THERAPEUTIC ALTERATION OF T CELL DEVELOPMENT: MODULATING DIABETOGENIC AND REGULATORY T CELLS IN THE TREATMENT OF TYPE 1 DIABETES MELLITUS

by

Todd Christopher White

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entitled Therapeutic Alteration of T cell Development: Modulating Diabetogenic and Regulatory T cells in the Treatment of Type 1 Diabetes Mellitus

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Dominick DeLuca	11/19/04 date
John J. Marchalonis	11/19/04 date
Samuel F. Schlueter	11/19/04 date
Douglas F. Larson	11.19.04 date
John W. Bloom	Nov 19, 2004 date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director: Dominick DeLuca — 11/19/04 date

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SIGNED: 

Todd C. White

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This dissertation is dedicated

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throughout his life to find a cure for Type 1 Diabetes.

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I want to thank Dr. DeLuca for the respect he has shown me

and hope that I can honor his confidence in me

by excelling as a scientist and a professional.

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PREAMBLE

The field of immunology has been considered a burgeoning branch of biology since Edward Jenner used immunization with cowpox to protect against human smallpox in 1796. Although this was one of the first modern uses of immunization (there is also evidence that early Egyptians understood the benefits of immunization), it was several decades before the processes that conveyed protection through immunization were recognized. Now, in modern times, immunology has become an explosive field where new insights into the immune system are being discovered every day and the phrase "Immunology" covers a vast amount of information. That being said, the research presented in this dissertation is primarily focused on the concepts of T cell development, autoimmunity, and T cell responses in the periphery. Although great care has been taken to explain the concepts and theories behind this research, some of the detailed mechanisms of interaction and T cell activation in the immune system are not discussed. For those who are interested additional information is available from many sources, including the references listed in this text. Those interested in learning more about basic immunology can find good background information in text such as: *Immunobiology* by Charles A. Janeway, Paul Travers, Mark Walport, Mark Shlomchik or *Kuby Immunology* by Richard A. Goldsby, Thomas J. Kindt and Barbara A. Osborne; which are the major immunology texts at the time of this publication.

ABSTRACT

In this dissertation we investigate the role of avidity in the T cell selection process by examining the impact of signal modulation on T cell and/or NKT cell development. Projects discussed herein (including peptide, anti-CD1d, and hydrocortisone (HC) therapy) examine how changes in avidity can be used to explore potential therapies for Type 1 diabetes mellitus (T1DM).

In the case of peptide therapy, we find that fetal thymic organ culture (FTOC), treated with exogenous diabetes related GAD peptides, lose their ability to generate T cell responses to GAD treatment peptides. Also, peptide therapy is shown to inhibit T1DM *in vitro* (ivT1DM) and *in vivo*. The abnormally high level of GAD peptides that are presented during peptide therapy treatment are thought to increase avidity between peptide specific T cells and selecting cells during thymic education, leading to increased negative selection of those T cells.

In the case of anti-CD1d, FTOC from C57BL/6 (B6) and non-obese diabetic (NOD) mice, when treated with 10 μg/mL of anti-CD1d, show divergent responses to treatment. In response to anti-CD1d, "normal" B6 FTOC shows decreased T cell development and NKT production. Conversely, "poor signaling" NOD mice show no major impact on general T cell development but instead show increases in NKT cell production. Also, treatment with anti-CD1d is shown to inhibit diabetes in our ivT1DM model. These effects are thought to be due to increases in avidity generated through anti-CD1d related increased TCR expression. Changes in avidity caused by anti-CD1d treatment are thought to generate increased negative selection in B6 FTOC, while the

same avidity increases are thought to increase positive selection (without increasing negative selection) in "poor signaling" NOD FTOC.

In the case of HC treatment, B6 FTOC treated with HC show changes in T cell yield, maturity, and TCR Vβ usage. Research with HC indicates that signal inhibitors have the capacity to change T cell development in a dose and time dependent manner. Based on this work, selection signal inhibitors or enhancers may have the capacity to change T cell development in a fashion that decreases autoimmune T cells and/or enhances regulatory NKT cell development.

CHAPTER I: INTRODUCTION

Problem Defined

The work described in this dissertation was performed in order to determine if it is possible to change T cell development in a way that would be beneficial for those predisposed for autoimmune diseases. Although generally ignored until the last 20 years, autoimmune diseases have a major impact on our health as individuals and as a nation. More than 1 million Americans have been diagnosed with T1DM, which is an autoimmune form of diabetes. Those diagnosed with T1DM tend to die 20 years sooner then non-diabetic individuals. Type 1 diabetics are 2-4 times more likely to have heart/vascular disease and stroke. Sixty to seventy percent of diabetics suffer from mild to severe nerve damage. These vascular and neural problems can lead to larger problems, which is why more than half of the leg amputations in the United States are due to diabetes. Diabetes can also lead to blindness and renal failure. Perhaps this is why 1 of every 7 US health care dollars is spent on diabetes or diabetes related complications. That being said, T1DM is only one of many autoimmune diseases. When combined, autoimmune diseases have a massive impact on healthcare worldwide.

Work presented here is primarily focused on modifying T cell development to (1) eliminate autoreactive diabetogenic T cells during T cell development or (2) to increase the number of immune regulatory NKT cells, which can also influence T cell development and control autoreactive responses in the immune periphery. Although the majority of this work is focused on T1DM, the techniques used in these research projects are potentially applicable to several other types of autoimmune diseases. With the

majority of autoimmune diseases linked to MHC genes, which are responsible for the interaction and, indirectly, the activation of autoreactive T cells, techniques discussed in this dissertation could potentially lend themselves to the treatment of any T cell dependent autoimmune disease. For example, the peptide therapy techniques described in this dissertation may be applied to other autoimmune diseases simply by using peptide antigens that are associated with a particular autoimmune disease. Alternatively, the projects using anti-CD1d and glucocorticoids may be directly applicable to other autoimmune diseases, since they can induce an overall general change in T cell and NKT cell development. The specific aims for each project are briefly discussed below.

Specific Aims

Specific Aim 1. To determine the capacity of glutamic acid decarboxylase (GAD) associated peptides to generate proliferative responses in NOD mice. In addition, we will also examine the ability of these peptides to block T1DM through administration to our in vitro T1DM model (ivT1DM). Combinations of these peptides will then be tested in order to evaluate their collective ability to induce tolerance and inhibit ivT1DM. Single peptides or peptide combinations will then be tested in NOD mice to determine *in vivo/in utero* tolerance efficacy. These studies will provide important information on the efficacy of using peptides as a preventative modality for T1DM in humans. Hypothesis: Increased presentation of GAD peptides during T cell development will cause T cells specific for those GAD peptides to experience increased avidity and negative selection, leading to the inhibition of T1DM.

Specific Aim 2. To analyze the production of NKT regulatory cells, including DN and/or SP4 ($CD4^+CD8^-$) $\alpha\beta^{TCR+}$ $DX5^+$ or $CD1d^{tet+}$ NKT cells, in NOD FTOC after the addition of anti-CD1d. We will also attempt to analyze the impact of this treatment on the progression of T1DM. NOD FTOC will be treated with anti-CD1d monoclonal antibody in order to effect the production of NKT cells. NKT cell population changes in the cultures will be analyzed using flow cytometry. If treatment expands NKT populations, therapy will target optimizing anti-CD1d based enhancement of NOD NKT cell populations. Similarly treated FTOC will then be tested in our *iv*T1DM system to determine if therapy can mitigate T1DM. Hypothesis: If monoclonal anti-CD1d has the capacity to cause changes in the cell-to-cell signaling during NKT development then it will have the capacity to positively or negatively impact thymic NKT cell production, depending on the type of signal generated.

Specific Aim 3. To determine if glucocorticoids, specifically hydrocortisone, can impact the production of T cells and/or modulate the ratio of T1DM associated regulatory NKT cells to phenotypically mature T cells produced by the thymus. Through FTOC, hydrocortisone and/or hydrocortisone blocking agents will be investigated to determine if the effects of inhibitory glucocorticoids impact T cell development. Optimal dosage and time of administration will be established for each type of modulation. Additionally, the overall production of phenotypically mature T cells and impact of T1DM associated regulatory NKT cells will be investigated through flow cytometry. The future goal of this

study is to determine if glucocorticoids can impact T cell development in a manner that can prevent T1DM. Hypothesis: If HC can cause an inhibition of internal signaling in T cells, as seen in peripheral T cells, then HC will have the capacity to negatively impact signal-dependent T cell production.

Literature Review

In the simplest terms, the field of Immunology can be divided into two bodies of work: Developmental Immunology and Functional Immunology. Developmental immunology focuses on the generation of immune, and immune related, cell types from uncommitted pluripotent hematopoietic stem cells to their end stage cell types. In contrast, functional immunology examines the processes used to generate both the innate and adaptive immune responses that are used by the body to combat disease and infection. Often, research in subfields of immunology, such as Cancer/Tumor Immunology and Autoimmunity, require detailed knowledge of both developmental and functional immunology. To that point, the work presented in this dissertation focuses on the processes of T cell development and peripheral T cell functions that are thought to lead to the generation of autoreactive responses in T1DM. Specifically, this discussion will begin with the examination of one of the primary genes/molecules associated with T1DM, the major histocompatibility complex.

Peptide Therapy

The Major Histocompatibility Complex: A Type 1 Diabetes Mellitus Associated Gene

The major histocompatibility complex (MHC), originally referred to as immune response (IR) complex, is a polymorphic gene complex that encodes molecules that are crucial to the function of the immune system. MHC molecules encoded in this gene complex play a major role in the transplant rejection found in mammals and are key to antigen presentation that goes on during typical immune responses. MHC molecules

function during an immune response by presenting amino acid fragments (i.e. peptides) from proteins found in the body. These peptides may be from native proteins or from foreign antigen. There are two main types of MHC molecules: Class I and Class II. Class I MHC are used by all nucleated cells in the body to present peptides of intracellular cytoplasmic proteins that have been broken down by the proteasome. This class I MHC presentation occurs in an effort to identify cells that have viral infections or, in some cases, cancer. If there is a viral infection, or a cell has become cancerous, "foreign" peptides (including mutated self peptides) will be presented in class I MHC and, in theory, the cell will be destroyed by activated $CD4^{-}CD8^{+}$ (SP8) cytotoxic T cells that have T cell receptors (TCR) that recognize these peptides.

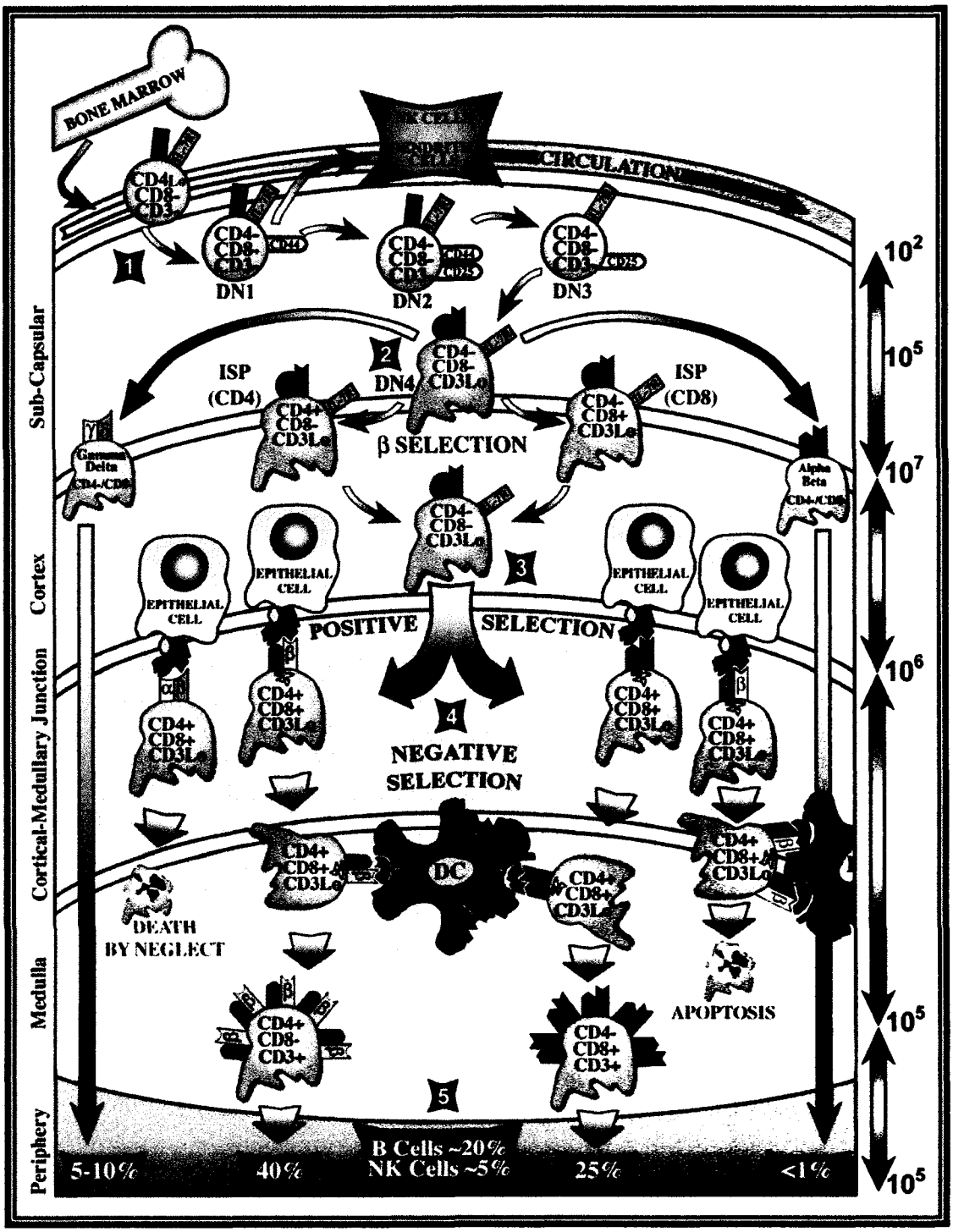
Class II MHC are used by antigen presenting cells (APC) to present peptides from proteins that have been brought in from the extracellular environment by phagocytosis or pinocytosis. This process occurs to allow the immune system to recognize foreign antigen (e.g. pathogens) that may be present in the extracellular environment of the body (i.e. the blood and interstitial fluids). Once the foreign antigen is broken down, and loaded into the class II MHC, these peptides are then subject to examination by the TCR of $CD4^{+}CD8^{-}$ (SP4) T cells. If the SP4 T cells can recognize the antigen they go on to activate the immune system in an appropriate fashion (i.e. a T cell based cellular response, a B cell based humoral response, or a combination of both). The type of response generated is based on the type of antigen, the amount of antigen, and the area of the body where the antigen was acquired.

In the case of T cell development, immature T cells differentiating in the thymus interact with the MHC on thymic epithelium, or hematopoietically-derived antigen presenting cells (APC), through their TCR as an integral part of development (1, 2). In order to complete development, T cells must undergo the processes of positive and negative selection; this is also termed "thymic education". The process of positive selection begins after the developing pre-T cell has first undergone rearrangement of the β and α chain of the TCR[a]. This process attempts to establish if the newly rearranged TCR can bind and recognize (i.e. receive a strong enough binding signal from) the self-MHC bound with self-peptide found on the surface of thymic cortical epithelial cells. This MHC/TCR interaction will become the major mechanism of specific pathogen recognition (i.e. the "adaptive" immune response) in the body. If the developing T cell is able to recognize class I or class II MHC it has passed "positive selection" and can continue development, moving from the cortex of the thymus to the cortical-medullary junction where it will be subjected to the process of negative selection. To prevent autoimmune responses, those T cells possessing TCR that are highly specific for self-antigens are eliminated by the process of negative selection (3, 4). During "negative selection", T cells interact with APC in the thymus. Those T cells that receive too strong a signal when binding to a self-MHC loaded with self-peptide will be anergized (i.e. enter a state of stasis) and eventually go through apoptosis, also known as programmed cell death (5). Although there are exceptions (6, 7), negative selection is thought to be mediated through a hematopoietically-derived APC (primarily through dendritic cells), and positive selection takes place through thymic epithelial cells. This entire process of

thymic education ensures that the T cells that mature and leave the thymus are all capable of recognizing peptides in the context of MHC but also, more importantly, that none of these T cells are capable of activating and responding against self. In a normal individual this process is a safe guard against autoimmune responses. For a more detailed illustration of T cell development see Figure 1.

During this process of "thymic education", appropriate presentation of self-antigens is crucial to self-tolerance. It has been shown that antigens that are inefficiently presented during thymic education can later lead to autoreactive T cell responses because of a lack of tolerance (8). This fact, coupled with evidence that activation induced cell death (AICD) of mature T cells requires higher levels of stimulation than is needed to induce T cell proliferation (9), indicates that cells that escaped negative selection may be able to respond to the higher levels of self-peptide that are presented during immune responses in the periphery, without being subject to peripheral tolerance. However, if autoreactive T cells do escape negative selection there are other mechanisms, such as regulatory T and NKT cells, used by the immune system to control autoimmune responses in the periphery. Some of these mechanisms will be discussed later.

Although alternate regulatory mechanisms of autoimmunity exist, it is still essential that thymic selection limit the number of autoreactive T cells that are generated during T cell development. From work generated over the last 20 years we know that signal strength provided by adequate peptide presentation is essential for appropriate immune function. In fact, when mice are incapable of loading their own class I specific peptides



Picture and Text by Aaron J. Middlebrook. Copyright 2004. From *NICOTINE AND TNF ALPHA, MODULATORS OF T CELL SIGNALING- EFFECTS ON T CELL DEVELOPMENT IN FETAL THYMUS ORGAN CULTURE (a dissertation, University of Arizona)*

Figure 1. T Cell Development. Hematopoietically derived precursors seed the thymus (1) and transition through several $CD4^{-}CD8^{-}$ (Double Negative (DN)) intermediate stages before undergoing b selection, which ensures successful TCR b chain rearrangement (2). Several rounds of proliferation characterize the early stages of development as these cells proceed through the immature single positive (ISP) stage and on to positive selection (3), which is mediated by cortical epithelial cells. Positive selection establishes an MHC-restricted T cell repertoire and is closely associated with the lineage commitment of thymocytes to either the SP4 or SP8 subset. Negative selection is carried out by dendritic cells (DC) residing within the cortical-medullary junction of the thymus (4) and safeguards against the development of high affinity and potentially autoreactive thymocytes. Ultimately these processes give rise to a diverse T cell population made up of $\gamma\delta$ T cells, SP4 $\alpha\beta$ T cells, SP8 $\alpha\beta$ T cells and DN $\alpha\beta$ T cells and NK cells (5). The thymus also produces a small population of B cells.

during selection, such as mice with their peptide transporter TAP^b genes (10) or their β-2 microglobulin genes deleted (5), their T cell development is severely affected. By modulating the levels of peptide added during T cell development in TAP or β-2 microglobulin knock out mice with transgenic peptide specific TCR, researchers were able to show that the positive or negative selection of peptide-specific $CD8^+$ T cells was solely dependent on the concentration of peptide used. Interestingly, research has shown that even a single amino acid change in the sequence of a nominal peptide used during selection can change the ability of T cells to be positively selected (11, 12). In this work it was shown that as long as the new peptide sequence could bind to the MHC then positive selection would be effected in a fashion that was independent of the MHC/peptide binding strength. This highlights the importance of TCR-MHC/peptide complex specificity vs. peptide/MHC binding strength during the process of positive and negative selection. These results have led to the proposal of a model in which positive and negative selection of T cells is regulated by (1) the total affinity of the TCR for the MHC/peptide complex; (2) the total number of TCR on T cell; (3) the affinity of the peptide for the presenting MHC molecule (for binding purposes) (4); and the total number of MHC-peptide complexes present on the selecting thymic stromal cell or APC (12). This concept has been called the total avidity of cell-cell interaction. Studies have shown that even the cell type presenting the antigen may not be as important as the total avidity of the cell-cell interaction (6). A graphic depiction of role of avidity in selection can be found in Figure 2.

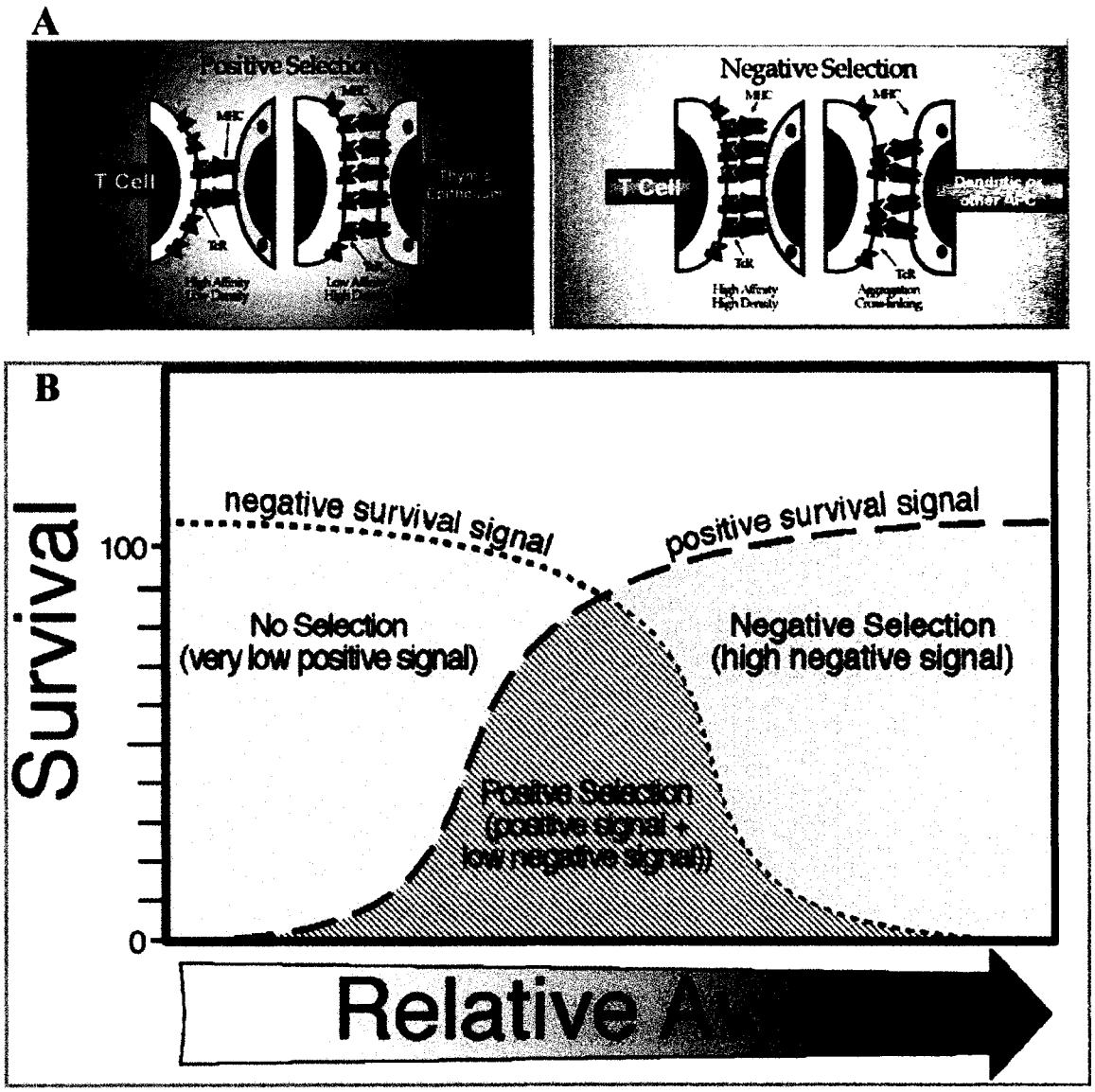


Figure 2. Requirements and Impact of Avidity on T cell Selection. Avidity of cell-cell interaction is essential for the thymic education of T cells. (A) depicts the basic requirements for each stage of selection. For a cell to pass positive selection, and have a chance at becoming a mature T cell, the cell requires a certain minimum avidity signal, which can be obtained through either high-affinity/low-density interactions or through high-density/low-affinity interactions. Once a cell has passed positive selection the

potential T cell is then subject to the process of negative selection. Negative selection occurs to remove autoreactive T cells, so cells that form high-affinity/high-density interactions or cells that have TCR, or other molecules, that form high-density aggregates with selecting cells are removed from the T cell repertoire. (B) shows an avidity based depiction of the selection process that leads to the formation of a functional, but not autoreactive T cell repertoire. As avidity increases, the percent of cells that pass positive selection increases. The process of negative selection also increases as avidity increases, causing cell survival to decrease. Since the avidity requirements for positive and negative selection are distinct, with a lower avidity for positive selection and a higher avidity for negative selection, these processes generate a T cell repertoire that is capable of responding to foreign peptides in the periphery, while eliminating the capacity for autoimmune responses.

