. Johnston. K. E. Hellstrom, H. Marquardt, & L. Chen. 1996. Use of combinatorial peptide libraries to construct functional mimics of tumor epitopes recognized by MHC class I-restricted cytolytic T lymphocytes. *J. Exp. Med.* 184: 121-130.

104. Hiemstra, H. S., G. Duinkerken, W. E. Benckhuijsen, R. Amons, R. R. P. de Vries, B. O. Roep, & J. W. Drijfhout. 1997. The identification of CD4+ T cell epitopes with dedicated synthetic peptide libraries. *Proc. Natl. Acad. Sci. USA*. 94: 10313-10318.

105. Koop, B. F., L. Rowen, K. Wang, C. L. Kuo, D. Seto, J. A. Lenstra, S. Howard, W. Shan, P. Deshpande, & L. Hood. 1994. The human T-cell receptor TCRAC/TCRDC (Cα/Cδ) region: organization, sequence, and evolution of 97.6 kb of DNA. *Genomics*. 19: 478-493.

106. Arden, B., S. P. Clark, D. Kabelitz, & T. W. Mak. 1995. Mouse T cell receptor variable gene subfamilies. *Immunogenetics*. 42: 501-530.

107. Serreze, D. V., E. H. Leiter, G. J. Christianson, D. Greiner, & D. C. Roopenian. 1994. Major histocompatibility complex class I-deficient NOD-β2m^{null} mice are diabetes and insulitis resistant. *Diabetes*. 43: 505-509.

108. Shastri, N. 1995. Single T cell probes for antigen/MHC expression. *Curr. Op Immunol*. 7: 258-262.

109. Sanderson, S. & N. Shastri. 1993. LacZ inducible, antigen/MHC-specific T cell hybrids. *Int. Immunol*. 6(3): 369-376.

110. Karttunen, J., S. Sanderson, & N. Shastri. 1992. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. *Proc. Natl. Acad. Sci. USA*. 89: 6020-6024.

111. Daniel, C., S. Horvath, & P. M. Allen. 1998. A basis for alloreactivity: MHC helical residues broaden peptide recognition by the TCR. *Immunity*. 8: 543-552.

112. Stryhn, A., L. O. Pedersen, T. Romme, C. B. Holm, A. Holm, & S. Buus. 1996. Peptide binding specificity of major histocompatibility complex class I resolved into an array of apparently independent subspecificities: quantitation by peptide libraries and improved prediction of binding. *Eur. J. Immunol*. 1996. 26: 1911-1918.

113. DiBrino, M., K. C. Parker, J. Shiloach, M. Knierman, J. Lukszo, R. V. Turner, W. E. Biddison, & J. E. Coligan. 1993. Endogenous peptides bound to HLA-3 possess a specific combination of anchor residues that permit identification of potential antigenic peptides. *Proc. Natl. Acad. Sci. USA*. 90: 1508-1512.

114. Kramer, A., T. Keitel, K. Winkler, W. Stocklein, W. Hohne, & J. Schneider-Mergener. 1997. Molecular basis for the binding promiscuity of an anti-p24 (HIV-1) monoclonal antibody. *Cell*. 91: 799-809.

115. Hemmer, B., M. Vergelli, B. Gran, N. Ling, P. Conlon, C. Pinilla, R. Houghten, H. F. McFarland, & R. Martin. 1998. Cutting edge: predictable TCR antigen recognition based on peptide scans leads to the identification of agonist ligands with no sequence homology. *J. Immunol*. 160: 3631-3636.

116. Soares, L. R. B., P. L. Orr, M. R. Garovoy, & G. Benichou. 1998. Differential activation of T cells by natural antigen peptide analogues: influence on autoimmune and alloimmune in vivo T cell responses. *J. Immunol*. 160: 4768-4775.