The processes of positive and negative selection, and the role of the MHC in these processes, have been of major interest to the field of developmental immunology since the late 1970s. In more recent times, the process of T cell selection has been more closely examined due to its perceived role in the development of autoimmune diseases. In regards to T1DM, the non-obese diabetic $(NOD)^c$ mouse model, used by our lab and others, has many genetic defects that correlate with the human model of the disease. The most apparent and most investigated of these genetic similarities are the structural abnormalities found in the class II MHC of both species. The NOD mouse has a single class II MHC allele, I-A^{g7}, with a β chain that is structurally nearly identical to the DQβ^8 allele that is found to have a high correlation with T1DM in humans. These two class II MHC share a very significant single point amino acid change in their β chain versus their non-T1DM associated homologues (I-A^b in mouse and DQβ^1 in humans). These and other T1DM susceptible MHC have a valine, serine, or alanine at position 57 instead of the typical aspartic acid that normally helps to form a salt bridge between the α and β chain of the typical class II MHC (Fig. 3). The lack of a salt bridge in the I-A^{g7} and DQβ^8 molecules leads to a more open conformation in the binding cleft where peptides are loaded for presentation, leading to poor peptide binding (13). Several binding studies have shown that these MHC molecules bind certain peptides poorly, and thus present poorly (14, 15). Indeed, since the diabetogenic class II MHC I-A^{g7} is "unstable" and binds many peptides poorly, it may not be able to bind enough of the self peptides that generate autoimmune responses in T1DM to induce the negative selection of diabetogenic T cells. An alternate theory is based on the poor tetramerization of I-A^{g7}

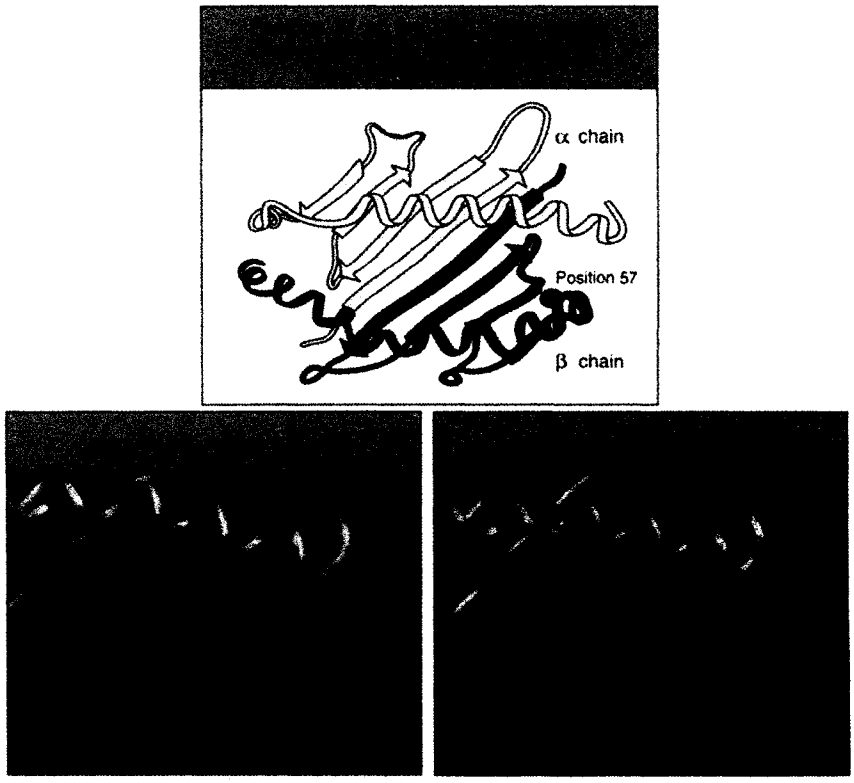


Figure 3. Role of the Class II MHC in Type I Diabetes Mellitus. DQβ1*0302 (a.k.a. DQβ8) and the I-Ag7 MHC of NOD mice share a common amino acid point change at position 57 (from aspartic acid to alanine or serine, respectively) that leaves the MHC β chain unable to complete the salt bridge found in their non-diabetic MHC counterparts. Without this bridge the MHC has a more open conformation, which has been shown to contribute to poor peptide binding. IMMUNOBIOLOGY: THE IMMUNE SYSTEM IN HEALTH AND DISEASE (PAPER) by JANEWAY, CHARLES A. JR./ . Copyright 2001 by GARLAND PUBG INC (B). Reproduced with permission of GARLAND PUBG INC (B) in the format Dissertation via Copyright Clearance Center.

(15-17). In this theory, the formation of MHC tetramers on the cell surface is thought to play an important role in T cell receptor (TCR) signaling by increasing overall signal strength during responses (18). Research has shown that I-A^{g7}, and the human analogue $DQ\beta^{8}$, fail to form SDS-resistant heterodimers when bound with peptide (due to the lack of a salt bridge), a trait not seen in their non-diabetic counterparts, this poor heterodimer formation may lead to poor tetramer formation during presentation (15, 16). What becomes apparent is that all of these defects may affect signal strength to a point where they allow diabetogenic T cells to survive negative selection, and migrate out of the thymus.

Glutamic Acid Decarboxylase and Other Autoantigens in Diabetes

Some supporting evidence to the theories discussed above is that the injection of islet cells, into the thymus of NOD mice, appears to induce a pancreatic β cell specific tolerance that prevents subsequent diabetes onset (19). This result suggests that high doses of the β cell specific antigens given to the thymus may prevent diabetes by overcoming the presentation defects in the NOD APCs. Presumably, in the presence of higher doses of the initiating β cell self-antigens, NOD APCs in the thymus can compensate for the I-A^{g7} presentation related defects by processing and presenting abnormally high levels of diabetogenic antigens.

Recently, several pancreatic β cell specific antigens, which may be responsible for the induction of T1DM, have been isolated using T1DM associated autoantibodies. These antigens include, but are not limited to, glutamic acid decarboxylase (10, 20, 21),

carboxypeptidase H (22), insulin (23) heat shock protein 60m/65h (24), islet cell antigen 69 (25)), and tyrosine phosphatase IA-2 (26-28). Research has shown that the immune response of NOD mice to GAD can be correlated with the onset of insulitis, which leads to T1DM in NOD mice (29). This is thought to occur because some GAD peptides are spontaneously recognized by helper T cells that mediate T_H1 associated responses (i.e. cell to cell killing). This recognition then leads to the onset of insulitis and, eventually, destruction of the pancreas and T1DM (30).

The initial T cell responses are confined to the carboxy-terminus of the molecule. The response subsequently spreads to other peptides within the molecule (31, 32). This "determinant-spreading" within the molecule is likely due to changes in the antigen processing systems of the cell, leading to the expression of "subdominant" and "cryptic determinants" not normally produced at high levels during processing. Long-term exposure to cytokines, which is thought to occur during T1DM and other long-term immune responses, has been shown to modify antigen processing in both the class I MHC presentation pathway (i.e. endogenous antigen) and class II MHC presentation pathway (i.e. exogenous antigen). INF-γ and TNF-α have been shown to initiate the production of three different proteasome subunits (LMP2/LMP7/MECL-1) that can be combined in different ways to substitute for three of the regular subunits (Delta, MB1, and Z), which are responsible for certain catalytic activities of the proteasome (33). These substitutions cause changes in the specificity for cleavage sites on proteins being processed by the proteasome, and thus alters the type of peptides being produced for the endogenous class I MHC presentation pathway. Similarly, the proteases that are used to breakdown

exogenous peptide in the lysosomes of APCs, for loading in class II MHC, can change based on exposure to certain cytokines. In fact, the type of APC that is processing the antigen has been shown to have an influence on the type of peptides generated (34). These protease changes can lead to the expression of knew "cryptic determinants" later in the response. This may suggest why autoimmune responses can later spread to other β cell specific antigens through the process know as determinant spreading (30). This is currently the most probable reason why tolerance to the carboxy-terminal peptides of GAD prevents NOD responses to the rest of the molecule, as well as, to the other β cell specific antigens. NOD mice tolerant to GAD also do not show the normal levels of insulitis seen in untreated NOD mice after 12 weeks. These data suggest that GAD is an important target antigen in T1DM, and it is thought that it may be the trigger of a T cell response cascade that results in T1DM. Therefore, it is possible that the effector cells responding to islet antigens, represented, for example, by SP4 clones specific for late stage antigens in the β cell granule membrane (35), would not be typically considered autoreactive because the self-peptides are not normally present. As suggested, multiple antigens may be involved in the autoimmune destruction of the pancreas that leads to T1DM, so it may be necessary to tolerize the immune system to several key initiating antigens in order to slow or stop this autoimmune process from occurring.

Recent evidence using transgenic mice that express lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) in their pancreatic β islet cells suggests that specific tolerance to LCMV-GP can prevent the T1DM that artificially occurs in this model (36). Also, in the experimental autoimmune encephalomyelitis (EAE) model, a single amino

acid change in the Ac1-11 peptide alters the response of susceptible animals from inducing EAE to tolerizing against EAE. Even in the face of subsequent immunization with the native Ac1-11 peptide these animals are protected (37), although the presence of 4 native amino acids in a 6 amino acid peptide derivative of Ac 1-11 can again induce EAE (38). These data, along with GAD tolerance data suggest that T1DM may be prevented with the appropriate immunotherapy given either early in T cell development or later in development when peripheral regulatory responses have matured. Work based on this premise, "*Therapeutic Alteration of IDDM Progression by T cell Tolerance to GAD65 Peptides in vitro and in vivo*", can be found in Appendix A.

Regulatory cells: Controllers of Autoimmune Responses and T cell Development

Another point of interest when investigating T1DM is that T cells capable of responding to β cell antigens have been detected in non-diabetic humans and animals (39) suggesting that the presence of certain regulatory T cells may prevent T1DM in these individuals. This concept has been suggested for the BB rat diabetic model (36, 40, 41). To date, several different types of immune regulatory cells have been identified or suggested, including: Natural Killer T (NKT) cells (including $DX5^+$ T cells), $CD4^+CD25^+$ regulatory T cells, and γδ T cells (42-44). Here we will focus on NKT cells.

NKT cells represent a subset of lymphocytes that bear both TCR and the natural killer (NK) cell receptor NK1.1 (CD161)(45). NKT cells are typically restricted to recognizing ligands in the context of the MHC like non-polymorphic class Ib molecule,

CD1d. The CD1d molecule has a single-chain structure similar to Class I MHC but is thought to bind phospholipids instead of peptides and has a wider binding cleft than Class I MHC. These regulatory cells are shown to express a very limited TCR repertoire that is capable of recognizing CD1d. These TCR, in mouse models, are typically composed of an α chain containing Vα14 with either Jα15, 18, or 281 (Vα24-JαQ in humans) and a β chain with Vβ2, 7, or 8.2 (Vβ11 in humans)(46-48). The natural ligands that bind to CD1d and that are recognized by NKT cells are still unknown, although recently cellular glycosylphosphatidylinositol has been proposed as a natural CD1d ligand (49, 50). Despite the fact that natural ligands for CD1d have yet to be established, these cells have been found to be responsive to the synthetic antigen glycolipid α-galactosylceramide (α-GalCer)(51, 52). CD1d restricted NKT cells capable of recognizing α-GalCer were first characterized in the mouse in 1997 and were found to express a highly restricted TCR repertoire, which has an invariantly rearranged Vα14/Jα281^{+} TCR α chain paired mainly with a Vβ8.2^{+} TCR chain (53, 54). Since that time, activation of NKT cells using α-GalCer has been used to expand NKT populations. Recently, treatment with α-GalCer has been shown to mitigate diabetes in NOD mice (55, 56).

Initially, it was believed that all NKT cells expressed the natural killer cell marker NK1.1. This made NK1.1 an ideal marker for NKT population staining, but recently some NK1.1 negative NKT cell populations have been identified (57) and the NOD mouse model, one of the primary models for NKT studies, expresses NK1.2 not NK1.1. This inconsistency in cell receptor expression implies that NK1.1 or any other pan NK cell marker (e.g. DX5) cannot be used as a reliable marker for the assessment of the

whole NKT cell population. The generation of CD1d-αGalCer loaded tetramers has solved some of the problems that occur with the ambiguous characterization of NKT cells by staining cells with a ligand specific for the most prevalent TCR types found on NKT cells. These CD1d tetramer ($CD1d^{tet}$) molecules make the identification of NKT cells easier and more accurate, as they identify these cells based on their TCR specificity rather than their phenotype (58-60). This TCR specific staining technique is beginning to become the standard for NKT cell staining, despite the fact that it also has its drawbacks. Essentially, NK1.1 and DX5 are found on NK and most NKT cells, making it an overly inclusive stain, while $CD1d^{tet}$ is not fully inclusive, only identifying a subset of NKT cells with a specific TCR type.

NKT cells are currently considered important regulatory cells in the control of autoimmune responses because they have the ability to secrete both T_H1 and T_H2 cytokines (45, 61, 62), which can change the type of immune response that is generated to a specific antigen. A deficiency in the function and number of NKT cells has been suggested as one of the major contributing factors in T1DM onset in NOD mice (63-65). A similar deficiency in NKT cells has also been identified in the human form of T1DM (66). These deficiencies have been shown to affect the development of both the NKT and T cell populations through the impact of T_H1 and T_H2 cytokine secretion (46, 67). Although the exact mechanism by which the NKT cells are involved in immune regulation is not known, they have been shown to suppress T1DM in NOD mice. This is thought to take place, primarily, through the secretion of IL-4 and IL-10, which is thought to shift T cell responses to a T_H2 humoral response (68). Adoptive transfer of

enriched NKT cells (69) or transgenic expression of the invariant Vα14/Jα281 TCR α chain used by some NKT cell types (70) can result in decreases in insulitis and diabetes incidence in NOD mice. Hence, it is widely believed that the defects in NKT cells in both NOD mice and humans may contribute to the pathogenesis of the disease (66, 71).

A possibly more important revelation is that NKT cells are found early in thymic development and are thought to play a role in T cell development, possibly by controlling cytokine levels during thymic education. "Normal" NKT cells produce high levels of IL-4 and IFN-γ in adult life but preferentially produce IL-4 during early stages of neonatal development, which is thought to influence neonatal T cell responses in a manner that will help control T_H1 autoimmune responses from occurring (72). Imbalances in cytokine responses of NKT cells towards IFN-γ (i.e. T_H1 responses), similar to those seen in NOD mice, have been correlated with T1DM (73, 74) and may play a major role in T1DM disease induction. Further work is still needed to define the exact roles of NKT cells during development but it has clearly been proven that their regulatory capacity extends beyond the mature responses in the periphery. Research examining the impact of NKT cell development on T1DM in NOD mice can be found in "*Differential Effects of Antibody to CD1d on T cell Development in NOD and C57BL/6 Fetal Thymic Organ Culture*", in Appendix B.

Avidity in T cell Development: A Developmental Role for Glucocorticoids in Autoimmunity

Hydrocortisone (HC) is a glucocorticoid (GC) steroid hormone produced by the adrenal glands after cytokine stimulation, typically produced during an immune response. HC has long been identified as a modulator of the immune inflammatory response (75). GCs are highly lipophilic and act by crossing the cell membrane and binding to the glucocorticoid receptor (GR, a transcription factor) in the cytoplasm of the cell, where the GR is bound by 2 subunits of an inhibitory chaperone (HSP90) in the inactive state. Once HC binds the GR, it releases HSP90, revealing nucleus-targeting sequences, and the now functional GR translocates to nucleus. After entering the nucleus, the GR can perform its transcriptional functions (Fig 4). Although the GR can activate some immune associated genes, including IL-7Rα (76-79), the GR is thought to cause the majority of its immune suppressive effects through negative interference with other transcription factors, such as NF-κB (80, 81) and AP-1 (82). In the case of NF-κB several different models for the interference caused by GCs have been presented.

One of these models is the IκB-α inhibitory model. This model proposes that GR can lead to a transcription level increase in IκB-α production (80, 81). IκB-α is a known inhibitor of NF-κB that functions by sequestering the nuclear transcription factor in the cytoplasm of the cell, not allowing NF-κB to perform its normal functions in the nucleus. Some of the "normal" functions of NF-κB are the activation of genes for the pro-inflammatory cytokines IL-1, TNFα, IL-2, IL-6, and several other inflammation

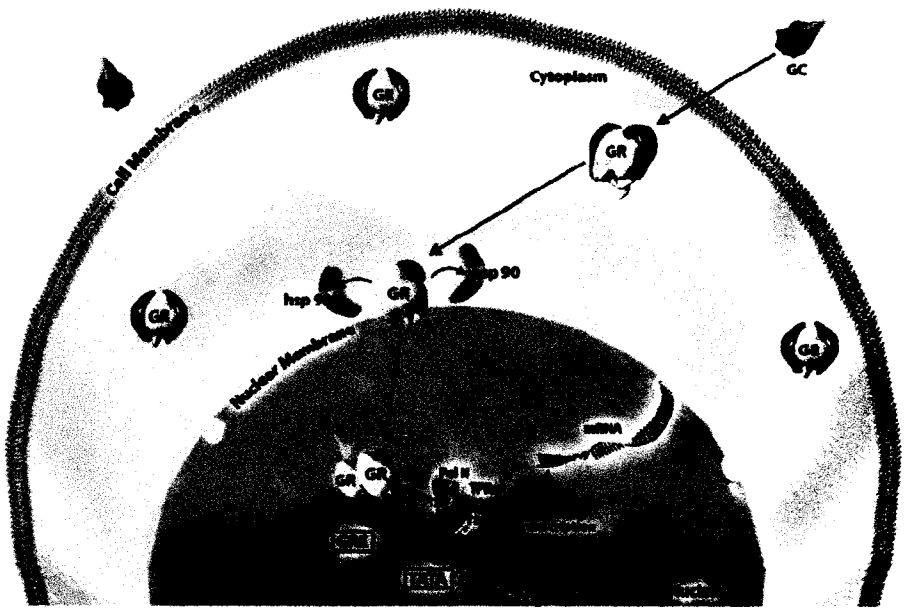


Figure 4. GC-Mediated Transcriptional Activation. Before GC binding, the GR exists as a large multiunit complex in the cytoplasm, which includes 2 molecules of hsp 90. After activation by binding of GC hormone (GC), the GR dissociates from the chaperone proteins and translocates to the nucleus. In the nucleus the GR binds as a homodimer to a specific palindromic DNA sequence, termed a GRE, located in the regulatory regions of target genes. The bound GR homodimer interacts with the basal transcriptional machinery shown bound to the TATA box. The basal transcription complex includes TATA-binding protein, associated transcription factors (TAFs and TFIIs), and RNA polymerase II (pol II). The interaction between GR and the basal transcription complex enhances transcription of the GR target gene.

associated genes (75). Without the transcription of these genes and their products the inflammatory immune response is severely hampered. The inflammatory immune response is known to enhance the initiation of antigen specific adaptive immune responses, thus GCs can also lead to the inhibition of the adaptive immune system.

A second model of suppression is the protein-protein interaction model. Protein-protein interactions can have various effects on protein function. Effects can often be inhibitory and in other cases synergistic. This is especially true in the case of transcription factors that form functional synergistic homodimers (e.g. GR) or heterodimers (e.g. the retinoic acid receptor (RAR) with the retinoid X receptor (RXR))(83). Conversely, several studies have shown that the GR and NF-κB can interact and associate *in vitro* (80) and *in vivo* (84) in a manner that is not mutually beneficial. These interactions can lead to the inhibition of NF-κB transcription and, thus, the inhibition of the genes it regulates. The effects of this model are similar to those discussed above (See Fig. 5 for these suppression models).

A third model by which GR suppression of genes may take place is the competition model. This model is based on more recent information that nuclear receptors and other transcription factors share several co-activator molecules that can become limited at times of high activation (85). Co-activators such as CREB (c-AMP response element binding protein) binding proteins (CBP), p300, and steroid receptor co-activator-1 (SRC-1) can become scarce and only bind to the nuclear receptors for which they have the highest affinity. Without these co-activators the nuclear receptors function less effectively (86). If the GR has a higher affinity for co-activators it shares with NF-κB

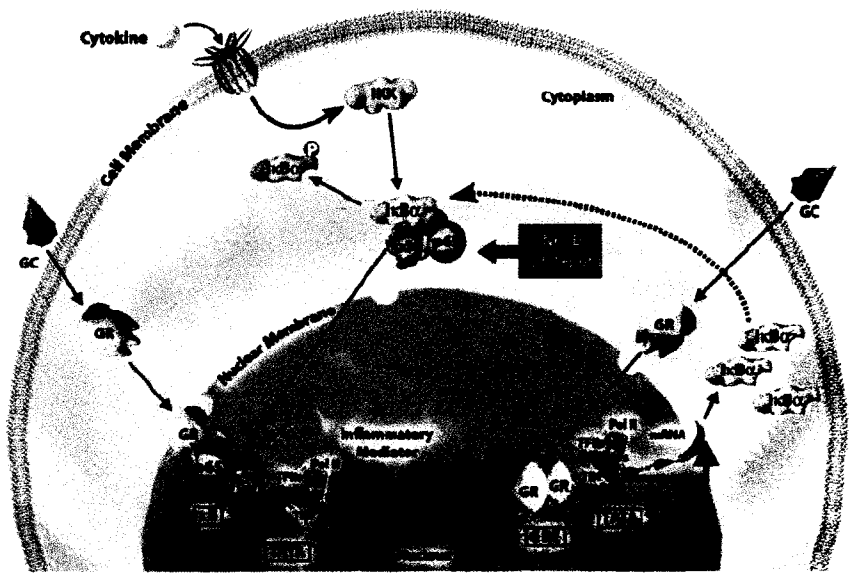


Figure 5. GR-Mediated Repression of NFκB Activity. In the inactive state NFκB (heterodimer of p65 and p50) is anchored in the cytoplasm by IκBα. Activation signals through cell-surface receptors result in activation of IκB kinase, which phosphorylates IκBα. After phosphorylation, IκBα undergoes proteolytic degradation, and the NFκB heterodimer (p65/p50) is free to pass into the nucleus, where it binds to B sites in the promoter regions of inflammatory mediator genes and enhances transcription. The GR might block NFκB activity by either of 2 mechanisms. Inhibition might occur through protein-protein interactions between the ligand-activated GR and NFκB. A second less plausible mechanism for GR-mediated inhibition of NFκB is activation of the *IKBA* gene by the GR. The enhanced synthesis of IκBα replaces the degraded IκB and neutralizes the free NFκB.

or AP-1, this could explain the poor transcription of NF-κB and/or AP-1 controlled genes when GR is present in the active form.

More recently, HC has been recognized for its role in thymic education (87). Although not essential for T cell development (88), HC appears to play a critical role in thymic education by dampening the cell to cell signaling in developing thymocytes through similar processes to those discussed above (89-93). HC can also differentially modulate responses of other autoimmune associated cell types, such as regulatory NKT cells. Peripheral and developing NKT cells and NK cells in B6 mice have shown high levels of resistance and expansion during HC administration when compared to their normal T cell counterparts (94). Thus, increasing hydrocortisone levels during certain times in development could lead to an increase in the ratio of NKT cells to T cells (i.e. suppressor/effector ratio) by more severely inhibiting T cell development. As discussed above, enhancing the number of NKT cells, or changing the ratio of NKT cells to T cells, in NOD mice has been shown to inhibit T1DM (65, 95, 96).

In the case of T cell development, it is also possible that HC, by decreasing signals transmitted during selection, would prevent the intrathymic deletion of diabetogenic T cells in the already poor signaling NOD mice and certain humans (97). As previously discussed, the processes of positive and negative selection during T cell development are defined and decided by the concept of avidity. Avidity itself can be defined as the total signal generated through cell-to-cell interactions. The implications of the avidity model of T cell development are that by changing the signal strength in part of the avidity equation (i.e. altering TCR expression, inhibiting cytokine signaling, or

interfering with adhesion, etc), it is possible to change the type of mature T cells that are developed. The concept of "T cell type" in this case refers to the individual T cell identity based on the TCR types generated that pass positive and negative selection, this does not necessarily refer to SP4 vs. SP8 cells specifically (although this can be debated based on your developmental model of choice). Thus, based on the avidity theory, it is possible that by adding exogenous GCs at levels that cause partial signal inhibition, or by removing or endogenous GC, one can change the type of T cells that pass thymic education. Through a better understanding of the role that signal strength plays in selection, it may be possible to eliminate certain autoreactive T cells and/or increase NKT cell production (based on their resistance to the effects of GCs), leading to the mitigation of certain autoimmune diseases. Work based on these principles, "*The effects of low level glucocorticoids on T cell Development*", is found in Appendix C.

NOD Fetal Thymic Organ Culture: Examining the Development of Regulatory and Autoreactive T cells

As discussed previously, when individuals are destined to become diabetic the altered selection of T cells may be responsible for their over-production of autoreactive T cells. Alternatively, we must consider that these individuals may suffer from a failure to produce adequate levels of the regulatory T cells that are thought to play a role in controlling autoimmune disease. In the case of the NOD mouse model of T1DM, both of these defects seem to play a role in disease susceptibility. Our laboratory has developed an *in vitro* fetal thymus organ culture (FTOC) system that allows us to evaluate the

development of diabetogenic T cells in NOD mice in only 14-21 days (98). Using this FTOC system we are also able to examine the thymus and its cell production at various developmental stages. Work discussed in the appendix uses this model to study the mechanisms underlying the production of diabetogenic T cells in the thymus, and to allow for rapid screening of potential therapies for T1DM.

One cell type found in the thymus that can be monitored with the FTOC system is the NKT cell. The majority of work performed on NKT cells characterizes them as capable of producing T_H1 (IL-2, IFN-γ and others) and T_H2 (IL-4, IL10 and others) cytokines, which makes them capable of skewing the phenotype of developing T cells and modulating the responses of mature T cells in the periphery (99). Although most of these cells can be found in the liver and spleen of adult mice they are not produced in athymic mice, indicating that these cells are thymus dependent and possibly thymus derived (100). As previously mentioned, it has been shown that NOD mice have functional and numerical deficiencies in their NK and NKT cell populations, which aids in the induction of spontaneous diabetes in these mice. The ability of NKT cells to produce IL-4 is deficient in NOD mice as compared to the "normal" C57Bl/6 (B6) mouse (74). It has also been shown that the transfer of these NKT cells into NOD recipients results in prevention of the onset of T1DM (74). Also, a subgroup of regulatory NKT cells, identified as $CD4^-/CD8^-$ DN $\alpha\beta^{TCR+}/DX5^+$ or $CD4^-/CD8^-$ DN $\alpha\beta^{TCR+}/CD1d^{tet+}$ cells, appear to be enriched in FTOC. Thus, FTOC has significant advantages for examining potential NKT modulating treatments since the normally almost undetectable

levels of NKT cells in NOD mice can be more easily examined for the positive and negative effects of treatment.

While regulatory cells have an important role in controlling autoimmune responses, the primary cell type generated by the thymus and major contributor to autoimmunity is the T cell. The FTOC system used by our lab has allowed us to make unique and unusual observations regarding T cell development. Because the thymus is isolated from fetal mice before the first wave of T cell development has occurred, the system allows us to freely manipulate development, through the timed addition of substrates, without the difficulties of *in vivo* work. Additionally, since the thymus has been separated from the body, the maturing T cells accumulate in the thymus, allowing for not only the examination of T cell development but also the investigation of long-term effects of treatment on mature T cells generated from the culture. These concepts were realized in the glucocorticoid work performed in Appendix C, in which we were able to lend support to the theoretical T cell activation threshold (TAT) model of T cell development proposed by Zvi Grossman and Alfred Singer (101). Using FTOC we were able to see the short-term effects of GC treatment and then follow the effects to their conclusion (for further information see Appendix C).

A more recent advancement in the use of FTOC was the development of the ivT1DM. With the development of this *in vitro* diabetes assay we were able to further assess the effects of T cell development based treatments in a functional assay. By co-culturing treated FTOC with fetal pancreas, we can now assess the effects of treatment on diabetes onset and severity *in vitro*. ivT1DM allows us to screen the possible disease

specific effects of treatment rapidly and without the complications of *in vivo* work. Research performed using this system can be found in the peptide therapy and anti-CD1d based work in Appendix A and B, respectively.

The development of our current FTOC and ivT1DM systems has allowed for the rapid screening and assessment of potential therapeutic techniques for autoimmunity. Due to the highly complex nature of the body these systems cannot currently replace *in vivo* research, but these systems have accelerated the number of potential therapeutic techniques being researched in our lab. The capacity to modulate T cell development in FTOC is not only suited for T1DM but can be used to assess treatments for other autoimmune disease types. Defects in the MHC structure, cell signaling, regulatory cell development, and positive/negative selection are all known to be common themes in several autoimmune disease types. Ideally, through FTOC, we may be able to discover more broad-spectrum techniques that are able to correct these common autoimmune defects and pre-treat those with the genetic potential for autoimmunity.

Dissertation Format

This dissertation is broken into three main parts. The first section, Chapter I, is an introduction and literature review that is aimed at generating adequate background knowledge to further understand the research as it is presented.

The second section is Chapter II, which is a discussion of the present course of our work on the projects discussed. This section will contain our insights and additional data on our current work, speculative discussions of mechanisms and theoretical impact of these mechanisms, as well as our future course of work. Since the discussion is based on the work presented in the Appendix, it would be most beneficial to read the sections A-C of the Appendix before reading the discussion in Chapter II.

Finally, the third section is the Appendix. Included in this appendix are three papers that deal with modulation of T cell development. Appendix A contains "*Therapeutic Alteration of IDDM Progression by T cell Tolerance to GAD65 Peptides in vitro and in vivo*", which was published in the Journal of Immunology in the July 1st edition of 2001. Appendix B contains an expanded version of the paper "*Differential Effects of Antibody to CD1d on Fetal NKT cell Development in NOD and C57BL/6 Thymic Organ Culture*", which was submitted to the Journal of Immunology in May of 2004. Although the paper was not accepted for publication we believe it has great merit. New data has been generated regarding cell receptor expression changes, which is included in the expanded version, and more data is in the process of being generated. The final paper, "*Modulation of T cell Development in Fetal Thymus Organ Culture through Exogenous Glucocorticoids*", deals with the effects of inhibitory Glucocorticoids on T

cell development. The work in this last paper was performed in B6 mice, but we currently have some data in the NOD mouse model. The ramifications of this work for T1DM are discussed in Appendix C and, to some extent, in Chapter II.

CHAPTER II: Present Study

Peptide Therapy

Work presented in this dissertation is targeted at manipulating T cell development in a way that will help prevent or delay the onset of T1DM. Each of the three projects described below attempts to eliminate T1DM through different mechanisms. The first project addressed deals with using peptide therapy to remove autoreactive T cells during T cell development in a peptide specific manner, thus inhibiting diabetes at the source. Dr. Steven Wilson originally began this project before my arrival at the University of Arizona. Early in my graduate career I completed the *in vivo* portion of this research, and with the guidance of Dr. DeLuca, completed the final manuscript. This work was published in *The Journal of Immunology*, in July of 2001, under the title of "*Therapeutic Alteration of IDDM Progression by T cell Tolerance to GAD65 Peptides in vitro and in vivo*" (found in Appendix A).

Research performed in the mid to late 1980s showed that MHC/peptide binding specificity and the concentration of peptide available could influence T cell development (102). This information, combined with research showing that autoantigens from pancreatic islets could generate T cells responses (103, 104), made the concept of peptide therapy a theoretical possibility. In the early to mid 1990s, it was discovered that the MHC molecules associated with T1DM ($DQ\beta^8$ in humans and I-Ag7 in mice) were poor peptide binders (16), spawning a high level of interest in peptide therapy as a possible mechanism to overcome MHC associated autoimmunity (105-107). The concept of peptide therapy is based on the idea that it is possible to correct for poor peptide binding

MHCs by overwhelming the APCs with copious amounts of peptide that lead to a higher than normal presentation of peptide on the surface of the cell. With this additional peptide presentation during selection, it is thought that the TCR specific binding, and subsequent signal generated, between the T cell and the selecting APC, is increased. This increased signal between the two cells will then lead to enhanced negative selection of any pre-diabetogenic T cells that are specific for the peptide that was added.

The work in appendix A was performed using simple i.p. peptide injections into pregnant NOD mothers. On the surface this technique appears to have many difficulties because the peptides must be absorbed, pass the placental barrier, and reach the developing fetal thymus without being cleared or destroyed. Despite these difficulties, the technique is effective at inhibiting T1DM in NOD mice. However, we currently do not have good immunological techniques that can directly detect whether these peptides have reached the thymus. The use of highly radioactive amino acids or tracer molecules could be used to prove that substances can reach the fetal thymus through this i.p. injection technique. Unfortunately, these types of experiments would still leave the question of whether the peptides were still intact when they reach the thymus. However, it may be possible to elute enough peptide from the APCs for analysis, if a huge bolus of peptides is given to the mother. Needless to say, these difficulties are still being worked on.

Based on these initial peptide therapy experiments we learned that 1) the i.p. *in utero* technique works (in regards to diabetes prevention), 2) the effects of peptide therapy in FTOC were peptide specific (based on proliferation), and 3) that the efficacy of multiple peptide mixtures did not correlate with the efficacy of the individual peptide

profiles (see Appendix A). The ability of mixtures of peptides to change the effectiveness of treatment versus single peptide mixtures is thought to be due to cumulative tolerance in beneficial mixtures (e.g. p34+p35) or competition for the MHC binding sites in mixtures that were less effective than a single peptide alone (e.g. p34+p35+p17 vs. p17). In a mouse model like the NOD, which only has one type of class II MHC expressed, there is a high level of competition for peptide presentation. In order for the addition of exogenous peptide to impact presentation, the peptide must either be pino/phagocytosed and loaded through the usual exogenous antigen processing pathway or exogenous peptides must displace peptide from the class II MHC already on the surface of the cell (108, 109). The implications of this competition for MHC binding site is that peptides with higher specificity for MHC can out compete peptides with lower specificity, when they are competing for the same binding site. Thus, using multiple peptides could have a spectrum of effects on the inhibition of T1DM. These effects could range from an enhancement of T1DM inhibition (when multiple peptides are still able to bind well and negatively select for their peptide specific clones) to the complete loss of T1DM inhibition (when multiple peptides dilute presentation to a point where avidity is no longer effectively enhanced). An example of these effects is seen in Appendix A, where GAD p17 inhibits well alone, but GAD p34 and GAD p35 do not inhibit well alone. However, a mixture of GAD p34 and p35 protects much better than either did alone, but a mixture of all three (p17, p34, and p35) did not protect well. These results can be explained if T cells specific for GAD p17 are strong/early/predominant T1DM responders and T cells for GAD p34 and p35 are weaker/late/less predominant T1DM

responders. When GAD p17 specific T cells are eliminated they may induce significant T1DM inhibition, however in order to create significant T1DM inhibition with p34 or p35, both peptides must be used to eliminate enough autoreactive T cells to generate protection. However, when all 3 peptides are used, the peptides may dilute and/or compete with each other enough to cause the presentation levels of the peptides to drop below the optimal point of enhanced negative selection. Although further investigation into these theories is warranted, it was not essential to the publication of the initial paper or to the assessment of the efficacy of peptide therapy treatment.

In recent years, several other IDDM associated antigens/peptides have been discovered in autoimmune diabetes responses (including carboxypeptidase H (CPH, (362-382 and Y-440-464) (22)), insulin β chain ((9-23)(23)), heat shock protein 60m/65h (HSP 60m/65h, (437-460 with valine for cysteine replacements at 442 and 447 (105)), islet cell antigen 69 (ICA69, whole protein (25)), and tyrosine phosphatase IA-2, (IA-2, 805-820 (26-28)), and newly discovered GAD peptides ((206-220, 221-235) (21)), all of which are immunogenic in NOD mice. Our current research in peptide therapy has moved to this larger pool of potential diabetes related peptides. We wish to examine the tolerance induction generated by these new peptides and their capacity to mitigate IDDM through early stage addition to our ivIDDM cultures, as well as through *in utero* peptide therapy. Once the therapeutic value is determined for these peptides independently, we will generate mixtures of the highest tolerance inducing peptides to evaluate if disease mitigation can be improved further through the use of certain peptide combinations.

Anti-CD1d NKT Expansion & Signal Modulation

With the discovery, in recent years, of several types of regulatory T and NKT cells, investigation into the role of these regulatory cells in autoimmune diseases was quick to follow. Once it was realized that there were definable defects in the number and function of certain types of regulatory cells in autoimmunity, research turned to devising ways to enhance cell number or correct functional defects. As discussed in Chapter 1 and the Appendix, NOD mice, as well as some Type 1 diabetics, have genetically linked signaling problems that so far include the MHC, components of the IL-2 signaling pathway, and more recently discovered "defects" in TNF-α. These deficiencies, and those yet to be discovered, have a major impact on the signal strength dependent development of T and NKT cells. Thus, the majority of research currently under investigation focuses on expanding NKT cells through the generation of additional signal either early in development or in the periphery.

Because of the ease of manipulation in the FTOC system, and its propensity to generate larger numbers of NKT cells, we felt there was an ideal opportunity to investigate the development and modulation of NKT cells. Thus, we began research, using anti-CD1d, to look at the effect of changes in signal strength on NKT development. Anti-CD1d was chosen because 1) it had the potential to block or perhaps generate signal (based on the quantity used, as defined in research by Nakayama et al and others (110, 111)), 2) it was readily available, and 3) α-GalCer, the only defined NKT ligand, was not initially available. By using a titration to establish effective dose in the thymus, we were able to show that appropriate amounts of anti-CD1d can lead to an expansion of NKT

cells in the NOD model of diabetes and that this expansion can mitigate ivT1DM. Currently, we are investigating the mechanism of action that leads to the NKT expansion in NOD FTOC.

Based on the initial data generated, it appears as if the addition of antibody to the Class I MHC like CD1d molecule leads to excess signaling, similar to that seen when anti-CD3 or anti-αβTCR are used. This initial assessment is based on the phenotype profiles generated in several signal modification experiments performed in our lab (discussed below). In these experiments, several known and proposed signal modifiers have been studied in normal B6 mice to determine the overall effect of signal change on development. By comparing the effect of signal strength from these different projects, it appears that anti-CD1d may inhibit but also causes leads to an increased signal to NKT cells and, to some extent, to T cells during development. This increased signal could be generated through Fc crosslinking of antibody by APCs, or perhaps through an inhibition of CD1d access, leading to higher levels of TCR and/or CD1d expression. Similar, enhancements in TCR expression have been reported when using anti-CD4 on developing thymocytes (110). Regardless of the mechanism, the profile generated through anti-CD1d is that of an enhanced signal, which causes increased negative selection of NKT cells in normal B6 model and increased positive selection, without increased negative selection, of NKT cells in the poor signaling NOD model.

Anti-CD1d appears not only to have an effect on NKT cells but also on general T cell types. B6 FTOC, and to some extent NOD FTOC, show enhanced negative selection profiles in the general T cell population when treated with the higher concentration of

anti-CD1d. This fits well into the signal enhancement profile. Essentially, at these higher levels of anti-CD1d treatment, excess signal is generated into any cells expressing and/or interacting through CD1d. Therefore, it is possible that regular T cells could receive marginal signals either directly through CD1d on there own surface (112), through secondary signals generated by APCs that are being engaged by the antibody, or through TCR upregulation due to an inhibition temporary inhibition of cell to cell interaction. Despite the effects seen in the general T cell repertoire, the effects of anti-CD1d are much more severe for NKT cells. In hindsight, the choice of anti-CD1d as a modulator appears to have been a blessing in disguise since not only does it affect typical NKT cells but it also appears to affect any cell type that is selected on CD1d (including several other possible autoimmune regulators such as $DX5^+$ and γδ T cells). This capacity for broad based expansion may make anti-CD1d a more effective and desirable treatment for autoimmune diseases than the current single peptide/ligand NKT expansion treatments being investigated (113).

Our current research has moved further into defining the mechanism of anti-CD1d treatment. Through the analysis of more surface markers, including CD1d, we hope to better define the effects of anti-CD1d on development. We would also like to investigate the differences in NKT development when anti-CD1d F_{ab} or FcR blockers are used, which could verify if signaling from cross linking is occurring. Ultimately, this research is focused on investigating the ability of anti-CD1d to induce NKT expansions *in vivo,* as a treatment for T1DM. To do this we must first identify an effective method of delivery and determine how well the antibodies can reach the thymus. If anti-CD1d is effective at

expanding NKT cells in the immune periphery then it could be used for treating ongoing disease. However, a recently completed trial using oral insulin for peripheral therapy suggest that treatment of early stage diabetic patients has no effect on disease induction, and may also have the potential to accelerate disease, especially in younger patients (114). Results similar to these were seen in our late stage peptide therapy data presented in Appendix A. Essentially; post-development treatments have more difficulty than thymic-based treatments because it is much easier to activate then tolerize once a peripheral response has started. Hopefully, treatments of this type can have a post-partum *in vivo* impact on NKT development, while thymic production is still ongoing, because unlike peptide therapy we are only trying to expand a population instead of eliminating it.

Signal Modulation in T cell Development

Our lab, over the last 5-6 years, has been investigating the impact of several different substrates on T and NKT cell development. These projects have included looking at the impact of antibodies (CD3, αβ TCR, and CD1d), cytokines (TNF-α, IL-7), hormones (hydrocortisone), and pseudo-neurotransmitters (nicotine). Although each of these substrates deal with distinct mechanistic pathways, and in some cases multiple mechanisms of action, they tend to fall into more general categories based on overall impact on T cell development; Selection Signal Enhancers and Selection Signal Inhibitors. The signal, in this case, is the overall avidity of interaction experienced by a T cell going through the processes of selection. In other words, will the addition or

elimination of this substrate have the overall effect of either enhancing or inhibiting the signals experienced by T cells during thymic education?

Through the use of experiments with known inhibitors or enhancers we have found that changes in signal strength can have distinct effects on cell yields and phenotype shifts over the course of FTOC development. Figure 6 shows the general T cell development impact of several substrates, grouped based on their impact on T cell development. Previous research has defined both hydrocortisone and TNF-α as signal inhibitors (115), while antibodies to CD3 or TCR have the capacity to be either inhibitors or enhancers based on the antibody concentration used (111, 116).

High-level inhibitors (microgravity and "stronger" HC ($1x10^{-7}$ M)) generate decreases cell yield, viability (possibly due to increased failure of positive selection), and maturity (except in DP stage) in cultures, while lower levels of inhibition (TNFα and "strong" HC ($1x10^{-9}$ M)) generate increases cell yield, increases in DP cell maturity, and increases in SP4 production. High-level signal enhancers ("stronger" anti-CD3 (5.0 μg/mL), and metyrapone) also generate decreases in cell yield but viability is not as effected (possibly due to clearance ability after negative selection). Low-level signal enhancers ("strong" anti-CD3 (5.0 μg/mL) and TNFα receptor inhibitor) tend to decrease "maturity" in the DP group and skew the SP4-SP8 ratio towards SP8 cells. The most important changes in this process occur in the DP group where "maturity" is more easily understood as the expression of αβTCR, since DP cells are by definition immature. The point of emphasis is that changes in the perceived avidity within the developing T cell can lead to adjustments in T cell (and perhaps the selecting cell) receptor expression to

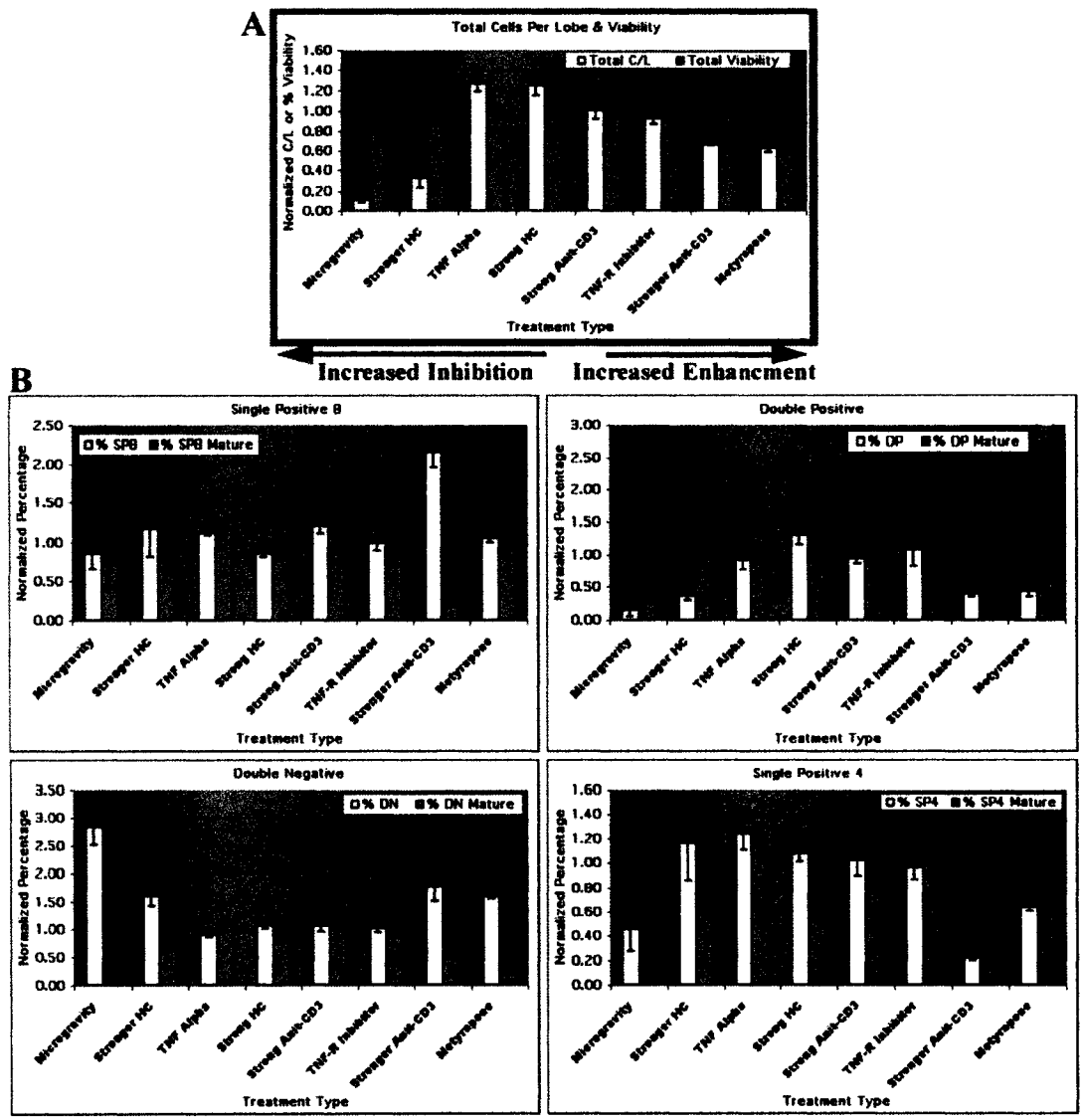


Figure 6. Impact of Signal Inhibitors and Signal Enhancers on T cell Development. Cells from FTOC, derived from ~13-15 dg thymi, were analyzed for cell yield and viability through trypan-blue exclusion (A) and for general T cell phenotype and mature phenotype distribution (B). FTOC were treated with microgravity (produced through rotation on a clinostat (117)), HC, TNFα, an inhibitor of the TNFα receptor, anti-CD3, or metyrapone (an inhibitor of HC production). The open columns represent the changes in

total C/L yield in (A) and the percent of general T cell phenotype changes in (B). The shaded columns represent the changes in culture viability in (A) and changes in phenotype maturity, based on αβTCR expression, in (B). Values were normalized to the untreated control for direct comparison

compensate for signal changes. In other words, selection signal inhibitors force T cells to up regulate receptor expression to increase signal, while selection signal enhancers force T cells to down regulate receptor expression. Better dose dependent examples of these responses can be seen in Figure 2 and 8 in Appendix C. Results shown here in Figure 6 are representative of work performed in our lab and are for example purposes only. The relative ability to inhibit or enhance signaling by these substrates has not been determined and distribution was set up strictly based on the profiles.

As a side note, it is apparent from the results in Figure 6 and work performed in Appendix C that time of administration can play an important role in the outcome of these treatments. Work with metyrapone, an inhibitor of cortisol production by the thymus (i.e. an inhibitor of inhibitory HC), shows that early stage impact of signal enhancers can generate unusual profiles when compared to other signal enhancers used. This is likely due a time dependent ability to impact development. Aside from other necessities for cortisol during cell development in general, when metyrapone is given early in development (i.e. before TCR β selection) it has the capacity to remove the inhibitory effects of cortisol immediately. This would generate increased signals to the T cell during β selection, which has been shown to have unique impacts on development. Ito et al. have shown that increased signal through the pre-TCR complex can lead to the generation of mature SP8 cells, SP4 cells with decreased TCR expression, and limited DP cells (118). These results are strikingly similar to those produced by the early addition of metyrapone. In contrast, treatments using antibodies (e.g. anti-CD3, anti-TCR, anti-CD1d etc) or receptor inhibitors would only be highly effective when the surface receptors that

these treatments target become available or relevant to selection. For most of the receptor types important for T cell selection (e.g. anti-CD3, anti-TCR, anti-CD1d etc), we only see higher levels of expression late in development. This may explain why high-level anti-CD3 cannot impact T cell development the way metyrapone does. Indeed, work with HC, metyrapone, TNFα (which has been shown to play a role early in development), and TNFα receptor inhibitor has shown the ability to impact selection early in development as well as late (see Appendix C for HC work. TNFα work (by Aaron J. Middlebrook) is in submission).

The Big Picture

Although the projects presented in the appendix are distinct, they have several common threads that tie them together. Clearly, these projects deal with T cell development and substrate induced changes in T cell development. However, the true underlying theme of this work is the use of the avidity-based models of T cell development to impact the types of cells that are generated by the thymus. Ideally, in our research, manipulations of T cell development are performed with one specific purpose in mind: The cure, delay, or inhibition of T1DM in humans. Still, beyond these lofty ideals is the quest for empirical knowledge, including a better understanding of the immune system and its development.