117. Vanderlugt, C. J. & S. D. Miller. 1996. Epitope spreading. *Curr. Op. Immunol.* 8: 831-836.

118. Kawasaki, E., L. Yu, M. J. Rewers, J. C. Hutton, & G. S. Eisenbarth. 1998. Definition of multiple ICA512/phogrin autoantibody epitopes and detection of intramolecular epitope spreading in relatives of patients with type 1 diabetes. *Diabetes.* 47: 733-742.

119. Elliott, J. F., H-Y. Qin, S. Bhatti, D. K. Smith, R. K. Singh, T. Dillon, J. Lauzon, & B. Singh. 1994. Immunization with the larger isoform of mouse glutamic acid decarboxylase prevents autoimmune diabetes in NOD mice. *Diabetes.* 43: 1494-1499.

120. Tian, J., M. A. Atkinson, M. Clare-Salzler, A. Herschenfeld, T. Forsthuber, P. V. Lehmann, & D. L. Kaufman. 1996. Nasal administration of glutamate decarboxylase peptides induces Th2 responses and prevents murine insulin-dependent diabetes. *J. Exp. Med.* 183: 1561-1567.

121. Duarte, E. A., G. Eberl, & G. Corradin. 1996. Specific tolerization of active cytolytic T lymphocyte responses in vivo with soluble peptides. *Cell. Immunol.* 169: 16-23.

F. APPENDIX I: List of IDDM-Associated Autoantigens

Antigen	Antibody Response	T-Cell Response
Proinsulin/Insulin	➢ Found in 60% of pre-diabetics patients	➢ Recognized by peripheral and islet-derived T-cells ➢ T-cell responsiveness inversely related to levels of anti-insulin antibody
GAD65 & GAD67	➢ Present in 70-80% pre- and recent onset IDDM patients and NOD mice ➢ Present in other autoimmune diseases	➢ Peripheral T-cell response observed in recent onset IDDM patients and NOD mice
Carboxypeptidase H	➢ Found in 10% of pre-IDDM patients	➢ Recognized by peripheral T-cells in humans
Islet Cell Antigen (ICA) 69	➢ Detected in 43% of pre-IDDM patients ➢ Cross-reactivity observed between anti-ICA69 and bovine serum albumin	➢ Peripheral T-cell response observed in recent onset IDDM patients
Tyrosine Phosphatase IA2 (ICA512)	➢ Present in 62% pre- and recent onset IDDM patients	➢ Peripheral T-cell response in recent onset IDDM patients
Phogrin (1A2β, 37 kD)	➢ Present in 62% pre- and recent onset IDDM patients	➢ Peripheral T-cell response observed in recent onset IDDM patients
Heat Shock Protein (hsp)-60	➢ Observed in pre-diabetic NOD mice	➢ Recognized by CD4+ T-cells in the NOD mouse ➢ T-cells reactive with the hsp60 peptide p277 transfer diabetes in NOD mouse

Glima38	➢ Present in 19% of recent onset IDDM patients ➢ Most of these patients are negative for anti-GAD or anti-IA2 antibodies	➢ Peripheral T-cell response observed in humans
non-GAD 64 kD Antigen	➢ Islet-associated autoantibodies detected in 80% pre-IDDM patients	➢ Unknown
Peripherin	➢ Found in NOD mice	➢ Peripheral T-cell response observed in NOD mice
Imogen (38 kD)	➢ In humans, anti-38 kD protein antibodies detected which may recognize Imogen	➢ Present in sera of recent onset IDDM patients
Ganglioside GM2-1	➢ Detected in 80% of pre-IDDM patients and NOD mice	➢ Unknown
Ganglioside GT3	➢ Observed in humans	➢ Unknown
β-granule antigen	➢ Unknown	➢ Pathogenicity of islet-derived T-cells in the NOD mouse suggest the existence of an antigen which has been localized to the β-cell granules
Islet-associated Sulphatide Antigen	➢ Observed in humans	➢ Unknown
52 kD Protein	➢ Observed in humans and the NOD mouse	➢ Unknown
155 kD Protein	➢ Observed in humans	➢ Unknown
Chymotrypsinogen-like 30 kD pancreatic antigen	➢ Observed in humans	➢ Unknown