The concept of avidity-based T cell development; as described in Chapter 1, 2, Appendix B, and C; is in its relative infancy. Over the last 20 years, with a better understanding of the interaction of T cells/TCR with APCs/MHC, the avidity-based

model of T cell development has gained ground and is currently accepted by some immunologists as a fact. But, until we have the capacity to actively monitor the interior signaling systems of T cells during T cell/APC interactions, the avidity theory will never be wholly proven. Regardless, work investigating T cell signaling and development continues to accumulate and support the avidity-based model of T cell development as fact. Our work, as those before us, also lends great support to this avidity-based model of T cell development. Each project presented here (peptide therapy, anti-CD1d treatment, and hydrocortisone treatment) adds its own unique look at how changes in avidity can impact T cell development.

In regards to avidity-based models of T cell development, research using peptide therapy has shown that the addition of exogenous peptides during thymic education leads to the negative selection of T cells specific for those peptides (Fig. 4 in Appendix A). This can occur regardless of the peptide type (self and non-self (119, 120)) and is thought to only require a peptide that can bind to the available MHC types. With self-peptides, the elimination of peptide specific autoreactive T cells can be achieved through the exogenous addition of self-peptides at concentrations that increase peptide/MHC presentation on the surface of the selecting APC. This increase in presentation leads to an actual increase in avidity when developing T cells, specific for the treatment peptide, and the selecting APCs interact. This increase in avidity, if strong enough, causes T cells specific for the treatment peptide to receive too strong a signal during development, leading to negative selection and elimination from the immune repertoire. Essentially, peptide therapy of this type generates "holes" in the T cell repertoire by removing peptide

specific T cells. If the T cells removed are specific for all the autoimmune antigens, associated with a certain autoimmune disease type, then the disease will not occur.

In the case of non-self peptides, peptide therapy is more likely to have a wider range of effects because of the absence of those peptides during normal development. For example, peptide therapy could theoretically cause increased positive or increased negative selection, based on concentration. At lower concentrations, non-self peptides could lead to increased positive selection, without increased negative selection, of treatment peptide specific T cells. Mild increases in avidity between T cells specific for non-self treatment peptides and the selecting APCs could lead to the positive selection of certain T cell/TCR types that are not typically seen in the immune system. Although not currently seen in the literature, peptide therapy of this type (i.e. using non-self peptides) could correct natural occurring holes in the immune repertoire that allow certain pathogenic diseases to persist. However, immune modulation through non-self peptides may not always work since some non-self peptides "mimic" our own self-peptides and TCR types that can recognize cannot exist due to normal negative selection. Also, research on positive selection shows that not all peptides are capable of inducing positive selection (121).

Attempting to use the avidity-based model of T cell development to generate additional cells, similar to the non-self peptide therapy as discussed above, is the basis for our research using anti-CD1d treatment. In the NOD mouse, poor signaling has an impact on the perceived avidity between developing T cells (or NKT cells) and the cells interacting with them during thymic education. It is thought that these signaling problems

cause decreases in the generation of certain cell types, such as the regulatory NKT cells, due to a lack of positive selection. These signaling problems are also thought to cause increases in the generation of autoreactive T cells due to a lack of negative selection. In our work, we have shown that anti-CD1d treatment can generate increases in the number of NKT cells produced by the thymus and that these increases have the ability to inhibit diabetes.

The exact mechanism through which anti-CD1d treatment works is not currently known, but several possibilities exist. It is possible that the binding and/or crosslinking of CD1d on the surface of selecting cells leads to activation of the selecting cell, which in turn leads to increases in TCR expression on interacting NKT cells. Conversely, the blocking of CD1d by the antibody may inhibit contact between the selecting APC and the T and NKT cells through steric hindrance or inhibiting basal interactions between CD1d and TCR, causing a drop in the interaction avidity in the cells undergoing selection. This would lead to an upregulation in TCR expression on developing NKT or T cells in order to produce adequate positive selection signals for the cell to survive. As anti-CD1d dissipates over time, NKT cells with increased TCR expression would experience increased avidity versus a normal NOD NKT cell, leading to increased negative and/or positive selection. Finally, developing T cells express CD1d on their surface and may be signaled directly through crosslinking of CD1d. However, the data shows that anti-CD1d treatment tends to specifically expand NKT cells and other $CD1d^{tet}$ binding cell types instead of all T cell types, making this direct crosslinking scenario less feasible.

Although the mechanism of anti-CD1d is not known, treatment does generate the upregulation of TCR expression on developing NKT cells (Fig. 13 in Appendix B) and increases in the number of NKT cells developed (Fig. 7 in Appendix B). The increases in NKT TCR expression are thought to generate increased interaction avidities between NKT cells and the selecting cells. This increase in avidity is compensatory in the poor signaling NOD mouse, leading to increases in the positive selection of NKT cells. Conversely, this increase in avidity is excessive in the normal signaling B6 mouse, leading to the negative selection of NKT cells (and some normal T cells as well). The divergent effects generated by anti-CD1d in NOD and B6 FTOC shows the double-edged sword of avidity, having the power to both positively and negatively impact T or NKT cell development. Research of this type also shows that it may be possible to correct for developmental signaling defects through the addition of antibodies or other signal blocking or inducing compounds.

The avidity-based model of T cell development has been modified and expanded over time to include new developmental and peripheral signaling research. In recent years research by Zvi Grossman, William Paul, Alfred Singer, and others has shown that avidity can impact T cell development not only during stages associated with positive and negative selection but throughout the T cell developmental process. This information led to proposal of a new avidity-based model of development termed "the tunable activation-threshold (TAT) model". The TAT model of T cell development is more thoroughly discussed in Appendix C.

Essentially, the TAT model proposes that the gradual upregulation of several receptor types (i.e. TCR, CD3, and perhaps others) during development is an intentional process by the cell to find the most appropriate expression/avidity levels for cell survival. The implications of this theory is that the pool of TCR types that pass selection is much larger than it would be without this tuning process. In other words, T cells with moderately self-specific TCR, which might be destined for negative selection, can down regulate TCR to a level where they are no longer capable of responding to self-peptides. Also, T cells with TCR that fail to receive adequate avidity signals during selection can upregulate TCR expression in an attempt to pass positive selection. This may explain why it is possible to find T cells with autoreactive TCR in normal individuals. Another facet of this theory is that severe perturbations in signaling (either strong increases or decreases in avidity) at any point during development can lead to elimination of T cells through processes that are essentially equivalent to the current concepts of positive and negative selection. Finally, it is also thought that the expression levels that are used to pass TCR β selection are used to establish the basal receptor expression point for the tuning process to begin. The implications of this role of β selection in tuning is that perturbations during β selection can effect the avidity levels experienced by the T cell as the tuning process proceeds. To take this concept a step further, if there is a range of acceptable avidities within any given T cell during selection, then changes in basal TCR expression levels during β selection could impact the avidity/receptor expression levels that the T cell is selected at, as well as the receptor expression levels it uses in the periphery.

These concepts proposed by the TAT model are beginning to be proven through research in developmental and peripheral T cell signaling (see (101, 122) for more information). Our research using selection signal enhancers (e.g. anti-CD3), which increase perceived avidity, and selection signal inhibitors (e.g. HC), which decrease perceived avidity, support the TAT model of T cell development. Comparative work with anti-CD3 and HC have shown that the addition of selection signal enhancers leads to a decrease TCR/CD3 expression in the developing T cell, while the addition of selection signal inhibitors has the opposite effect, inducing the upregulation of TCR/CD3 expression. These changes would be expected based on the TAT model, which predicts that T cells will compensate for changes in avidity using surface receptor expression. In other words, when an extra signal is generated, causing perceived avidity increases, T cells will respond in an attempt to survive by decreasing surface receptor expression. The inverse is true when a selection signal inhibitor is given (i.e. receptor expression increases). These results are further supported by data that shows that when HC is given to mature cells from FTOC, which have completed selection, they only show down-regulations in receptor expression, similar to what happens in the periphery.

The TAT model of T cell development is further supported by our work using time dependent administration of HC, which shows that the impact of selection signal inhibitors is time dependent. Early administration, when the majority of T cells are going through β selection, has a different impact on T cell development than later administration, when the majority of cells are going through positive/negative selection.

The time dependent changes induced by HC, from the perspective of the TAT model, are explained in detail in Appendix C.

Again, the research projects presented in the Appendix are distinct and the treatments used therein generate distinct outcomes, but all of these projects use the manipulation of avidity to modulate T cell or NKT cell development. In peptide therapy and anti-CD1d research, we see that the manipulation of avidity can lead to therapeutic treatments for autoimmune diabetes. In the case of peptide therapy this impact is T cell specific, affecting only certain TCR types. In the case of anti-CD1d treatment the majority of this impact tends to be on NKT cells and other cell types that interact with CD1d, perhaps including other potential regulatory cell types (γδ T cells and others). Finally, research performed using HC has shown that HC or HC blocking may have the potential to make large-scale changes in T cell or NKT cell development through changes in perceived avidity (i.e. internal modification of signal strength). Work of this type has just begun in the NOD mouse to determine the impact on diabetes. Initial experiments with HC in NOD FTOC have shown the ability to mitigate diabetes in vitro, but whether this change in disease onset is due to positive changes in immune development (i.e. the elimination of diabetogenic T cells, a shift towards a more T_H2 profile, or the enhancement of regulatory cells) or negative changes in immune development (i.e. a lack of functional immune cells) is yet to be determined. Nevertheless, it has become apparent in recent years that avidity-based changes during T cell development may hold the key to finding treatments that can simultaneously correct a large portion of the autoimmune diseases that currently exist.

Conclusion

Research presented in this dissertation suggests a slightly different way of evaluating and analyzing the impact of substrates on T cell selection. Analysis of this type is in its infancy. There are still many more questions to be asked, including: How do these changes in avidity truly impact T cell development in the human model which has multiple MHC types (in terms of all the functional phenotypes (SP4, SP8, NKT, γδ T cells, etc)? How do these changes effect the TCR usage within mature T cell groups? What is the impact of these changes on immune function and autoimmunity? Preliminary research, using the simple 1 MHC NOD model, indicate that 12 day anti-CD1d treatment and HC treatment have the potential to inhibit disease in our *in vitro* diabetes model (Appendix B for anti-CD1d. For HC data not shown). However, the true test of these treatments will be *in vivo*, which is currently where the most difficulties reside.

Treatment *in vivo*, even in mouse models, is more difficult because of the "thymic-blood barrier" found in the thymus. Research performed investigating GCs has shown that increases in corticosterone in pregnant mothers does not effect corticosterone levels in the fetuses (123). This would make the in utero treatments, used in our peptide therapy work, obsolete. It is also difficult for treatments to penetrate the thymus through the intra venous addition of substrates, as shown in work by Le Gros et al (124). However, therapy of the type presented here may have better therapeutic potential/safety for autoimmunity than treatments targeted at T cells in the periphery, which have shown limited benefits and potential to accelerate disease (114, 125).

Thymus based elimination of autoreactive T cells and enhancement of regulatory T cells, as seen in the peptide therapy and anti-CD1d research, have shown a the ability to mitigate T1DM without the risk of autoreactive cell expansion or cytokine based toxic effects that have been seen with treatment types in the periphery. Research has shown that just shifting the ratio of T_H1/T_H2 cells, through IL-10 cytokine addition in the periphery, or enhancing NKT production from the thymus can slow disease progression or stop the induction of T1DM entirely. This makes avidity-based treatments a viable option, since treatment may not be needed at the initiation of T cell development. In other words, avidity based treatments could be effective during the juvenile phases of life, when T cell production has already begun but is still ongoing, because treatments would have the capacity to effect the production of new autoreactive T cells and/or the production of autoimmune regulators. Continuing research in this area can help to ensure that we develop a better understanding of how T cell development occurs on a larger scale, beyond the system specific changes within the cell. From a therapeutic standpoint, understanding the underlying minutiae that induces changes, although empirically important, will not be as important as the overall effect of therapeutic treatment.

Final Notes

The years I have spent here at the University of Arizona and in the DeLuca Lab have taught me many things about science and about myself. My personal revelations I will keep to myself, but in terms of science I have learned that to be accurate and fair you must always honestly question everything (i.e. dogmas are made to be broken). Also,

your own expectations and predictions can sometimes get in the way of honest data analysis. Therefore, carefully consider all possibilities and not just the ones you have proposed. Much of the insight and models generated in this dissertation were the result of experiments that were focused on answering different hypotheses than those that were answered in the end. Finally, no experiment is ever truly finished and no experimental setup is ever perfect. There is always another experiment to be performed. For those of you reading this, I would like to thank you for your time and hope you have taken the time to analyze, understand, and question everything that has been presented here.

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FOOTNOTES

[a] Successful TCR rearrangement leads to the formation of a novel and very specific TCR type which is capable of interacting with only a very limited number of MHC + peptide combinations in a MHC class restricted manner. For many years the dogma, which has been more recently disproved, was "one TCR type for one MHC + peptide combination".

[b] TAP-Transporters associated with antigen processing: The TAP-1 molecule is thought to be responsible for transport into the endoplasmic reticulum (ER) and minor cleavage of peptides that have been processed by the proteasome. These peptides will be bound to class I MHC as part of the endogenous protein presentation pathway.

[c] The NOD mouse is used as an animal model for human IDDM due to the fact that they share many key features of diabetes and diabetes induction. These include the presence of insulitis, heritable major histocompatibility complex (MHC) Class II alleles, and T cell dependent pathogenesis.

APPENDIX A

THERAPEUTIC ALTERATION OF IDDM PROGRESSION BY T CELL TOLERANCE TO GAD65 PEPTIDES *IN VITRO* AND *IN VIVO**

by Stephen S. Wilson†, Todd C. White and Dominick DeLuca††

Department of Microbiology and Immunology

The University of Arizona, Tucson, Arizona, 85724

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† La Jolla Institute for Allergy and Immunology, San Diego, California, 92121. (858) 558-3500

††To whom correspondence should be addressed. Department of Microbiology and Immunology, Life Sciences North, Room 648, The University of Arizona, Tucson, Arizona, 85724. Office: (520) 621-8941, FAX: (520) 626-2100. E MAIL: deluca@u.arizona.edu.

ABSTRACT

We have reported previously that NOD fetal pancreas organ cultures (FPOC) lose the ability to produce insulin when maintained in contact with NOD fetal thymus organ cultures (FTOC). Initial studies indicated that exposure to glutamic acid decarboxylase (GAD65) peptides *in utero* resulted in delay or transient protection from the IDDM in NOD mice. We also found that exposure of young adult NOD mice to the same peptides could result in acceleration of the disease. To more closely examine the effects of early and late exposure to diabetogenic antigens on T cells, we applied peptides derived from GAD65 (GAD amino acids, 246-266, 509-528, and 524-543), to our "*in vitro* IDDM" (ivIDDM) model. T cells derived from NOD FTOC primed during the latter stages of organ culture, when mature T cell phenotypes are present, had the ability to proliferate to GAD peptides. IvIDDM was exacerbated under these conditions, suggesting that GAD responsiveness correlates with the ivIDDM phenotype, and parallels the acceleration of IDDM we had seen in young adult NOD mice. When GAD peptides were present during the initiation of FTOC, GAD proliferative responses were inhibited, and ivIDDM was reduced. This result suggests that tolerance to GAD peptides may reduce the production of diabetogenic T cells or their capacity to respond, as suggested by the *in utero* therapies studied in NOD mice.

INTRODUCTION

Type I Diabetes (IDDM) is the clinical result of immune-mediated beta islet cell destruction. Because IDDM is due, in part, to one's genetic background (1), a cure may be affected by manipulation of the genome. However, this approach is not heritable, and may cause serious harm to the patient via as yet unknown mechanisms. A potentially safer approach might be to remove (or severely dampen) the capacity of a pre-disposed individual's T cells to respond to the pancreatic antigen(s) that are most directly involved in the initiation and/or chronic activation that leads to the destruction of islet cells. Several candidate islet antigens have already been identified. Given more extensive research, IDDM related immune responses to a limited number of these antigens may be correlated with the specific MHC backgrounds found in given individuals (2, 3). This information has been used to develop a strategy for preventing the induction of IDDM by treating patients with diabetogenic antigens before their disease had fully manifested itself (4-8). However, in some initial clinical studies, IDDM onset was not altered by diabetogenic antigen treatment, and evidence suggested the possibility that such treatment may accelerate disease induction (9).

Because of their similarities to patients with human IDDM (10), Non Obese Diabetic (NOD) mice have been studied to determine what islet associated antigens are recognized early in disease. In two studies, the earliest spontaneous responses in pre-clinical mice were directed against proteins or peptides of human glutamic acid decarboxylase (GAD65) (11, 12). The fact that mice spontaneously developed reactivity

to these peptides prior to the development of reactivity to other islet associated components lead some researchers to hypothesize that GAD65 may play a role in disease initiation (11, 12) through the process of determinant spreading (13). More recently, additional immunogenic epitopes from GAD have been described (13); their role in disease pathogenesis remains unclear, but they appear to be responsible for a large part of the T cell response to GAD65. Neonatal mice treated with tolerizing doses of GAD65 protein were protected from spontaneous IDDM induction (11). Further, NOD mice were protected from IDDM by administration of GAD at 3 weeks of age (12). Together, these studies suggest that autoimmune reactivity to GAD65 is an important step in the progression towards clinical disease in NOD mice.

One potential problem when using the NOD model is the ability of general immune stimulation to block efficient IDDM induction. Reports have demonstrated IDDM-protection by treatment of NOD mice with LPS (14) or poly I:C (15). Indeed, NOD mice that are not kept in largely pathogen-free environments succumb to IDDM at significantly reduced rates (10). Thus, a second interpretation of GAD65 induced IDDM modulation may be that the high dose of antigen used to stimulate the immune system might prevent animals from becoming clinically diabetic in a manner independent of the specific determinant. Some researchers, for example, have found that while GAD peptide immunization could not prevent IDDM in NOD mice, Diphtheria-Tetanus toxoid-Acellular Pertussis alone or insulin B chain peptide could mitigate IDDM (16). Furthermore, recent work also suggests that intrathymic injection of NOD mice with

whole pancreas tissue or insulin B chain peptides could prevent IDDM, but that certain GAD peptides accelerate disease (17).

To explore the potential role of GAD65 reactivity in the inhibition or acceleration of NOD IDDM, several experiments were performed using peptides from GAD65 to affect the generation of diabetogenic T cells *in vivo* as well as in the more controlled NOD FTOC "*in vitro* IDDM" (ivIDDM) system.

NOD mice exposed only *in utero* to GAD peptides were found to develop IDDM at a significantly lower rate. This limited protection is postulated to be due to increased intrathymic presentation of GAD peptides, leading to a reduction in the initial number of GAD auto-reactive T cells that would reach maturity. Thus, experiments to directly measure the effects on islet responsiveness after early T cell tolerance to GAD were performed. Previously, we had shown that FTOC, which has been pre-cultured for 14 days, can inhibit the production of insulin in subsequently added fetal pancreas, as well as provide T cells which migrate into the co-cultured FP causing insulitis-like lesions (18). This system allows for the evaluation of the effects of immunomodulators on the development of diabetogenic T cells without the involvement of peripheral regulatory mechanisms. The ivIDDM activity of these cultures can be attenuated by co-culturing the developing FTOC while in contact with NOD FPOC from the first day of culture. Co-culturing presumably induces tolerance by exposing pre-T cells to high doses of islet antigens, leading to their inactivation or deletion. To determine if GAD65 peptides could confer the same protective effects (and mimic those of *in utero* exposure), FTOC were treated with increasing doses of soluble peptide from the initiation of culture. Those T

cells that were cultured in the presence of GAD65 peptides were compared to those from NOD FTOC that were cultured with control peptides or in the absence of high dose peptides to determine if GAD65 treatment could alter the ability of NOD FTOC to inhibit insulin production by the subsequently added FP. Indeed, FTOC that are normally ivIDDM "diabetogenic" were rendered benign by early exposure to certain GAD peptides, suggesting that this specificity is critical to efficient T cell involvement in recognition and islet cell destruction.

A similar approach was taken to evaluate the acceleration of IDDM in NOD mice treated with GAD peptides as young adults. In this case, NOD FTOC were treated with GAD peptides after the production of mature T cells, increasing their ability to react with these antigens. When ivIDDM was subsequently performed, those FTOC that had been primed to GAD peptides demonstrated an increased induction of this *in vitro* correlate to diabetes.

MATERIALS AND METHODS

Peptides

GAD65 246-266 (NMYAMMIARFKMFPEVKEKG), GAD65 509-528 (IPPSLRYLEDNEERMSRLSK) L for N at position 8 as reported in (2), GAD65 524-543 (SRLSKVAPVIKARMMEYGTT), PSA 95-109 (SFPHPLYDMSLLKNR), MBP 123-137 (PRTPPPSQGKGRGLS), and HEL 11-25 (AMKRHGLDNYRGYSL) peptides were synthesized by Biosyn Biotechnology and Research Genetics using standard and proprietary Fmoc chemistry, and a purity of >80% was established by HPLC and mass spectrometer measurements. All peptides were dissolved in HPLC grade water prior to addition to FTOC in sterile organ culture DME (*in vitro*). The GAD peptides and the control HEL peptide are recognized by the IA^{g7} MHC class II molecules expressed in NOD mice, while the PSA and MBP control peptides are not.

Mice

Breeding pairs of NOD/Lt mice were obtained as a kind gift from the laboratory of Dr. E. Leiter at The Jackson Laboratory (Bar Harbor, ME). Our colony was maintained in a specific pathogen free vivarium at the University of Arizona Central Animal Facility, and propagated by brother-sister mating. Mice are allowed free access to standard breeder chow (S-2335 irradiated breeder chow, Harlan Teklad, Madison WI) and autoclaved drinking water. The incidence of IDDM in NOD/Lt females in our colony at the University of Arizona is >80-90% by 40 weeks of age. NOD/Lt mice were then bred to

produce timed-pregnant females. The fetuses were removed from pregnant females at the indicated time points (plug date = day 0). We consistently found that our animals were variable with regard to their stage of development, even though they had been vaginally plugged on the same day. We therefore re-staged the pups that we obtained based on their developmental characteristics (such as digit separation on the paws) as given in "The Mouse, its Reproduction and Development" (19).

Fetal Thymus/Pancreas Organ Culture

The organ culture methods used have been described in detail by our laboratory and elsewhere (20) Briefly, at least 6 thymus lobes and/or equal numbers of pancreata (usually 10) dissected from 13-16 day gestation (dg) fetal mice were placed on the surface of Millipore (25 mm thick, 0.45 mm pore size) filters which were supported on blocks of surgical Gelfoam (Upjohn Co., Kalamazoo, MI) in 3 mL of medium in 10 x 35 mm plastic Petri dishes. Dulbecco's modified Eagle's (DMEM) medium (4.5 g/L D-glucose), supplemented with 20% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) was used. The medium also contained streptomycin (100 mg/mL), penicillin (250 mg/mL), gentamycin (10 mg/mL), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (2 x 10^{-5} M), as well as 3.4 g/liter sodium bicarbonate. The cultures were grown in a fully humidified incubator in 5% CO_2 in air at 37°C. Cells were harvested from FTOC and FPOC using collagenase digestion, as has been reported previously (20) or by manual dissociation by teasing tissue from the

millipore strips in HBSS + 5% FCS solution. Viability was consistently high (>90%), for both thymic and pancreatic tissue, as determined by trypan blue exclusion.

Supernatant recovery and insulin radioimmunoassay (RIA)

At the specified days of culture, 300 µl of culture supernatant was removed from each 3 mL culture dish for insulin RIA. MicroMedic® Insulin RIA kits were obtained from ICN MicroMedic Systems, Inc. (Horsham, PA) and used to measure the quantity of insulin in culture supernatants as directed by the manufacturer's instructions, and standardized to bovine insulin. After measuring the amount of insulin in a 300 µl sample, the amount of insulin in the 3 mL culture was determined. This value was divided by the number of fetal pancreata to give the amount of insulin secreted per pancreas (µIU/pan). The total amount of insulin in each culture was measured without regard to the degradation of insulin in culture, and represents the total accumulation of insulin during the time interval reported.

Proliferation Assays

NOD FTOC were cultured for 14 days in standard FTOC, as previously reported (20), to produce phenotypically mature populations of TCR-bearing T cells (18). Except for studies of spontaneous responses, after the initial culture period of 14 days either in the presence from day=0 ("tolerance"-inducing), or absence (*in vitro* "priming") of synthetic peptides (see Results), FTOC were treated with a mixture of soluble GAD peptides (10 µg/mL), the control peptide (10 µg/mL), or fresh medium. After a final additional 48

hours in organ culture, cells were removed from FTOC, dissociated, counted, and plated in triplicate at 2-4 x 10^5 cells per well in 96 well plates. Cells were then immediately challenged in 10 μg/mL of GAD65 peptides 246-266, 509-528, and 524-543 or 10 μg/mL of the control peptides for 48 hours. After this stimulation period, FTOC cells were pulsed with 1 μCi ^{3}H-thymidine for an additional 24 hours, mechanically harvested and counted using a Packard Beta Scintillation Counter. In addition to GAD65 peptides and the non-immunogenic PSA peptide (10 μg/mL), identical FTOC populations were also challenged with Concanavalin A (Con A, 2.5 μg/mL) as a positive control. Stimulation index (SI) was calculated as the test counts divided by the background control standard (as defined in the text).

Peptide Treatment of Pregnant and Young Adult Mice and Assessment of IDDM In vivo

Pregnant NOD/Lt mice, at 14-15 days gestation, received a single injection (intra-peritoneal) consisting of 50 mg of peptide in 250mL sterile Phosphate Buffered Saline (PBS). Treatment consisted of one of the following peptides or peptide combinations: (1) GAD65 246-266 (p17), (2) GAD65 509-528 (p34), (3) GAD65 524-543 (p35), (4) GAD65 509-528 AND GAD65 524-543 COMBINED (50mg of each peptide per mouse) (5) GAD65 246-266, GAD65 509-528 AND GAD65 524-543 COMBINED (50mg of each peptide per mouse), (6) HEL 11-25, (7) MBP 123-137, (8) MBP 123-137 AND PSA 95-109 COMBINED (50mg of each peptide per mouse). Injection groups 1-5 consisted of auto-reactive GAD65 IA^{g7}-binding peptides. Injection group 6 consisted of HEL 11-

25, an IA^{g7} binding peptide known to produce proliferative responses in NOD mice. Injection groups 7 and 8 consisted of peptides unable to be bound by IA^{g7}.

Age-matched 3-4 week old littermate pre-diabetic female NOD mice were injected once IP with a mixture of GAD65 509-528 and 524-543 at a low (0.5 µg of each peptide per mouse, n=5) or high (50.0 µg of each peptide per mouse, n=5) dose, or 50 µg/mouse of an irrelevant control peptide (PSA 95-109, n=5) in PBS.

Mice were assessed for the development of IDDM by testing every 10-14 days, (after 10 weeks of age for the offspring of pregnant mice and after 15 weeks for young adults) for glycosuria. Those mice that were found to have a urine glucose reading >200 mg/dL (GlucoStix) were removed from the colony and re-tested for hyperglycemia using a digital blood glucose monitor. Those mice with a confirmed blood glucose level >250 mg/dL were considered diabetic.

Statistical Analysis

Data were analyzed using StatView 4.5 from Abacus Concepts (Berkeley, CA). Experimental differences in IDDM incidence in treatment groups were assessed by Kaplan-Meier life table analysis using log rank (Mantel-Cox) test for significance. Unpaired Student's t-test was used for final determination of the significance of the effects of treatments given to organ cultures. A p value < 0.05 was considered statistically significant for all statistical analyses.

RESULTS

Low dose immunization with GAD65 peptides increases IDDM incidence in NOD mice.

Early reports have established that GAD65 responsiveness is found in 3 week old NOD mice (11, 12), and that tolerance to GAD65 protein is protective (11). However, some reports suggest that GAD peptides can accelerate IDDM (17). Thus, to determine if IDDM severity or onset in NOD mice could be increased or accelerated, GAD65 peptides were given in tolerogenic and stimulatory doses to pre-diabetic mice.

Age-matched 3-4 week old littermate female NOD mice were injected once IP with either 50 μg each of a mixture of GAD65 peptides or 50 μg of the control peptide in saline. Mice were then followed for the development of hyperglycemia (Figure 1). Pre-diabetic female NOD mice treated with a 0.5 μg dose of GAD65 peptides ($n = 5$) developed IDDM at an accelerated rate as compared to control peptide ($n = 5$) treated groups ($p = 0.046$). The high dose treatment appeared to have protective effects on treated NOD mice that, in stringent analysis, were nearly statistically significant in Kaplan-Meier survival analysis ($p = 0.084$).

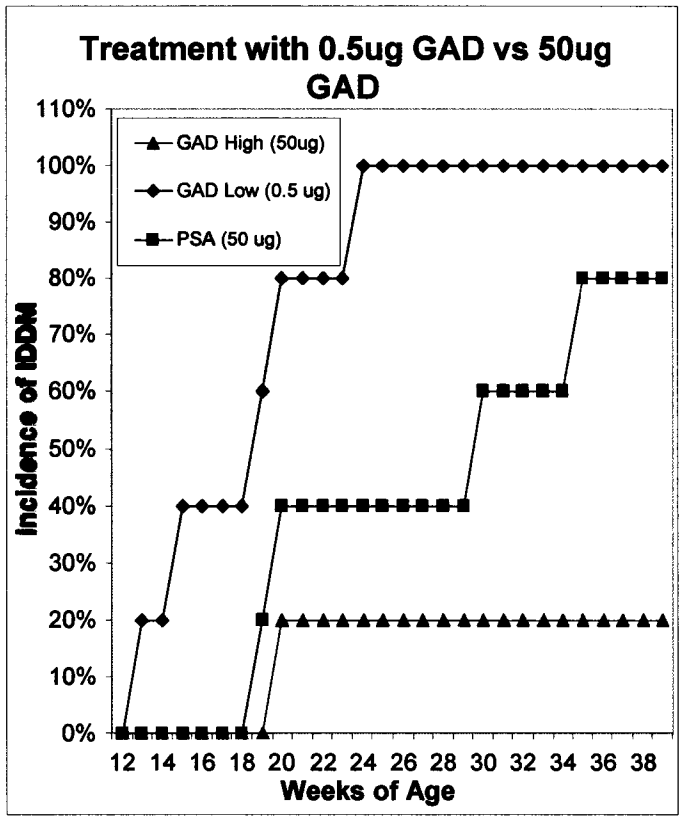

Figure 1. A low dose of a mixture of GAD65 509-528 and GAD65 524-543 injected into pre-diabetic NOD mice accelerates IDDM. Age-matched 3-4 week old littermate pre-diabetic female NOD mice were injected once IP with a mixture of GAD 509-528 and 524-543 at a low (0.5 μg/mouse of each peptide, diamonds) or high (50.0 μg/mouse of each peptide, triangles) dose, or 50 μg/mouse of an irrelevant control peptide (PSA 95-109, squares). Those mice treated with a low dose of GAD peptides (n = 5) developed IDDM at an accelerated rate as compared to control mice ($p = 0.046$). Despite the appearance that high dose treatment had protective effects on treated NOD mice (n =5), the curve was not found to be statistically significant ($p = 0.084$) when compared with the PSA peptide treated control.

Offspring of GAD-treated pregnant NOD mice display a significantly delayed onset of IDDM.

Because the initiation of IDDM seemed so critically dependent upon the responsiveness to GAD65 initially, we examined the ability of individual and mixtures of GAD peptides 246-266, 509-528, and 524-543 to alter the clinical effects of IDDM in NOD mice at the earliest possible time point.

At 14-15 days gestation, three groups of pregnant NOD mice were injected once intraperitoneally (IP) with either 50 μg of GAD65 246-266, or 509-528, or 524-543 (GAD65 p17, p34, or p35 single), 50 μg of a mixture of GAD65 509-528 plus 50 μg GAD65 524-543 (GAD65 p34+35 mix), or 50 μg of a mixture of GAD65 246-266, 50 μg 509-528 and 50 μg GAD65 524-543 (GAD65 p17+p34+p35 mix). Controls consisted of 50 μg of HEL 11-25 (control HEL single, a tight binding peptide to IA^{g7} which elicits a strong immune response), 50 μg of MBP 123-137 (control MBP single, which does not bind to IA^{g7}) and a mixture of 50 μg MBP 123-137 plus 50 μg of PSA 95-109 (control MBP+PSA mix). After weaning, male NOD mice were culled and female offspring mice were monitored for the development of hyperglycemia (see Materials and Methods). This protocol was chosen in order to examine the potential benefits of using a wide range of GAD determinants for tolerance induction and disease prevention, as compared with single peptides alone.

As compared to offspring of NOD mothers treated mid-gestation with either a single control peptide (the IA^{g7}-binding HEL 11-25 or the non-binding MBP 123-137) or a mixture of control peptides (PSA 95-109 + MBP 123-137), offspring of mice given

some GAD65 peptide treatment regimens clearly had a reduced incidence of IDDM (Figure 2). NOD offspring treated with GAD65 246-266 singly (p=0.035, n=14) developed IDDM at a reduced rate when compared with HEL control peptide. Offspring treated with the GAD65 509-528/524-543 mixture (p=0.003, n=10) developed IDDM to a reduced overall level, and at a significantly reduced rate of onset, as compared to age-matched control (PSA+MBP) mix-treated animals. However, offspring of mice treated with GAD65 509-528 (p=0.202) or GAD65 524-543 singly (p=0.388) were not significantly protected from disease when compared with animals treated with HEL. Offspring of mice treated with a mixture of GAD65 246-266, 509-528 and 524-543 were also not significantly protected (p=0.226, n=9) when compared with animals from dams treated with MBP+PSA. Of interest, however, is that the initial incidence of IDDM in the 3 peptide treated group is similar to the controls at 18 weeks, but this value failed to climb until week 37, and then only slightly. Animals given the mix with two peptides or single peptides developed disease more slowly, but after 27 weeks, the incidence increased rapidly to a value similar to that of the animals treated with three peptides. Clearly, the rapid rise of disease incidence in the 3 peptide treated animals is responsible for the lack of significance of disease protection in this group. However, some animals in all GAD peptide treated groups never developed disease. The GAD 509-528/524-543 mixture group has been carried out for over 45 weeks with 40% of the animals still disease free. Some of the GAD65 246-266 treated mice have also been followed for 45 weeks, with a similar IDDM incidence.

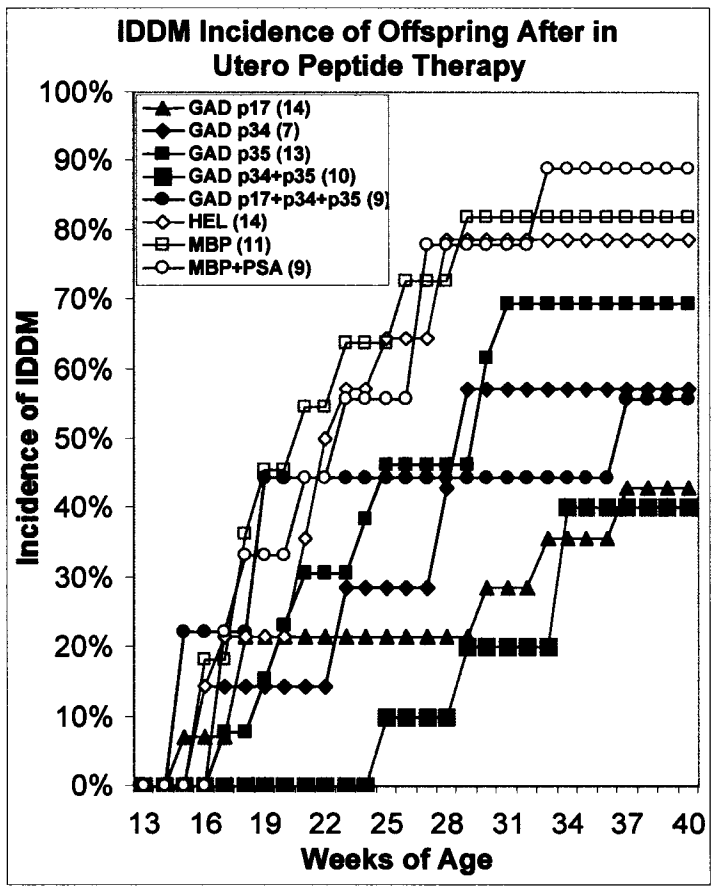

Figure 2. *In utero* treatment with GAD65 peptides delays the onset of IDDM in offspring mice. 14-15 day gestation NOD female mice were injected once IP with either a 50 μg mixture of each of GAD65 peptides 246-266, 509-528 and 524-543 (p17+p34+p35, closed circles), a 50 μg mixture of each of GAD65 peptides 509-528 and 524-543 (p34+p35, x's), 50 μg of GAD65 peptide 246-266 (GADp17, closed triangles), 50 μg of GAD65 peptide 509-528 (GADp34, closed diamonds), 50 μg of GAD65 peptide 524-543 (GADp35, closed squares), 50 μg of a mixture of each control peptide (PSA+MBP, open circles), or a single control peptide (HEL, open diamonds or MBP, open squares). Offspring female mice (the number in each treatment group is shown in parentheses in

the figure legend) were monitored for the development of diabetes. IDDM in offspring of mice treated with GAD65 246-266 (n=14) was significantly inhibited as compared to mice treated with a single control peptide (HEL, p=0.035) or the control peptide mixture (MBP+PSA, p=0.014). IDDM induction in offspring of mice treated with a mixture of GAD 509-528 and 524-543 (n=10) were also significantly inhibited as well as delayed as compared to mice treated with control peptides (HEL, p=0.011 and MBP+PSA, p=0.003). However, offspring from females treated with GAD 509-528 (p=0.203) and 524-543 (p=0.388) were not significantly inhibited in their IDDM induction compared with the HEL single peptide control. Offspring from mice treated with a mixture of GAD65 246-266, 509-528 and 524-543 (n=9) were also not significantly different from the MBP+PSA (p=0.226) control peptide mixture or the HEL single peptide control (p=0.448).

The acceleration of IDDM observed in the young adult NOD mice given low doses of GAD peptides coupled with the diminished incidence in IDDM observed in the pups exposed to the same peptides *in utero* suggested mechanisms that involve activation of diabetogenic T cells in the former case and immune tolerance (albeit transient) in the latter case altered IDDM initiation or propagation. However, IDDM in NOD mice appears to be quite sensitive to any if not most immune perturbations, thus we wished to directly measure the effects of T cell activation and tolerance to GAD peptides upon islet destruction in the highly controlled ivIDDM system. We also wished to determine the ability of the ivIDDM system to reflect the induction of IDDM *in vivo.*

The response in NOD FTOC is not specifically or spontaneously enhanced to GAD65 peptides.

To determine if NOD FTOC was spontaneously primed to GAD65 peptides without any exogenous stimuli, NOD FTOC were cultured to produce phenotypically mature populations of TCR-bearing T cells. After this initial culture period, cells were removed from FTOC and placed in 96 well plates. Cells were then challenged to proliferate in response to GAD65 peptides 246-266, 509-528, and 524-543 (10 μg/mL). In addition to GAD65 peptides, identical FTOC populations were also challenged with a non-immunogenic peptide (PSA 95-109) and Concanavalin A.

NOD FTOC-derived cells did not proliferate in response to GAD65 246-266, 509-528 or 524-543, significantly ($p > 0.3$) above the level of the non-immunogenic control peptide PSA (SI = 1.0) or FTOC cells left unchallenged (not shown). The cultures did

respond to Con A stimulation (SI=4.5±0.5), and were thus considered competent to respond in a receptor-mediated fashion. The response to Con A, which is lower than that of mature splenic T cells, is typical of the immature cells produced by organ cultures (21), and the response is roughly equivalent to those made by thymocytes during the first month after birth (22).

T cells in NOD FTOC can be primed to GAD65 peptides.

Based on data described above, it appeared that a significantly enhanced spontaneous response to GAD65 peptides was lacking in FTOC. We wished to determine if mature phenotype NOD FTOC indeed produced self-reactive cells in low abundance and could be enriched or activated/primed to produce GAD65-responsive T cells as this might normally occur in the NOD periphery.

NOD FTOC were cultured for 14 days in standard FTOC, as described previously (18) to produce phenotypically mature populations of TCR-bearing T cells. Peptide solutions of either a mixture of GAD65 246-266, 509-528, and 524-543 peptides, fresh medium, or the control PSA peptide were then added to FTOC to produce a final concentration of 10 μg/mL. After an additional 48 hours in organ culture, cells were removed from FTOC and challenged versus each peptide individually in proliferation assays.

As shown in Figure 3, NOD FTOCs that were primed using the mixture of GAD65 peptides were significantly more responsive to GAD65 524-543 ($p < 0.02$) as compared to identical cultures that were primed with the control peptide ($p < 0.01$) or left

untreated ($p < 0.01$). Those cultures primed using GAD65 peptides were also significantly more responsive to GAD 509-528 than cultures that were not primed ($p = 0.045$). In contrast, the response to GAD65 246-266, was not significantly enhanced compared to control peptide ($p = 0.51$) or untreated cultures ($p = 0.19$). Cultures primed with PSA and then challenged remained unresponsive to PSA ($p = 0.10$), while the Con A response was positive.

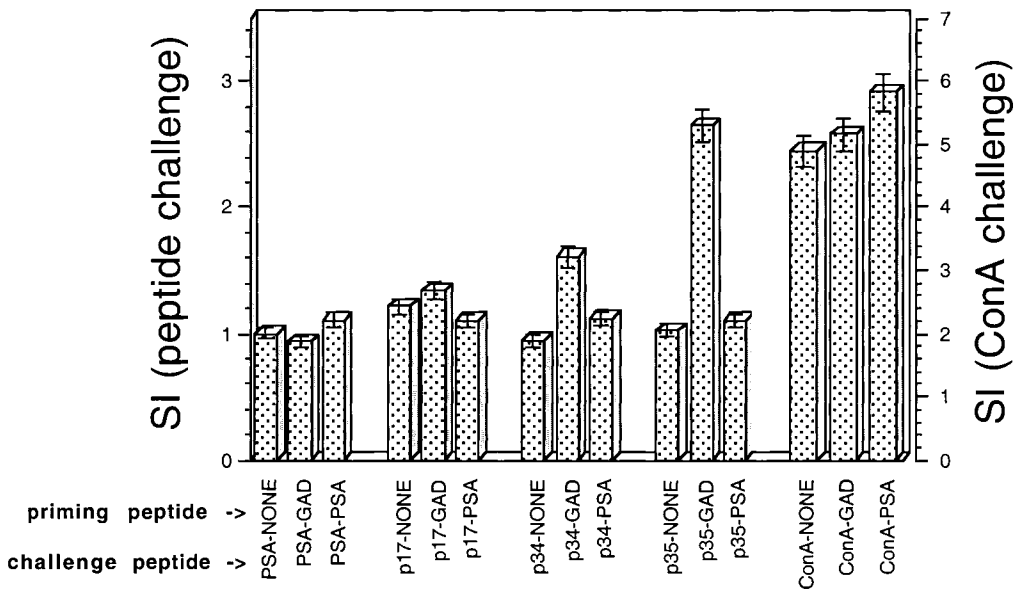

Figure 3. Mature NOD FTOC can be primed to GAD65 peptides 509-528 and 524-543. NOD FTOC were cultured for 14 days and then primed *in vitro* with either a mixture of GAD65 peptides, a non-immunogenic control peptide, or untreated. After an additional 48 hours of culture, FTOC cells were dissociated and challenged with 10 μg/mL of each peptide, a control peptide (PSA), left unchallenged, or challenged with 2.5 μg/mL ConA (n=5). Error bars represent the SEM. All groups have a stimulation index as compared to control peptide "primed" cultures. The range of CPM responses used to calculate the average SI in these experiments was 3371 CPM to 48,765 CPM.

FTOC that is primed specifically to GAD65 524-543 displays greater "ivIDDM" activity.

To determine the functional impact of specific autoantigen stimulation on the ivIDDM model system, FTOC were primed with synthetic GAD65 peptides after the development of phenotypically mature T cells, at the initiation of co-culture with FP (usually 14 days of culture). Peptide solutions of either a mixture of the three GAD65 peptides at 10 or 100 μg/mL, or 100 μg/mL of the control PSA peptide were then added to FTOC, as was done previously to "prime" FTOC. This treatment increases FTOC proliferative responses to GAD65 peptides 509-528 and 524-543 (Figure 3). At this time, freshly procured NOD FP were added to each of the GAD65-treated and control FTOC. At 7, 14, and 21 days post-stimulation/co-culture, supernatants were removed from the cultures and used to determine the effect stimulated and unstimulated FTOC had on insulin production in the FP in co-culture.

As shown in Table 1, at the peak of ivIDDM (day 21), priming NOD FTOC to GAD65 peptides 524-543 increased the ivIDDM activity (bold type). Interestingly, this "priming" protocol did not produce an enhanced ivIDDM effect when using GAD65 246-266 or 509-528, even though the latter was clearly an antigenic peptide for the FTOC-derived cells (Figure 2). Indeed, in one experiment, both 10 μg/mL and 100 μg/mL of GAD65 246-266, and 100 μg/mL of GAD65 509-528, prevented ivIDDM at 21 days of FP co-culture (italics). This effect, however, was modest, and not reproducible. These data suggest that there can be a parallel between the ivIDDM system and the diabetogenic response *in vivo*, as both can show an increase in disease if T cells are activated to diabetogenic antigens prior to exposure to pancreatic tissue.

Table 1. Addition of GAD65 peptide 524-543 at the time of FP co-culture induces a greater degree of "*in vitro* IDDM" activity from NOD FTOC.

TYPE	PRIMING PEPTIDE	INSULIN (μIU/pan) iv		IDDM (% CONTROL)	
		EXP.#1	EXP.#2	EXP.#1	EXP.#2
FP ALONE	INS. CNTRL (NONE)	326.0	212.8	100%	100%
FT/FP	IDDM CNTRL (NONE)	192.1	125.9	59%	59%
FT/FP	100mg/mL 509-528	212.1	272.8	65%	*128%*
FT/FP	10mg/mL 509-528	196.0	129.8	60%	61%
FT/FP	100mg/mL 524-543	65.4	65.4	20%	31%
FT/FP	10mg/mL 524-543	61.3	45.4	19%	21%
FT/FP	100mg/mL 246-266	ND	279.4	ND	*131%*
FT/FP	10mg/mL 246-266	ND	228.8	ND	*108%*

NOD FTOC were cultured 14 days to produce phenotypically mature populations of TcR-bearing T cells. Peptide solutions of either a mixture of the three immunodominant GAD65 peptides at 10 or 100 μg/mL, or 100 μg/mL of the control PSA peptide were then added to FTOC. At this time, freshly procured NOD FP were added to each of the GAD65-treated and control FTOC. At 21 days post-stimulation/co-culture, supernatants were removed from the cultures and used to determine the effect stimulated and unstimulated FTOC had on insulin production in the FP in co-culture. GAD 524-543 induced greater ivIDDM as shown by a decreased production of insulin by the cultures

(underlined and bold). GAD 509-528 (at the high dose) and 246-266 (at both doses) slightly inhibited ivIDDM, as indicated by an increased production of insulin (shown in italics).

NOD FTOC can be rendered specifically unresponsive to GAD65 peptides.

In parallel with the "priming" experiments detailed above and in Figure 3, we wished to determine if priming and recall to GAD65 peptides could be specifically inhibited by treatment with GAD65 peptides during the initial development of NOD FTOC. These studies are of particular interest in light of recent reports that propose the use of peptides to induce protective tolerance to early auto-antigens by treatment of pre-diabetic individuals (23, 24).

NOD FT were placed in standard FTOC, and some cultures were immediately treated (day=0) with peptide solutions of either a mixture of GAD65 509-528 and 524-543, the control PSA peptide at 10 μg/mL, or standard organ culture media. FTOC was then cultured for 14 days to produce phenotypically mature populations of TcR-bearing T cells. During the final 48 hours of FTOC, as with "primed" cultures above (Figure 3), FTOC were treated with a mixture of GAD65 peptides (10 μg/mL), control peptide (10 μg/mL) or fresh medium. At the end of this period, the cells were removed from FTOC and assayed for specific proliferative capacity in response to GAD65 and control peptide challenge.

A representative experiment is shown in Figure 4. NOD FTOC that develops in the presence of GAD65 peptides is no longer reactive to peptides 524-543 and 509-528 of GAD65. In summary of these experiments, the response to 524-543 (SI = 1.11 ± 0.11, n=3) was much less in GAD65 peptide pre-cultured FTOC after priming than in cultures that had not been pre-cultured with GAD65 peptide mixtures (SI= 2.64 ± 0.20, n=5), or as compared to FTOC challenged with control peptide (SI= 1.1 ± 0.04, $p > 0.3$, n=3 for

all peptides). As a positive control of responsiveness (25), Con A stimulation of identically treated cultures remained significantly higher than all peptide-challenged FTOC (SI= 2.23 ± 0.42, $p < 0.05$, n=3).