G. APPENDIX II: Results of the Search for NRP-homologues

Query Sequence	Protein	Organism	Target Sequence
KYNKANWFL	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. lactis*	KYEKSNWFM
	putative protein	*A. thaliana*	CYNKATWFG
	phospholipase A2 isozyme PA-15	mulga snake	AYNKANWNI
	phospholipase A2 isozyme PA-13	mulga snake	AYNKANWNI
	phospholipase A2 isozyme PA-10A	mulga snake	AYNKANWNI
	MG350	*M. genitalium*	LVNKANWFD
	unknown	*M. genitalium*	LVNKANWFD
	ferredoxin oxidoreductase proprotein	*T. vaginalis*	KYNKAEWLN
KYNKANWFI	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. lactis*	KYEKSNWFM
	phospholipase A2 isozyme PA-15	mulga snake	AYNKANWNI
	phospholipase A2 isozyme PA-13	mulga snake	AYNKANWNI
	phospholipase A2 isozyme PA-10A	mulga snake	AYNKANWNI
	putative protein	*A. thaliana*	CYNKATWFG
	ferredoxin oxidoreductase proprotein	*T. vaginalis*	KYNKAEWLN
	MG350	*M. genitalium*	LVNKANWFD
	unknown	*M. genitalium*	LVNKANWFD
KYNKANWFV	aminopeptidase C	*L. lactis*	KYEKSNWFM
	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	putative protein	*A. thaliana*	CYNKATWFG
	phospholipase A2 isozyme PA-15	mulga snake	AYNKANWNI
	phospholipase A2 isozyme PA-13	mulga snake	AYNKANWNI
	phospholipase A2 isozyme PA-10A	mulga snake	AYNKANWNI
	MG350	*M. genitalium*	LVNKANWFD
	T04F8.2	*C. elegans*	KYNPEHWFV

	RNA polymerase	Murine hepatitis	IFNRCNWFV
	ferredoxin oxidoreductase	*T. vaginalis*	KYNKAEWLN
	proprotein		
	unknown	*M. genitalium*	LVNKANWFD
KYNKXNWFL	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. lactis*	KYEKSNWFM
KYNKXNWFI	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. lactis*	KYEKSNWFM
KYNKXNWFV	aminopeptidase C	*L. lactis*	KYEKSNWFM
	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	T04F8.2	*C. elegans*	KYNPEHWFV
KYNKXNXFL	no sequence found	n/a	n/a
KYNKXNXFI	no sequence found	n/a	n/a
KYNKXNXFV	no sequence found	n/a	n/a
KYNKXXWFL	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
KYNKXXWFI	no sequence found	n/a	n/a
KYNKXXWFV	hypothetical protein	*P. horikoshii*	KYSKEVWFV
KYNKXXXFL	no sequence found	n/a	n/a
KYNKXXXFI	no sequence found	n/a	n/a
KYNKXXXFV	no sequence found	n/a	n/a
XYNKXNWFL	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. lactis*	KYEKSNWFM
XYNKXNWFI	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
XYNKXNWFV	no sequence found	n/a	n/a
KYXKXNWFL	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. lactis*	KYEKSNWFM
KYXKXNWFI	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. lactis*	KYEKSNWFM
KYXKXNWFV	aminopeptidase C	*L. lactis*	KYEKSNWFM
	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
XYXKXNWFL	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
XYXKXNWFI	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
XYXKXNWFV	no sequence found	n/a	n/a
XYXKXXXFL	no sequence found	n/a	n/a
XYXKXXXFI	no sequence found	n/a	n/a
XYXKXXXFV	no sequence found	n/a	n/a
KFNKANWFL	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. helveticus*	KFEKSNWFF
	aminopeptidase C	*L. lactis*	KYEKSNWFM