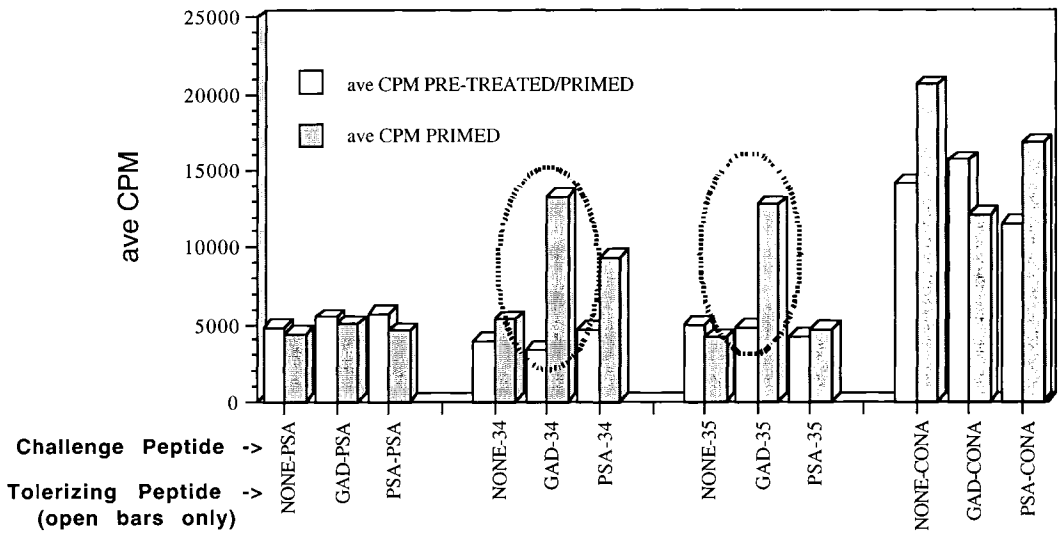

Figure 4. Mature NOD FTOC treated since day=0 (open bars) of culture are no longer responsive to challenge when compared with untreated cultures (closed bars). NOD FTOC was treated with 10 μg/mL of either a mixture of GAD65 peptides, a control peptide, or left untreated at the initiation of culture. NOD FTOC were cultured for 14 days and then primed *in vitro* with a mixture of GAD65 peptides. After an additional 48 hours of culture, FTOC cells were dissociated and challenged with 10 μg/mL of each peptide, a control peptide (PSA), left unchallenged, or challenged with 2.5 μg/mL ConA (shown is a representative experiment of n=3). The responses of cultures to GAD 509-528 and 524-543 that are specifically reduced by pre-treatment with the relevant peptide are circled.

Unresponsiveness to GAD65 peptides prevents ivIDDM.

Reports from *in vivo* studies have indicated that selective tolerance to GAD65 protein prevents the onset of IDDM in NOD mice (11, 12). We wished to determine if rendering FTOC unresponsive by peptide treatment during the development of mature T cells would ablate ivIDDM.

NOD FT were placed in standard FTOC and then immediately treated with peptide solutions of either a mixture of GAD65 peptides (see Materials and Methods) at a total of 0.1 μg/mL to 100 μg/mL, or 100 μg/mL of the control PSA peptide. FTOC was then cultured for 14 days in standard FTOC, to produce phenotypically mature populations of TCR-bearing T cells (in the presence of GAD65 and control peptides). At this time, freshly procured NOD FP were added to each of the treated and untreated control FTOC. At 21 days of co-culture, supernatants were removed from the cultures and used to determine the effect stimulated and unstimulated FTOC had on insulin production in the FP in co-culture.

Figure 5 shows two experiments in which there was a dose-dependent reduction in ivIDDM caused by early "tolerogenic" treatment of FTOC with GAD65 peptides.

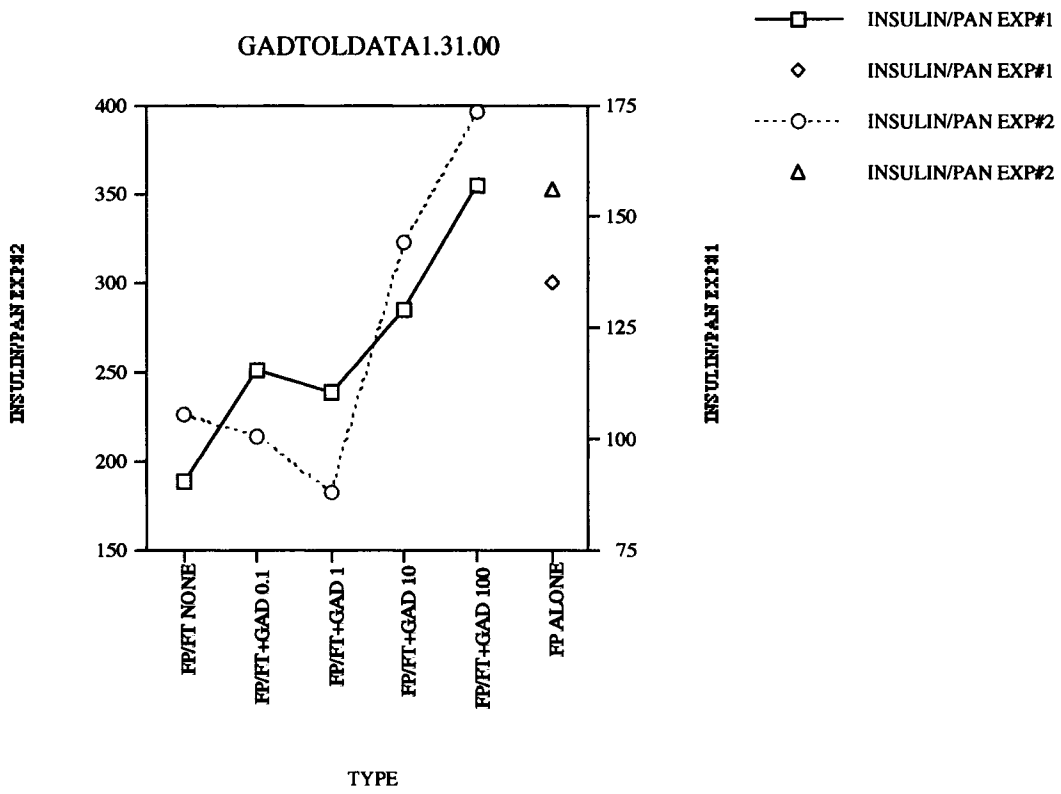

Figure 5. Possible tolerogenic activity of GAD peptides. At the onset of FTOC, cultures were treated with the indicated levels of a mixture of GAD65 peptides (246-266, 509-528 and 524-543), or left untreated. After 14d of culture, NOD FP were added. After an additional 21d of FT/FP co-culture, insulin produced by FP was measured by RIA in culture supernatants (μIU/FP see materials and methods and (18)). The data from 2 separate experiments are shown as a line graph to demonstrate the dose-dependent protective effect of the peptide treatment on the induction of ivIDDM.

DISCUSSION

Experiments designed to test the efficacy of GAD or GAD peptides to prevent IDDM in NOD mice have given mixed results. Regimens that diminish GAD responsiveness in NOD mice prevented the development of IDDM (11, 12, 26). Those results suggest that the therapeutic effect mediated by the *in utero* treatment in the experiments presented here is due to antigen-specific immune tolerance at a very early developmental stage. However, T cell clones specific for GAD peptides p35 and p17 (the latter being very similar to Coxsackie virus (27)) neither responded to islet cells nor did they accelerate IDDM onset when transferred into NOD mice (28); thus disputing the need for specific T cell activity directed to particular GAD antigens. In addition, recent work using intrathymic injections of whole GAD65 into young NOD mice showed that IDDM could be retarded, but similar treatment with some GAD65 peptides accelerated IDDM (17). It is imperative that we determine the role and capacity of the earliest T cells specific to islet antigens such as GAD65 that are released into the periphery if they are to be targets for IDDM remediation. This concern is heightened by recent data in clinical trials in which treatment with islet cell antigens has the potential to accelerate, rather than prevent, IDDM (9). Our results using the ivIDDM model suggest that indeed, tolerance to GAD peptides has immediate effects upon the most primary population of T cells and the resultant immune capacity to inhibit islet function.

We have previously reported on the use of a modified *in vitro* FTOC system for the study of the development of diabetogenic T cells in NOD mice (18). Here, we wished to determine if the ability of our *in vitro* system to decipher how the response to GAD

peptides might alter IDDM in NOD mice treated *in utero* as well as those *in vivo* systems in which GAD protein was therapeutic, as reported by others. If substantial correlations exist between *in vitro* activity and disease outcome *in vivo*, the FTOC system could provide new insights and predictions about therapies to prevent IDDM, and possibly be used for rapid analysis of the efficacy of various T cell-directed antigen treatments.

Our ability to alter the induction of IDDM *in vivo* with GAD peptides is in agreement with some studies, but not with others. The reason for these different results is unclear, but it is possible that the induction of IDDM resistance with GAD peptides may be more difficult to achieve in adult NOD mice with the dose and route of administration used in some experiments. The ability of the same peptides that delay IDDM *in vivo* to prevent IDDM onset when given during early thymic development *in vitro*, suggests that these peptides may be used to mitigate disease. From our *in utero* data, and that from other studies in which GAD65 peptides accelerated IDDM in young adult NOD mice (17), we would postulate that protection and acceleration of IDDM are both age and dose-dependent. Thus, a fetal NOD mouse given a high dose of GAD65 peptide may very well be protected during the early part of its life when it is using the repertoire established during pre-natal development. As an adult, the same NOD mouse that is now more immunologically advanced and that has continued to produce T cells that are no longer under specific tolerogenic pressures, may be more resistant to disease prevention using the same peptide regimen. Indeed, such a mouse may become diabetic at an increased rate when given the same peptide therapy, especially if a relatively low dose of peptide were delivered. Dose dependent tolerance induction to islet antigens has already been

reported (29). In the experiments reported here, we have found that injection of single peptide preparations, or a mixture of GAD 246-266, 509-528, and 524-543 into 15 day gestation pregnant NOD mice can significantly delay IDDM onset in the pups. It is noteworthy that the mixture of GAD 509-528 + GAD 524-543 was considerably better in preventing IDDM than the single peptides given alone, suggesting that treatment with a broader range of epitopes of GAD could alter the course of IDDM, although the mixture of all 3 GAD peptides did not seem to do any better. In contrast, the same treatment given to young adult NOD mice can either have a marginal inhibitory effect or accelerate disease depending on the dose of GAD peptides used. The latter results confirm earlier reports suggesting that some GAD treatment regimens can, indeed, induce disease (30). This result is consistent with the clinical data mentioned above.

It is of interest that, in the *in utero* studies, the peptides were, apparently, effectively retained in the fetal pups long enough to alter the production of diabetogenic T cells. Presumably, the delay in the induction and overall incidence of IDDM in mice treated *in utero* with GAD peptides, rather than complete inhibition of disease, is due to the continued production of new T cells by the thymus after the introduced high dose of peptides have been degraded and lost. It would seem likely that treatment with higher (or multiple) doses of peptide, over the entire late gestation and early neonatal period, will improve protection against disease. These experiments are currently underway.

We found that NOD FTOC could be induced to respond to a nominal antigen (GAD65 524-543) at a level approximately 3 times greater than background proliferation levels. This is of interest because reports have demonstrated the presence of allogeneic

CTL precursors in FTOC (31, 32), and the ability of FTOC to respond in MLC and IL-2 production assays (31, 33) but not the ability of these cells to be specifically reactive to nominal antigen. As compared to spleen cell preparations from adult mice, a three-fold proliferation index, as shown in Figure 3, is low. However, our results compare quite favorably with FTOC studies that showed a 2 to 28 fold (usually 3-5 times control) response in allogeneic MLC (33, 34). The finding that cells recovered from FTOC (that are roughly equivalent in function and gestational age to 1 week after birth) are able to respond to nominal antigen is in agreement with a report which suggests that neonatal immune populations are responsive to antigenic stimulation (35). In fact, in our proliferation assay system, 10 μg/mL were used to challenge and re-stimulate NOD FTOC; a level one-half to one-tenth that of other reported proliferation assays. Using greater amounts of peptide antigen (or > 2.5 μg/mL ConA) caused uniformly depressed proliferation index values (not shown).

We found that NOD FTOC could not be effectively primed to the immunogenic GAD65 peptide 246-266 versus a control non-immunogenic peptide (Figure 3). The response to 509-528 was also weak. Other work showing early reactivity to GAD 246-266, 509-528, and 524-543 from 3 week old NOD mice (11, 12) suggests that these mice have been primed to these peptides at an early age. The lack of a broad response to GAD peptides in NOD FTOC may indicate an inherent limitation in the response diversity or precursor frequency of the cultures during early fetal development. We chose the 14d time point because the maximum number of cells in FTOC are found at roughly 14 days of culture, and, longer cultures, to our present knowledge, do not produce cells with a

more mature phenotype. It will be of obvious importance to determine if responses to other nominal antigens at similar levels can also be generated by FTOC cells, as well as to determine which antigens FTOC is capable of responding to as compared to similarly aged NOD mice (1 week old). However, this report now indicates that 14 day gestation fetal thymus lobes cultured in FTOC for an additional 14 days are capable of responding to at least one early GAD antigen (524-543) to a level of statistical significance, and, that doses of antigen that are normally benign to adult T cell populations are inhibitory to FTOC-derived responder populations.

The need for priming to obtain a proliferative response to GAD peptides by organ culture derived cells requires consideration as to how the ivIDDM response is achieved. It may be that extrathymic events are critical in the induction of IDDM in NOD mice. Thus, *in vivo*, an event which causes the damage of islet cells with release of islet cell antigens may allow for the stimulation of GAD-specific T cells that have escaped deletion in the thymus due to poor activation machinery in NOD mice (36). This idea gains support from data which show that the GAD65 peptides that we used have been reported to be presented in a class II-restricted manner (23), down-stream of a more primary (possibly class I-restricted) event (37). Such events may be pancreas damage due to occult pancreas infections, or infections with viruses such as Coxsackie B4 which may possess epitopes that are identical to some GAD peptides; thus inducing immunoreactivity to GAD by cross-reaction (molecular mimicry) (27, 38-40). This endogenous priming may also be a result of a generalized inflammatory defect which is adjunct to viral infections (41). In either case, an early event (preceding the generation of

a response to GAD65) may lead to upregulation of processing and presentation of otherwise cryptic peptides. This process of upregulation of presentation leading to exposure of previously cryptic peptide determinants on a protein have been extensively characterized in other autoimmune systems such as the EAE model of multiple sclerosis (42-44). It is of some interest, however, that we were able to generate an ivIDDM response in our system in the present study and in earlier work (18) without overt islet damage in the organ cultured pancreas. The necessary priming of diabetogenic T cells in our system must occur by some process other that acute viral infection or inflammation; possibly by culture-induced release of pancreatic antigens.

We found that the priming of FTOC with GAD peptides in the manner required to induce a response to GAD 524-543 resulted in an increase in ivIDDM activity, but an increased response to GAD 509-528 did not increase ivIDDM. This result suggests that a peptide that is capable of eliciting a T cell response can cause diabetes in our system. Thus, peptides that have been shown to be antigenic in NOD FTOC (GAD 509-528 and GAD 524-543) were differentially capable of increasing ivIDDM. This result is important, since it shows that peptides that are antigenic for NOD FTOC are not necessarily capable of affecting ivIDDM. These peptides are internal controls for one another in this experiment, which also suggests that the mere activation of T cells by a peptide antigen in this system is not responsible for the increase in ivIDDM activity. In the present study, we used a mixture of GAD peptides to accelerate IDDM in young adult mice, but GAD 524-543 alone can accelerate IDDM in these animals (17), suggesting that the ivIDDM system can parallel results obtained *in vivo*.

However, our *in utero* data suggest that GAD 246-266 was capable of preventing IDDM, even though it was not recognized by FTOC-derived T cells. This peptide could not exacerbate ivIDDM, either. Therefore, FTOC is not a completely accurate indicator of the range of responsiveness of NOD mice *in vivo*. Perhaps, as discussed above, the response to GAD 246-266 matures later in the development of the thymus than can be determined in FTOC, preventing its involvement in ivIDDM. However, the apparent persistence of peptide in the pups of mice treated *in utero* may allow for the induction of unresponsiveness by GAD 246-266 in these experiments.

We also found that a relatively low dose (10 mg/ml *in vitro*) of a mixture of GAD peptides could mitigate ivIDDM if they were given to NOD FTOC at the beginning of the culture period, before the development of mature T cells. Thus, peptides that could induce ivIDDM could also prevent ivIDDM if they were given under conditions that could prevent their ability to induce a response (immune tolerance, Figures 4 and 5). This result would be expected if GAD peptides are responsible for some of the immune recognition of pancreas tissue, as pre-culture of NOD FTOC with the whole pancreas is also capable of preventing ivIDDM against another challenge with pancreas tissue in organ culture (18).

The *in utero* data suggest that mixtures of GAD peptides were the same as single peptides in preventing IDDM, if the final percentage of diseased mice is measured. It is interesting to note, however, that the induction of disease in mice treated with different combinations of peptides was different. The GAD 246-266 single peptide treated mice and the mice treated with all three peptides had a relatively large number of animals

become diabetic at the same time as the controls, but after about 20 weeks few mice treated with the three-peptide mixture became diabetic. The GAD 246-266 treated mice and the GAD 509-528 + GAD 524-543 treated mice eventually reached the same overall disease incidence, although the induction of disease in the latter was delayed. It is possible that a later induction of GAD 246-266 responsiveness in the developing pups may be responsible for late disease induction in adult progeny (e.g. >25 weeks in Fig. 2). Animals treated with this peptide may only be partially tolerant for this response, and those treated with GAD 509-528 and 524-543 would not be tolerant at all. These mice would develop disease later, and this response may account for the delayed disease induction seen in some of the treated mice. Presumably, the response to GAD 509-528 and 524-543 matures earlier in the developing pups, and these responses could be more easily prevented by treatment with these peptides *in utero* with the protocol used in our studies, especially if the peptides were used together. Apparently, however, mixing all three peptides diluted out the ability of GAD 509-528 and 524-543 to induce tolerance to the early IDDM response, as this response occurred in the groups of mice treated with all the peptides. These data are an indication of the complexity of peptide treatment to prevent IDDM, even with a genetically identical population of animals. These results suggest caution should be exercised in the use of these treatments in a clinical setting.

Other researchers, using *in vivo* systems, have shown that protection from IDDM by injection of insulin (16, 45, 46) or intra-nasal administration of GAD (24) involves "immune diversion" from a T_H1 to a T_H2 response. A similar mechanism has been proposed for the protection of NOD mice by intrathymic injection of islet cells, whole

insulin B chain, and whole GAD65 (17). While our data using the ivIDDM model do not preclude T_H1 to T_H2 diversion as the mechanism of protection by pre-treatment of the FTOC with GAD peptides, the decrease in the ability of the cells from treated FTOC to respond to these peptides in proliferation assays is also consistent with clonal deletion or efficient regulation/anergy of GAD responsive cells. Regardless, the loss of these cells through deletion caused by therapeutic high peptide concentrations during T cell development may prove an effective method for prevention of IDDM, should the critical initiating antigens be found.

Overall, our data suggest that GAD is an important target antigen in IDDM, and that it may be a trigger or required component of the T cell response cascade that results in IDDM. Work with other islet associated antigens such as insulin (45, 46) or insulin peptides (16), and heat shock protein (HSP60) peptides (47) to prevent IDDM are also promising. Recent clinical trials (9), however, support the work shown here and elsewhere (17, 48) that the adult NOD mice treated with these antigens later in development, when peripheral regulatory responses have matured, can exacerbate disease. The present data showing the efficacy of inducing tolerance to GAD peptides during fetal development in FTOC or *in utero*, suggest that IDDM may be prevented with the appropriate immunotherapy given early in T cell development. Our results also suggest that the ivIDDM model may be useful in the rapid screening of candidate antigens in various combinations for peptide immunotherapy to prevent IDDM. Promising combinations of peptides could then be tested in the longer-term *in vivo* models to establish the best time to treat with these antigens before use in clinical trials.

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Abbreviations: APC(s), antigen presenting cell(s); d, days; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FP, fetal pancreas; FPOC, fetal pancreas organ culture; FT, fetal thymus; FTOC, fetal thymus organ culture; GAD, glutamic acid decarboxylase; dg, gestation days; IDDM, insulin-dependent diabetes mellitus; mAbs, monoclonal antibodies; MHC, major histocompatibility complex; NOD, non-obese diabetic; RIA, radioimmunoassay.

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APPENDIX B

Differential Effects of Antibody to CD1d on T cell Development in NOD and C57BL/6 Fetal Thymic Organ Culture[1]

Todd C. White[2*], Vandana Chauhan[2*], Aaron J. Middlebrook[2*], Jin S. Im[†] and Dominick DeLuca[3*]

*Department of Microbiology and Immunology

University of Arizona, Tucson, AZ 85724

[†]Department of Microbiology and Immunology

Albert Einstein College of Medicine, Bronx, N.Y. 10461

[1] Supported by grants from The American Diabetes Association and The Juvenile Diabetes Research Foundation.

[2] These authors contributed equally to the publication of this article.

[3] Address correspondence and reprint requests to Dr. Dominick DeLuca, Department of Microbiology and Immunology, Life Sciences North, Room 648, University of Arizona, Tucson, AZ 85724. E-mail address: deluca@u.arizona.edu

[4] Abbreviations used in this paper: T1DM, type 1 diabetes mellitus; FTOC, fetal thymus organ culture; NOD, non-obese diabetic; B6, C57Bl/6; SP4/SP8, single positive ($CD4^+$ or

$CD8^+$ as indicated); DP, double positive ($CD8^+CD4^+$); DN, double negative ($CD8^-CD4^-$); TN, triple negative ($CD8^-CD4^-CD3^-$); C/L, cells per lobe; N, normalized.

ABSTRACT

Natural Killer T (NKT) cells are a population of regulatory immune cells that have been implicated as key players in regulating the balance of T_H1 and T_H2 cytokines during T cell development and during immune responses in the periphery. Non-Obese Diabetic (NOD) mice, an animal model for Type 1 Diabetes Mellitus (T1DM), are known to have numerical and functional deficiencies in their NKT cell populations compared to normal mice. Corrections of these deficiencies, either through expansion or enhanced function, have shown the ability to mitigate T1DM. In this research we show that the addition of antibodies specific for CD1d (anti-CD1d) have differential and divergent effects on the generation of potential NKT cell pools in "signaling deficient" NOD fetal thymic organ culture (FTOC) versus "normal" control C57BL/6 (B6) FTOC. Anti-CD1d treatment led to an expansion of NKT cell populations in NOD FTOC, while causing decreases in NKT cell populations in B6 FTOC. The effects of these increases on NOD T1DM were tested using the *in vitro* T1DM (*iv*T1DM) organ culture system. Increases in NKT cells resulted in increased insulin production versus controls, suggesting an inhibition or delay of *iv*T1DM.

INTRODUCTION

NKT cells represent a subset of lymphocytes that bear both TCR and the NK cell receptor NK1.1 (CD161) (1). NKT cells are typically restricted to recognizing ligands in the context of the non-polymorphic MHC-like CD1d molecule. These regulatory cells are shown to express a very limited TCR repertoire that is capable of recognizing glycolipid molecules bound to CD1d. The NKT TCR, in mouse models, are typically composed of an α chain containing Vα14 with either Jα18 (formerly Jα281 or Jα15, Vα24-JαQ in humans) and a β chain with Vβ2, 7, or 8.2 (Vβ11 in humans) (2-4). The naturally recognized ligands that bind to CD1d are still unknown, although recently cellular glycosylphosphatidylinositol has been proposed as a natural CD1d ligand (5, 6). Although natural ligands for CD1d have not been fully established, NKT cells are capable of responding to a marine sponge derived synthetic antigen, glycolipid a-galactosylceramide (α-GalCer) (7, 8). Activation of NKT cells using α-GalCer has been used to expand NKT populations and, recently, treatment with α-GalCer has been shown to mitigate diabetes in NOD mice (9, 10).

NKT cells are considered an important regulatory cell as they have the ability to secrete both T_H1 and T_H2 cytokines rapidly upon stimulation (1, 11, 12). A deficiency in the function and number of NKT cells has been suggested as one of the major contributing factors in T1DM onset in NOD mice (13-15). A similar deficiency in NKT cells has also been identified in the human form of T1DM (16). These NKT deficiencies have been shown to adversely effect T cell development through defective or inappropriate T_H1 versus T_H2 cytokine secretion (4, 17). Although the exact mechanism

by which the NKT cells are involved in immune regulation is not known, they have been shown to suppress T1DM in NOD mice. This is thought to take place, primarily, through the secretion of IL-4 and IL-10, which is thought to shift T cell responses to a T_H2 humoral response. (18). Adoptive transfer of enriched NKT cells (19) or transgenic expression of the invariant Vα14-Jα18 TCR α chain used by some NKT cell types (20) can result in decreases in insulitis and diabetes incidence in NOD mice. Hence, it is widely believed that the NKT cell related defects in both NOD mice and humans may contribute to the pathogenesis of the disease (16, 21).

Although the expansion, or increased function, of NKT cells can mitigate diabetes in NOD mice, several factors play a role in the NOD propensity for T1DM. Among these factors are signaling defects in the T cells of NOD mice, which are thought to lead to poor deletion of autoreactive T cells in the thymus. Similar defects in signaling may also be playing a role in NKT cell development. To investigate signaling differences, we used fetal thymus organ culture (FTOC) as an *in vitro* model for T cell development to determine the impact of anti-CD1d, a potential concentration-based signal inhibitor/enhancer, on the development of NKT cells in diabetic mice (NOD) versus non-diabetic mice (B6). In these experiments, the DN or SP4 cells derived from FTOC were analyzed for NKT populations using αβTCR, DX5, and α-GalCer loaded CD1d Tetramer ($CD1d^{tet}$). Through this research we found that the addition of anti-CD1d to NOD FTOC resulted in an increase in the number of $DX5^+$ and $CD1d^{tet+}$ cells in a dose dependent manner. Using an *in vitro* T1DM system we observed that these increases in the number of potential NKT cells were associated with a reduction in the severity of *iv*T1DM.

MATERIALS AND METHODS

Mice

Breeding pairs of NOD/Lt mice were obtained as a gift from the laboratory of Dr. Edward Leiter at the Jackson Laboratory (Bar Harbor, ME). Our colony is maintained in a specific pathogen free vivarium at the University of Arizona Central Animal Facility and propagated by brother-sister mating. Mice were allowed free access to standard breeder chow (S-2335 irradiated breeder chow; Harlan Teklad, Madison WI) and autoclaved drinking water. The incidence of T1DM in NOD/Lt females in our colony at the University of Arizona is 80-90% by 40 weeks of age. NOD/Lt mice were then bred to produce timed-pregnant females. The fetuses were removed from pregnant females at the indicated time points (plug date = 0 days of gestation (dg)). C57BL/6 mice were purchased from National Cancer Institute (Frederick, Maryland). Timed pregnant females were used. The fetuses were removed from the pregnant females at the indicated time points (plug date = 0 dg). We consistently find that our animals are variable with regard to their stage of development even when they are vaginally plugged on the same day. Therefore developmental assessment of mice used in these experiments was based on their characteristics as given in "The Mouse, its Reproduction and Development" (22).

Fetal Thymus Organ Culture

The organ culture methods used have been described in detail by our laboratory and others (23). Briefly, at least 6 thymus lobes, dissected from 13-15 dg fetal mice were

placed on the surface of Millipore filters (25 μm thick, 0.45 μm pore size; Millipore, San Francisco, CA), which were supported on blocks of surgical Gelfoam (Upjohn Co, Kalamazoo, MI) in 10 x 35 mm plastic Petri dishes with 3 ml of medium. Organ culture media consisted of Dulbecco's modified Eagle's medium (4.5 g/L D-glucose; JRH, Lenexa, KS) supplemented with 20% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), streptomycin (100 μg/ml), penicillin (250 mg/ml), gentamycin (10 μg/ml), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (2 x 10^{-5}), and 3.4 g/L sodium bicarbonate. The cultures were grown in a humidified incubator in 5% CO_2 at 37°C. Cells were harvested as previously described (24, 25). The thymus lobes were placed into a solution of Accutase (ISC BioExpress, Kaysville, Utah). The tissue was incubated at 37° C for 15 minutes. The lobes were then dispersed into a single cell suspension by gentle aspiration with a Pasteur pipette. This treatment disaggregates most of the lymphoid and non-lymphoid stromal cells from the tissue. After washing once in Hanks' balanced salt solution (HBSS) plus 5% FBS to prevent further enzyme action, cell viability in Accutase-extracted samples was determined by 1% trypan blue exclusion. Viability was always greater than 85%. The results are expressed as the total cells recovered x 10^4 per lobe. Unless otherwise noted, FTOC was carried out for 12 days.

in vitro Type 1 Diabetes Mellitus Co-culture Model

This co-culture *iv*TIDM model has been described in detail by our laboratory in previous publications (25). Briefly, at least 6 fetal thymus lobes (13-16 dg) from NOD

mice were cultured as described above for 14 days to ensure development of mature T cells. All anti-CD1d antibody additions were performed on day 0 of this 14-day culture period. On day 14, the pre-cultured lobes were transferred to a new culture dish to ensure that any remaining biologically active anti-CD1d antibody was not present for the co-culture period. After the transfer of the millipore strip (containing pre-cultured lobes), an equal number of syngeneic fetal pancreas fragments (15-17 dg) were placed in direct contact with each of the pre-cultured fetal thymus lobes. The addition of fetal pancreas marks day 0 of the co-culture period. On day 0, 7, 14, and 21 of co-culture, 500 μl of culture supernatant was removed from each of the 3ml culture dishes for insulin RIA. MicroMedic insulin RIA kits obtained from ICN MicroMedic Systems (Horsham, PA), were used to measure the quantity of insulin in culture supernatants as directed by the manufacturers instructions, and standardized to bovine insulin. After supernatant was removed, 500 μl of replacement OCDME + 20% FCS was added.

Reagents

Fluorescein (FITC), Tri-color (TC), and phycoerythrin (PE) hamster isotype controls, PE anti-mouse TCR β chain, PE anti-mouse CD3ε, PE anti-mouse DX5, FITC anti-mouse CD8, and TC anti-mouse CD4 were purchased from Caltag Laboratories (South San Francisco, CA) or Pharmingen (San Diego, CA). The CD1d tetramer PE was a gift from Dr. Mitch Kronenberg at the La Jolla Institute of Allergy and Immunology (LIAI). One of us (J. S. Im) also produced both unloaded and CD1d tetramer PE loaded with α-GalCer for these studies. Tetramers from both sources gave comparable results.

Purified NA/LE anti-CD1d (Clone 1B1, an IgG2bκ antibody) and control NA/LE IgG2bκ class antibody (Clone MPC-11) was purchased from Pharmingen (San Diego, CA). The IgG2b control antibody showed limited effects on the development of FTOC compared with the untreated control, and in consideration of time and space the data is not shown. At points of comparative importance the effects of anti-CD1d and control IgG2b antibody treatments are described in the text. For these treatments, antibodies were diluted in Hanks+5% FCS and directly added to the thymic lobes to generate the specified concentrations.

Flow Cytometry (FC) Analysis

Cell suspensions were stained with monoclonal antibodies directly conjugated with TC (CD4), FITC (CD8), PE (DX5), PE (TCR β), and PE (αGalCer/CD1d tetramer). The antibodies were used at 1 $\mu g/10^6$ cells. CD1d tetramer staining was performed using 1.0-0.05ug/10^5 cells (determined by batch analysis). After staining, cells were fixed in 1% para-formaldehyde before FC analysis. Three-color FC analysis was performed using a FACScan (BDIS, San Jose, CA) equipped with photomultiplier tubes and optical filters as recommended by the manufacturer. FITC, PE and TC were excited by a 488-nm Argon laser. Fluorescence data were collected using 3-decade logarithmic amplification on 10,000-50,000 viable lymphoid cells as determined by forward and 90° light scatter intensity to exclude stromal and other non-lymphoid elements (For an example of gates used in analysis see (26) and Figures 4,6,10, and 12). Data were collected with CellQuest (Santa Rosa, CA) and analyzed using FlowJo (Treestar) software.

Statistical Analysis

There is some variability in total cell production across FTOC preparations (i.e. across experimental setups). However, total cell production of replicate samples, from within a given experiment, were typically in close agreement. Concordantly, proportions and/or features of anti-CD1d's effects did not change markedly across experiments. However, because of variations in the end culture cell production percentages and total cell numbers, based on slight differences in the developmental/gestational states of tissue used in separate experiments (24), the values were normalized within each experiment to a percentage of the untreated control. Statistical analyses (mean and standard error of the mean) were then performed using these normalized values, eliminating developmental/gestational variance. This technique allows for a more accurate comparison when dealing with FTOC, which is constantly undergoing changes in T cell production based on developmental status, but may lead to slight discrepancies between the normalized and unmodified data. A paired student t test was used for within strain comparisons while unpaired student t tests were performed for between strain comparisons.

RESULTS

Anti-CD1d addition has minimal significant affects on the phenotype and maturity of T cells in FTOC.

6-10 NOD or B6 fetal thymus lobes, 14-15dg, were cultured for 12 days in the presence of logarithmically increasing doses of an anti-CD1d antibody or an IgG2b control antibody. The following titration levels were examined: 0.001 μg/ml (~$6.25x10^{-12}$M), 0.01 μg/ml (~$6.25x10^{-11}$M), 0.1 μg/ml (~$6.25x10^{-10}$M), 1.0 μg/ml (~$6.25x10^{-9}$M), or 10 μg/ml (~$6.25x10^{-8}$M). Antibodies were initially added directly to the thymus lobes on day 0 of culture and remained in the media until the end of culture (See *Materials and Methods* for details).

Cultures were initially analyzed for cell growth, viability, and phenotypic staining for the SP4, SP8 ($CD4^-/CD8^+$), DN and DP ($CD4^+/CD8^+$) cell populations. Initial analysis of cell growth in FTOC showed no statistically significant differences in overall cell yields or viability when comparing the untreated control dishes and those dishes receiving anti-CD1d (Fig. 1) or IgG2b (data not shown, see *Reagents* in *Materials and Methods* for details).

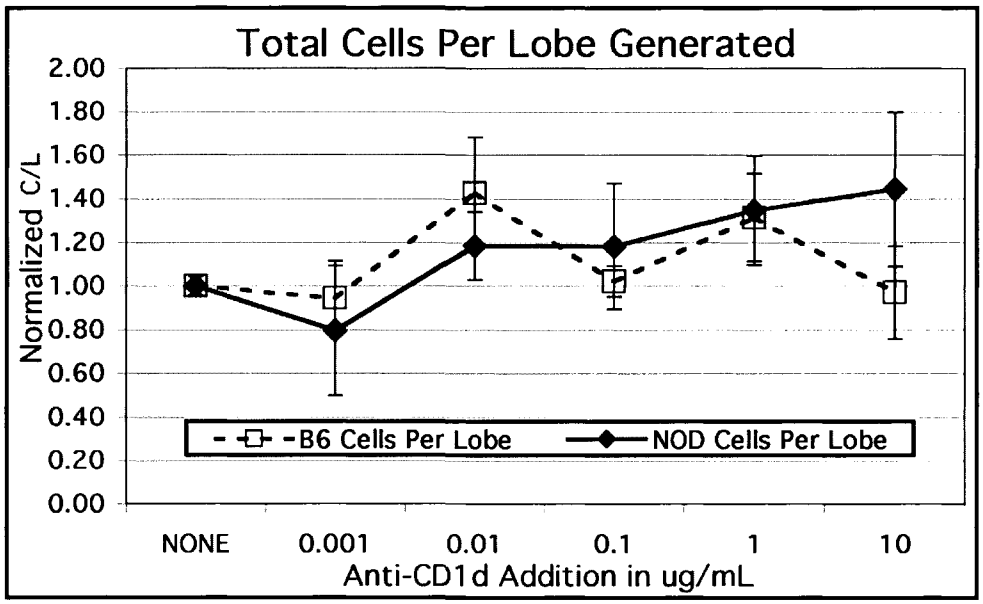


Figure 1. Cell Yields from FTOC. Cells from a 12-day FTOC, derived from 13-14 day gestation thymi, were analyzed for yield and viability through trypan-blue exclusion. FTOC were treated with a titration of anti-CD1d ranging from 0.001 μg/ml-10 μg/ml. The open squares (□) represent the control B6 FTOC and the closed diamonds (◆) represent the diabetic NOD FTOC. Values were normalized to the untreated control for direct comparison. Actual ranges for the C/L generated in the untreated control were 36.0-58.6 x 10^4 for B6 FTOC and 43.6-70.1.5 x 10^4 for NOD FTOC. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control within the mouse strain. One open circle (°) indicates $p \leq 0.10$ and two open circles (°°) indicate $p \leq 0.05$, when comparing the NOD vs. B6 strain. (n=6)

Analysis of the phenotype percentages of certain T cell populations (i.e. SP4, SP8, DN and DP) showed few significant differences in the anti-CD1d treated cultures. No significant differences were seen in the IgG2b treated cultures (not shown) when compared to the untreated control in either the NOD or B6 FTOC experimental groups. Impact of anti-CD1d on the NKT associated major phenotype groups (DN and SP4) can be seen in Figure 2. It is important to note that the percentage and number of cells per thymus lobe (C/L) produced in NOD FTOC DN populations tended to increase as the level of anti-CD1d treatment increased while the percentage and C/L generated in B6 FTOC appeared relatively unaffected (Fig. 2B). However, minor increases also occurred in the NOD FTOC when IgG2b was added (not shown), but these increases were relatively small and not found to be significant. In regards to cell expansions, only the NOD anti-CD1d treated cultures showed significant increases in the NKT associated cell populations (DN or SP4). Specifically, the NOD DN populations showed significant increases at the 1.0 μg/ml and 10.0 μg/ml level (Fig. 2B, a 35 ± 20% increase at 1.0 μg/ml (p=0.043) and a 28 ± 15% increase at 10 μg/ml p=0.016). These differences are believed to be partially due to the increase seen in the NKT populations, which will be discussed below.

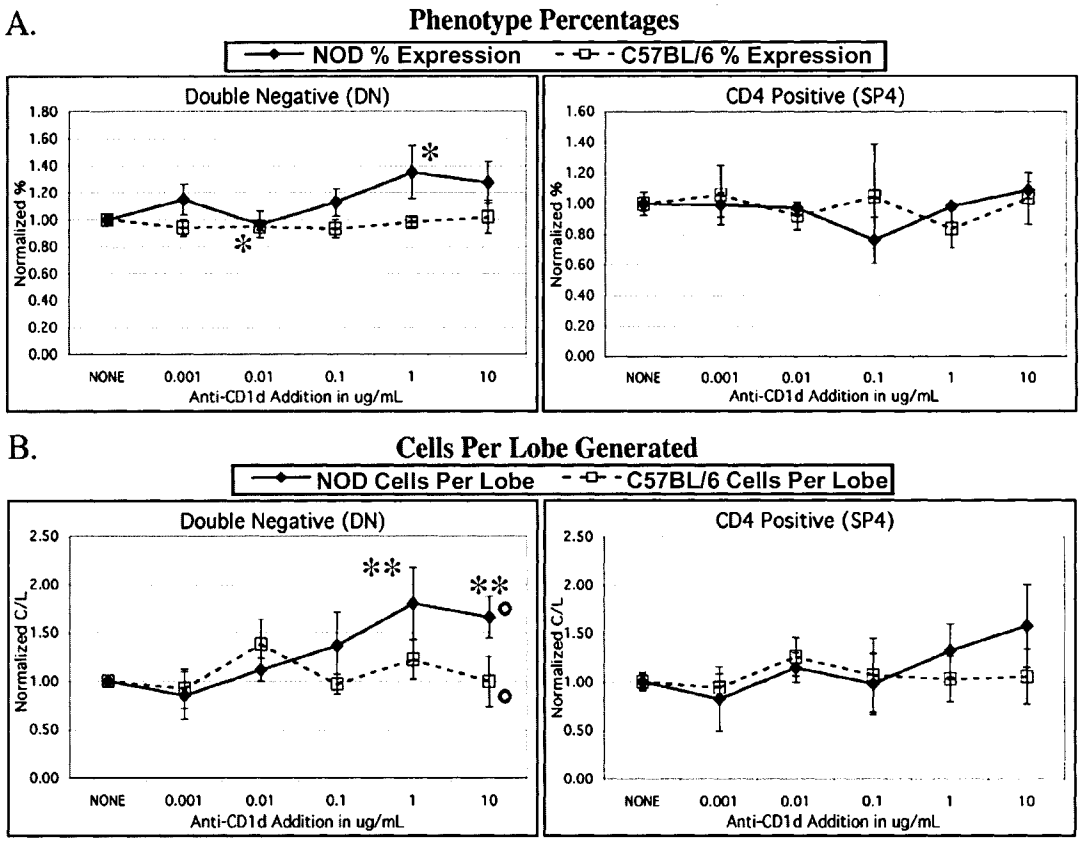


Figure 2. Profiles for SP4 and DN Cells from Anti-CD1d Treated FTOC. Cells from a 12-day FTOC, derived from 13-14 day gestation thymi, were analyzed for SP4 and DN thymocyte populations by using three-color flow cytometry. FTOC were treated with a titration of anti-CD1d ranging from 0.001 μg/ml-10 μg/ml. The open squares (□) represent the control B6 FTOC and the closed diamonds (◆) represent the diabetic NOD FTOC. Group A represents Phenotypic Percentages and Group B represents Cells per Lobe generated. Values were normalized to the untreated control for direct comparison. Actual ranges generated for untreated control in Group A, in overall percentage, were as follows: B6 CD4 8.9-13.0%, NOD CD4 15.8-22.8%; B6 DN 28.2-42.5%, NOD DN 10.2-

30.5%. Actual ranges generated for untreated control in Group B, in C/L, were as follows: B6 CD4 3.6-6.8 x 10^4, NOD CD4 6.3-13.9 x 10^4; B6 DN 11.0-22.5 x 10^4, NOD DN 6.2-14.0 x 10^4. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control within the mouse strain. One open circle (°) indicates $p \leq 0.10$ and two open circles (°°) indicate $p \leq 0.05$, when comparing the NOD vs. B6 strain. (n=6)

Comparison of αβTCR, DX5, or CD1d tetramer staining in NOD and B6 FTOC.

To date, there has been some controversy as to which markers are most accurate for the characterization of the NKT cell populations (27). Initially NKT cell populations were thought to be comprised of the DN and/or SP4 cells that were $\alpha\beta TCR^+$ and $NK1.1^+$ (28). However, more recently it has been discovered that NKT cell populations can be comprised of both $NK1.1^+$ and $NK1.1^-$ cells (29). Regardless of the significance of NK1.1 expression, studies performed using NOD mice have experienced difficulties because NOD mice do not express the NK1.1 marker found on B6 and other mice strains (30). For this reason, our initial experiments with NOD mice were analyzed for the presence of NKT cells with an anti-DX5 antibody, which is a pan NK/NKT cell marker. The DX5 marker itself is not an ideal indicator of NKT cell populations since it can be found on non-NKT populations and is not found on every NKT cell type (27), making it, in some regards, both overly inclusive and exclusive. Despite the lack of complete specificity, the DX5 marker has been shown to be capable of identifying representative changes in regulatory NKT populations, including those that can regulate diabetogenic T cell responses (31). More recently, the advent of NKT TCR specific CD1d tetramers ($CD1d^{tet}$) has made the identification of certain NKT subpopulations easier and more reliable (32). Despite the highly specific nature of CD1d tetramers, without a detailed consensus definition for NKT cells, it can still be argued that this staining technique is overly exclusive (i.e. does not include all NKT regulatory types).

Therefore, in an attempt to characterize the NKT cells generated in our organ cultures we examined the SP4 and DN sub-populations using several different

phenotyping reagents (αβTCR, DX5, and CD1d^{tet}). These results were then used to characterize the potential NKT subpopulations produced by FTOC and to examine the effects of anti-CD1d addition on these NKT subpopulations. It was important to establish baseline values for all cell types generated in FTOC since this culture system encompasses T cells types typically found in the thymus as well as mature naïve T cells that would normally be found only in the periphery. Previous work performed by our lab has shown that phenotypic percentages of thymocytes found in the later stages of FTOC (beyond 7d of culture) contain high percentages of mature SP4, SP8, and DN cell types when compared to age matched live offspring (24). Presumably, these high frequencies of mature T cells represent the maturation of a cohort of T cells in this closed organ culture system that cannot easily exit the tissue and accumulate there.

To examine the NKT population generated in FTOC, 6-10 NOD or B6 fetal thymus lobes, 14-15 dg, were subjected to anti-CD1d titrations as described above. Data generated were then used to establish comparative baseline values for the DN and SP4 populations for the NKT related phenotypic markers αβTCR, DX5, and CD1d^{tet}. NKT cells are thought to be primarily restricted to the DN and SP4 αβTCR^{+} population (33). Analysis of αβTCR^{+} cells revealed that NOD and B6 FTOC generate significantly different percentages of the DN αβTCR$^{mature+}$ thymocytes (cells that express αβTCR at moderate to high levels) in FTOC. In the control B6 FTOC, 13.3 ± 3.1% of the DN cells showed a mature αβ phenotype, while 29.38 ± 4.4% of NOD DN cells showed a similar phenotype (p=0.051, Table 1). However, similar analysis of the SP4 populations yielded no significant difference in the percentage of αβTCR$^{mature+}$ thymocytes when comparing

NOD and B6 cultures, which would be expected, considering the large number of non-NKT cell types found in this subpopulation. Despite the large number of DN and SP4 $\alpha\beta TCR^{mature+}$ cells found in NOD FTOC, the NOD $DX5^+$ and $CD1d^{tet+}$ percentages and C/L were significantly decreased when compared to B6 FTOC. This statement holds true for both the DN and SP4 populations. For example, in the DN population 44.8 ± 9.4% and 6.0 ± 1.0 C/L in B6 FTOC were $DX5^+$, while NOD FTOC showed 14.2 ± 2.9% and 1.6 ± 0.4 C/L of the DN population to be $DX5^+$. In other words, NOD FTOC generates 68% fewer DN $DX5^+$ cells, by percentage, and 73% fewer actual DN $DX5^+$ cells when compared to B6 FTOC (p=0.068 for % and p=0.024 for C/L; Table 1). Similar, but not significant, differences were seen in the SP4 $DX5^+$ population and the SP4 and DN $CD1d^{tet+}$ populations, where NOD mice showed a reduction in potential NKT cells. Differences found in the NOD are more impressive considering NOD FTOC generated a significantly higher percentage of DN $\alpha\beta TCR^{mature+}$ cells versus B6 FTOC, indicating that NOD FTOC generated less potential NKT cells despite having a larger DN $\alpha\beta TCR^{mature+}$ pool.

These NOD NKT results agree, in principle, with *in vivo* results found in other laboratories, which have shown deficiencies in the number and function of NKT cells in NOD mice (34). As discussed above, FTOC generates a higher level of DN and SP4 cells than found in the thymus *in vivo* and this is thought to lead to the higher baseline percentages of $DX5^+$ and $CD1d^{tet+}$ cells found in our experiments. In support of this idea, Pellicci et al have shown that organ cultures can generate higher levels of NKT cells than found *in vivo* (35).

NKT Analysis: Percentage Expression and Cells Per Lobe Generated(x 10^4)					
		POPULATION PHENOTYPE			
		$CD4^-CD8^-$ (DN)		$CD4^+CD8^-$ (SP4)	
		STRAIN		STRAIN	
STAIN TYPE		C57BL/6	NOD	C57BL/6	NOD
Alpha Beta TCR Mature$^+$	%	**13.25 ± 3.13**	**29.38 ± 4.40**	81.5 ± 3.65	83.78 ± 3.76
	C/L	1.82 ± 0.33	3.18 ± 0.67	4.00 ± 0.58	11.15 ± 2.94
DX5$^+$	%	**44.81 ± 9.36**	**14.22 ± 2.89**	14.40 ± 4.13	4.53 ± 0.90
	C/L	**6.01 ± 1.01**	**1.62 ± 0.40**	0.67 ± 0.21	0.48 ± 0.09
CD1d Tetramer$^+$	%	21.15 ± 10.42	3.40 ± 0.66	10.87 ± 6.00	3.86 ± 1.21
	C/L	2.90 ± 1.40	0.40 ± 0.10	0.65 ± 0.40	0.42 ± 0.12

Table I. NKT Analysis: Percentage Expression and Cells Per Lobe Generated (x 10^4). Percentage expression (%) and cells per lobe generated (C/L) in untreated "control" FTOC were analyzed and compared between B6 and NOD mouse strains. Unpaired student t tests were performed to obtain significance values on all data. Bolded text indicates $p \leq 0.10$ and bolded-underlined text indicates $p \leq 0.05$, when comparing results between the NOD and B6 strains.

Anti-CD1d addition has strain specific effects on the generation of αβTCR$^{mature+}$ cells in C57BL/6 and NOD Fetal Thymus Organ Culture.

The DN and SP4 populations generated in FTOC were examined for αβTCR, DX5, and CD1d^{tet} positive cells after anti-CD1d treatment. Analysis of B6 αβTCR$^{mature+}$ cells yielded few significant changes in the percent positive or C/L in the SP4 and DN populations, when treated with the various levels of anti-CD1d. However, the DN αβTCR$^{mature+}$ population did tend to experience decreases in both the percentage of positive cells and C/L generated when treated with higher levels of anti-CD1d (0.1 μg/ml- 1.0 μg/ml), leading to a significant decrease at the 1.0 μg/ml level (p=0.031 for % and p=0.089 for C/L; Fig. 3A & B, open square (□)). The B6 SP4 population showed no strong anti-CD1d induced trends.

Analysis of αβTCR$^{mature+}$ cells generated in NOD FTOC yielded no significant changes in the percentage of positive cells or C/L values at most of the anti-CD1d treatment levels. However, the DN population did show a significant increase in the number of αβTCR$^{mature+}$ C/L at the highest level of anti-CD1d treatment (10 μg/ml, p=0.019; Fig. 3A & B, closed diamond (◆)). This trend, when normalized and compared with normalized B6 values, suggests the two strains to have distinctly different responses to similar levels of anti-CD1d (DN graph in Fig. 3B).