	DNA methyltransferase	*H. pylori*	KFNENNWFE
	ATP synthase protein 8	*A. quadrimaculatus A*	KFNKLNWKW
	unknown	*M. genitalium*	LVNKANWFD
	MG350	*M. genitalium*	LVNKANWFD
	RNA polymerase	Murine hepatitis	IFNRCNWFV
KFNKANWFI	aminopeptidase C	*L. helveticus*	KFEKSNWFF
	RNA polymerase	Murine hepatitis	IFNRCNWFV
	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	DNA methyltransferase	*H. pylori*	KFNENNWFE
	MG350	*M. genitalium*	LVNKANWFD
	unknown	*M. genitalium*	LVNKANWFD
	aminopeptidase C	*L. lactis*	KYEKSNWFM
	ATP synthase protein 8	*A. quadrimaculatus A*	KFNKLNWKW
	C23D3.15	*S. pombe*	RFNKSNFFI
KFNKANWFV	RNA polymerase	Murine hepatitis	IFNRCNWFV
	aminopeptidase C	*L. helveticus*	KFEKSNWFF
	DNA methyltransferase	*H. pylori*	KFNENNWFE
	aminopeptidase C	*L. lactis*	KYEKSNWFM
	ATP synthase protein 8	*A. quadrimaculatus A*	KFNKLNWKW
	MG350	*M. genitalium*	LVNKANWFD
	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	unknown	*M. genitalium*	LVNKANWFD
KFNKXNWFL	DNA methyltransferase	*H. pylori*	KFNENNWFE
	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. helveticus*	KFEKSNWFF
	ATP synthase protein 8	*A. quadrimaculatus A*	KFNKLNWKW
	aminopeptidase C	*L. lactis*	KYEKSNWFM
KFNKXNWFI	DNA methyltransferase	*H. pylori*	KFNENNWFE
	aminopeptidase C	*L. helveticus*	KFEKSNWFF
	RNA polymerase	Murine hepatitis	IFNRCNWFV
	ATP synthase protein 8	*A. quadrimaculatus A*	KFNKLNWKW
	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. lactis*	KYEKSNWFM
KFNKXNWFV	DNA methyltransferase	*H. pylori*	KFNENNWFE
	RNA polymerase	Murine hepatitis	IFNRCNWFV
	aminopeptidase C	*L. helveticus*	KFEKSNWFF
	ATP synthase protein 8	*A. quadrimaculatus A*	KFNKLNWKW
	aminopeptidase C	*L. lactis*	KYEKSNWFM
	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
KFNKXNXFL	no sequence found	n/a	n/a

KFNKXNXFI	no sequence found	n/a	n/a
KFNKXNXFV	no sequence found	n/a	n/a
KFNKXXWFL	no sequence found	n/a	n/a
KFNKXXWFI	no sequence found	n/a	n/a
KFNKXXWFV	no sequence found	n/a	n/a
KFNKXXXFL	no sequence found	n/a	n/a
KFNKXXXFI	no sequence found	n/a	n/a
KFNKXXXFV	no sequence found	n/a	n/a
XFNKXNWFL	RNA polymerase	Murine hepatitis	IFNRCNWFV
XFNKXNWFI	RNA polymerase	Murine hepatitis	IFNRCNWFV
XFNKXNWFV	RNA polymerase	Murine hepatitis	IFNRCNWFV
KFXKXNWFL	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. helveticus*	KFEKSNWFF
	aminopeptidase C	*L. lactis*	KYEKSNWFM
KFXKXNWFI	aminopeptidase C	*L. helveticus*	KFEKSNWFF
	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
	aminopeptidase C	*L. lactis*	KYEKSNWFM
KFXKXNWFV	aminopeptidase C	*L. helveticus*	KFEKSNWFF
	aminopeptidase C	*L. lactis*	KYEKSNWFM
	aminopeptidase C	*S. thermophilus*	KYEKSNWFL
XFXKXNWFL	no sequence found	n/a	n/a
XFXKXNWFI	no sequence found	n/a	n/a
XFXKXNWFV	no sequence found	n/a	n/a
XFXKXXXFL	no sequence found	n/a	n/a
XFXKXXXFI	no sequence found	n/a	n/a
XFXKXXXFV	no sequence found	n/a	n/a