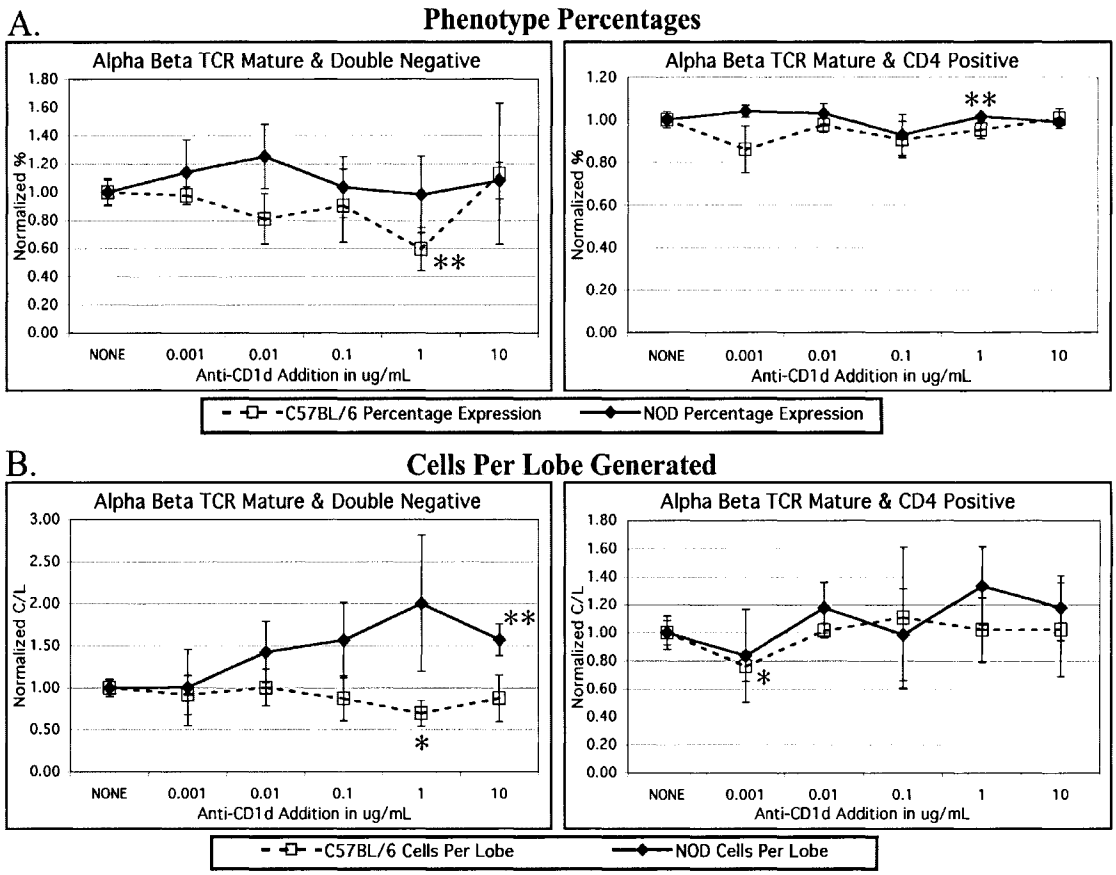


Figure 3. Profiles for αβTCR Mature Cells from Anti-CD1d Treated FTOC. Cells from the CD4 and DN subpopulations of 12-day FTOC, derived from 13-14 day gestation thymi, were analyzed for the presence of mature (moderate to high) levels of αβTCR using three-color flow cytometry. FTOC were treated with a titration of anti-CD1d ranging from 0.001 μg/ml-10 μg/ml. The open squares (□) represent the control B6 FTOC and the closed diamonds (◆) represent the diabetic NOD FTOC. Group A represents Phenotypic Percentages and Group B represents Cells per Lobe generated. Values were normalized to the untreated control for direct comparison. Actual percentages and C/L for the untreated control are listed in Table I. One asterisk (*)

indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control within the mouse strain. One open circle (°) indicates $p \leq 0.10$ and two open circles (°°) indicate $p \leq 0.05$, when comparing the NOD vs. B6 strain. (n=6)

Anti-CD1d addition has strain specific effects on the generation of DX5$^+$ cells in C57BL/6 and NOD Fetal Thymus Organ Culture.

For reference, a sample of DN and SP4 DX5 flow cytometric analysis can be seen in Figure 4. When examining this data and the data in Figure 6, it should be noted, that due to the developmental processes still occurring in FTOC at the time of harvest that receptor expression is spread over a much wider range than typically found in the periphery. This occurs with almost all types of receptor analysis in FTOC.

Analysis of B6 DX5$^+$ cells yielded few significant changes at any of the anti-CD1d treatment levels, in regards to percentage of cells positive or C/L in FTOC (Fig. 5A & B, (□)). The one exception is a significant increase in the percentage of DX5$^+$ cells generated at the low 0.001 μg/ml anti-CD1d level, in the DN cell population, where there is a 28 ± 11% increase versus the untreated control (p=0.021) (Fig. 5A, (□)). The B6 SP4 population did show a trend towards lower percentages and C/L of DX5$^+$ cells at the three higher levels of anti-CD1d treatment (i.e. the 0.1, 1.0, and 10.0 μg/ml levels), but these differences were not found to be significant versus the untreated control or versus the IgG2b control antibody treated FTOC (all $p \geq 0.23$).

Analysis of NOD FTOC DN DX5$^+$ cells yielded almost no significant changes in percentage at any of the anti-CD1d treatment levels as well. The one exception is a significant decrease in the percentage of DX5$^+$ DN cells generated at the high 10.0 μg/ml anti-CD1d level, where there is a 19 ± 3% decrease versus the untreated control (p=0.001) (Fig. 5A, (◆)). This treatment level in the NOD DN population is the lone point where anti-CD1d induced a decrease in DX5$^+$ cells. All other treatment levels

induced higher percentages and C/L for NOD $DX5^+$ cells in both the DN and SP4 populations, with several significant increases seen in the percentage and C/L values in the SP4 group (Fig. 5A & B, (◆)). These observations are highlighted by significant changes in the 1.0 μg/ml $DX5^+$ C/L groups, where the cell number increased by nearly 2-fold in the DN group and by 3-fold in the SP4 group versus the untreated controls (Fig. 5B, (◆); p=0.012 and 0.033, respectively). A slight expansion of the $DX5^+$ cells in the DN and SP4 populations also occurred when IgG2b antibodies were used, but these changes were not statistically significant compared with the untreated control FTOC.

Once again, comparison of normalized NOD and B6 percentage and C/L showed a divergent trend between the two strains. The NOD $DX5^+$ population increased while the B6 $DX5^+$ decreased or was unaffected. This divergent effect led to several significant differences when comparing the two strains. The most extreme of these differences was seen at the 1.0 μg/ml anti-CD1d treatment level in both the DN and SP4 populations in which $DX5^+$ C/L recoveries were significantly increased in the NOD versus the B6 (Fig. 5B; p=0.100 and 0.056, respectively).

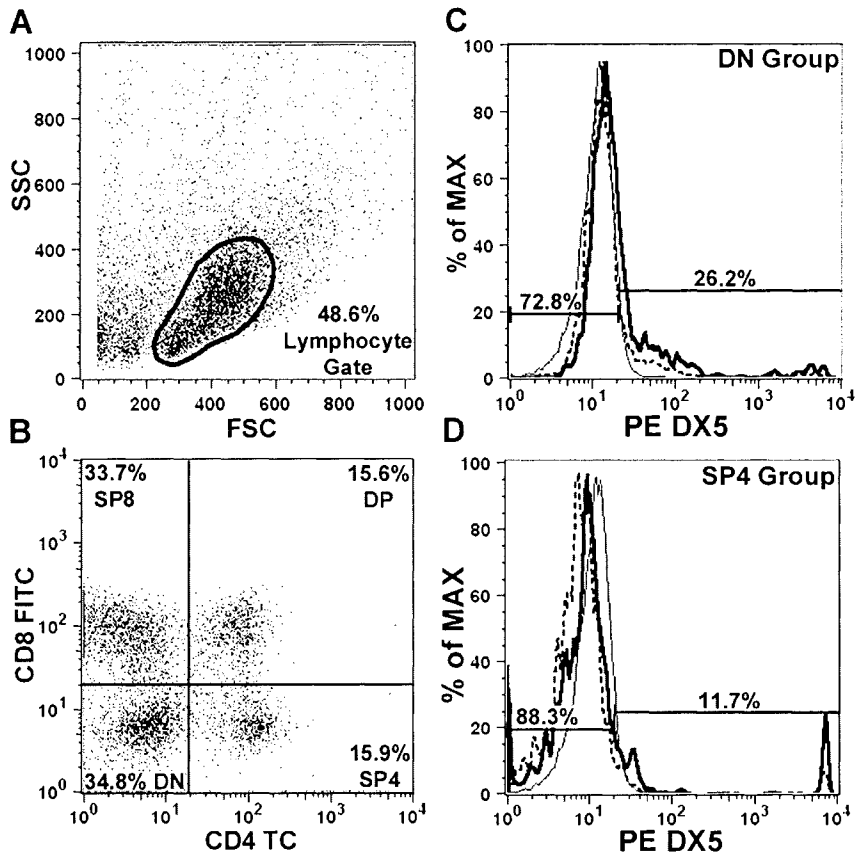


Figure 4. Flow Cytometric Analysis of Thymocytes Derived from Anti-CD1d Treated 12-day NOD FTOC. Cells shown in this figure were harvested from FTOC using 13-14 day gestation NOD fetal thymi treated with 1 μg/ml anti-CD1d. The contour plot (A) shows Forward versus Side Scatter and lymphocyte gate (48.6%). CD8 FITC vs. CD4 TC of the lymphocyte gate (B) reflects a similar phenotypic distribution when compared to the untreated control cultures. CD1d Tetramer histograms of the DN and SP4 compartments (C & D, respectively) indicate the percentage of $DX5^+$ cells (bold line) as they were determined by gating versus the untreated control and isotype control (dashed line and shaded histogram, respectively). All references to $DX5^+$ cells in this paper reflect a similar approach to flow cytometric analysis and DX5 gating.

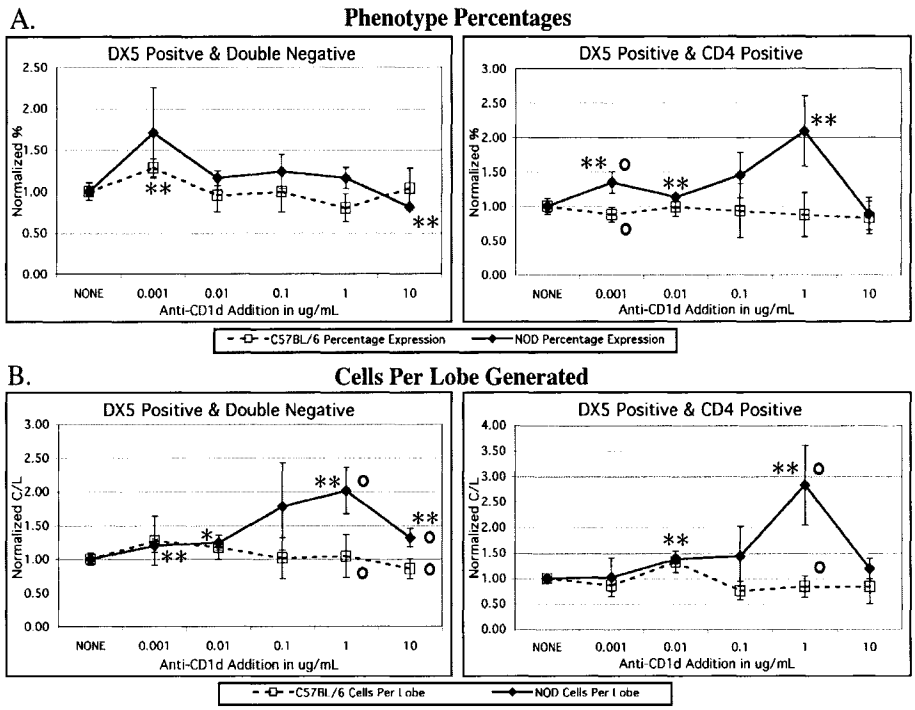


Figure 5. Profiles for $DX5^+$ Cells from Anti-CD1d Treated FTOC. Cells from the CD4 and DN subpopulations of 12-day FTOC, derived from 13-14 day gestation thymi, were analyzed for the presence of $DX5^+$ cells using three-color flow cytometry. FTOC were treated with a titration of anti-CD1d ranging from 0.001 μg/ml-10 μg/ml. The open squares (□) represent the control B6 FTOC and the closed diamonds (◆) represent the diabetic NOD FTOC. Group A represents Phenotypic Percentages and Group B represents Cells per Lobe generated. Values were normalized to the untreated control for direct comparison. Actual percentages and C/L for the untreated control are listed in Table I. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control within the mouse strain. One open circle (°) indicates $p \leq 0.10$ and two open circles (°°) indicate $p \leq 0.05$, when comparing the NOD vs. B6 strain. (n=6)

Anti-CD1d addition has strain specific effects on the generation of CD1d^{tet+} cells in C57BL/6 and NOD Fetal Thymus Organ Culture.

For reference, a sample of DN and SP4 CD1d^{tet} flow cytometric analysis can be seen in Figure 6. Previous *in vivo* work has shown these cells to account for ~1% of the total cell population, but these numbers are enhanced in FTOC, as explained above (27, 36). CD1d^{tet} staining of B6 FTOC revealed significant decreases in the percentage and C/L at virtually all anti-CD1d titration levels (Fig. 7). The most extreme decreases were seen at the highest anti-CD1d treatment level of 10 μg/ml. Both DN and SP4 CD1d^{tet+} cell types were affected. In the DN cell population, percentage and C/L values drop by 49 ± 16% and 59 ± 15% versus the untreated control, respectively. The SP4 CD1d^{tet} cell yield dropped by 39 ± 8% versus the untreated control (Fig. 7A, B & C, (□)). Figure 7C shows an expanded view of the 59 ± 15% DN population decrease in B6 CD1d^{tet} C/L. This data became compressed when graphed with NOD FTOC data.

CD1d^{tet} staining of NOD FTOC revealed significant increases in the percentage and C/L at several of the anti-CD1d titration levels. The greatest of these increases were seen at the highest anti-CD1d treatment level of 10 μg/ml. Both DN and SP4 CD1d^{tet+} cell types were affected. In the DN cell population, percentage and C/L increased by 431 ± 65% (p=0.001) and 776 ± 209% (p=0.012), respectively, versus the untreated control. The SP4 CD1d^{tet} cell population showed a similar expansion with the percentage and C/L increasing by 434 ± 128% (p=0.031) and 812 ± 419% (p=0.128), respectively, versus the untreated control (Fig. 7Aand B, (◆)). These DN and SP4 percentage and C/L increases were also found to be significant when compared against the IgG2b treated FTOC (DN %

p=0.001, DN c/l p=0.040, SP4 % p= 0.029, SP4 c/l p=0.141; not shown). Actual C/L generated for B6 and NOD FTOC at all CD1d titration levels are available in Figure 7D.

As might be expected from the $DX5^+$ data (Fig. 5), and the work of others showing that NKT cells in NOD are decreased *in vivo*, the production of $CD1d^{tet}$ cells in NOD FTOC was lower than that of B6 FTOC, especially in the DN population. A final comparison of normalized NOD and B6 FTOC $CD1d^{tet}$ data again shows a divergent trend between the two strains, with the NOD $CD1d^{tet+}$ population showing an impressive expansion while the B6 $CD1d^{tet+}$ population showed an equally impressive reduction (See Fig. 7C for an example of B6 cells per lobe decrease). This divergent effect led to several highly significant differences when comparing the two strains. In the case of $CD1d^{tet}$, the most extreme of these differences were seen at the 10.0 μg/ml anti-CD1d treatment level in both the DN and SP4 populations (Fig. 7A & B). The expansion seen in the DN $CD1d^{tet+}$ population resulted in the production of cells at levels similar to those found in the untreated control of the "normal" B6 control strain. Conversely, treatment of B6 FTOC, with 10.0 μg/ml of anti-CD1d, led to a severe reduction in the DN $CD1d^{tet+}$ population that approached the levels found in the untreated control in the "NKT defective" NOD strain. These changes were more extreme in the SP4 population where the NOD $CD1d^{tet+}$ values achieved 300% of the untreated control in the "normal" B6 strain.

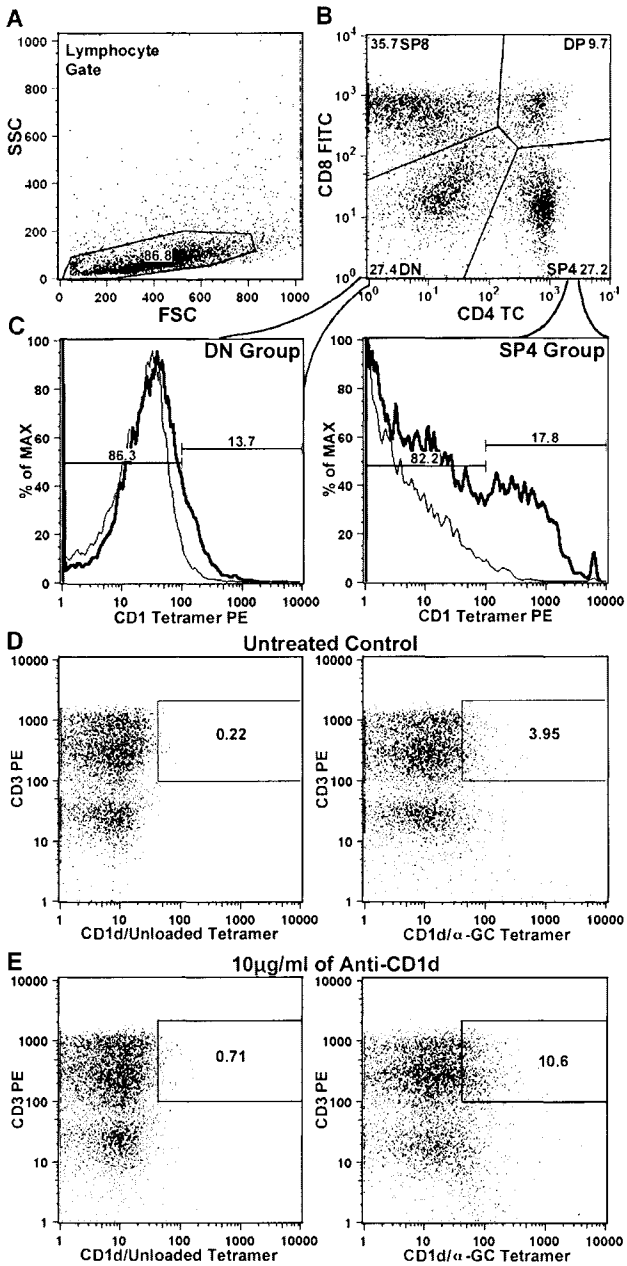
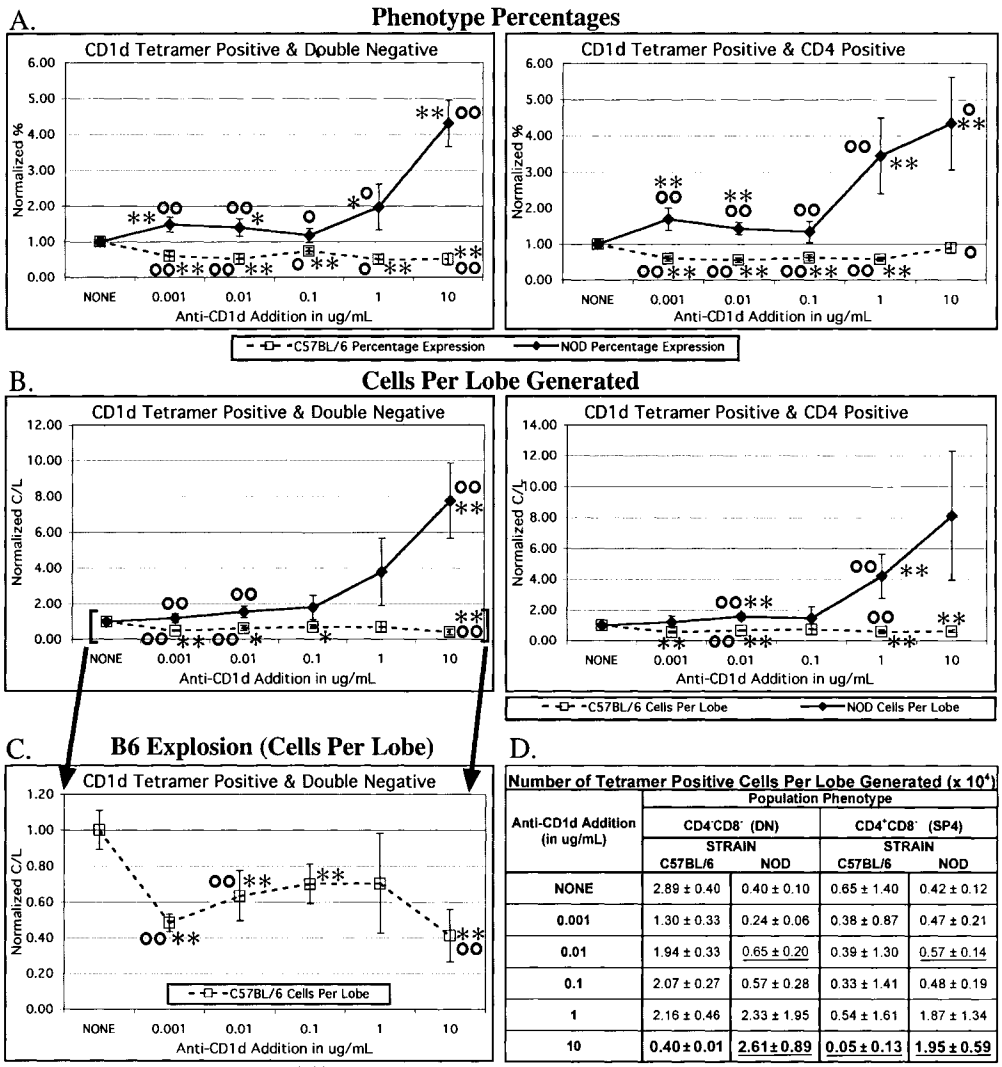


Figure 6. Flow Cytometric Analysis of Thymocytes Derived from Anti-CD1d Treated 12-day NOD FTOC. Cells shown in this figure were harvested from FTOC using 13-14 day gestation NOD fetal thymi treated with 10.0 μg/ml anti-CD1d. The contour plot (A) shows Forward versus Side Scatter and lymphocyte gate (86.8%). CD8 FITC vs. CD4 TC

of the lymphocyte gate (B) reflects a similar phenotypic distribution when compared to the untreated control cultures. CD1d Tetramer histograms of the DN and SP4 compartments (C) from treated cultures (bold line) are shown vs. the untreated control (shaded histogram). Percentages shown represent the number of $CD1d^{tet+}$ cells in a 10 μg/mL anti-CD1d treated culture. Positive values for the untreated control were 3.67% for DN and 1.74% for SP4. Gating was determined based on the unloaded CD1d tetramer. Positive values for the unloaded tetramer (not shown) after gating was established were 0.13% for DN and 0.08% for SP4. All references to $CD1d^{tet+}$ cells in this paper reflect a similar approach to flow cytometric analysis and gating. D and E show $CD1d^{tet+}$ staining based on CD3ε expression for unloaded and α-GalCer loaded tetramer in an untreated control and a 10 μg/mL anti-CD1d treated NOD FTOC, respectively. This technique or αβTCR staining is normally used to determine $CD1d^{tet+}$ cells, but does not allow for the isolation of the DN and SP4 compartments when using standard 3-color flow cytometry. Although the percentages varied based on the staining technique the respective trends were always consistent.



Number of Tetramer Positive Cells Per Lobe Generated (x 10^4)				
Anti-CD1d Addition (in ug/mL)	Population Phenotype			
	$CD4^-CD8^-$ (DN)		$CD4^+CD8^-$ (SP4)	
	STRAIN		STRAIN	
	C57BL/6	NOD	C57BL/6	NOD
NONE	2.89 ± 0.40	0.40 ± 0.10	0.65 ± 1.40	0.42 ± 0.12
0.001	1.30 ± 0.33	0.24 ± 0.06	0.38 ± 0.87	0.47 ± 0.21
0.01	1.94 ± 0.33	0.65 ± 0.20	0.39 ± 1.30	0.57 ± 0.14
0.1	2.07 ± 0.27	0.57 ± 0.28	0.33 ± 1.41	0.48 ± 0.19
1	2.16 ± 0.46	2.33 ± 1.95	0.54 ± 1.61	1.87 ± 1.34
10	0.40 ± 0.01	2.61 ± 0.89	0.05 ± 0.13	1.95 ± 0.59

Figure 7. Profiles for $CD1d^{tet+}$ Cells from Anti-CD1d Treated FTOC. Cells from the CD4 and DN subpopulations of 12-day FTOC, derived from 13-14 day gestation thymi, were analyzed for the presence of $CD1d^{tet+}$ cells using three-color flow cytometry. FTOC were treated with a titration of anti-CD1d ranging from 0.001 μg/ml-10 μg/ml. The open squares (□) represent the control B6 FTOC and the closed diamonds (◆) represent the diabetic NOD FTOC. Group A represents Phenotypic Percentages and Group B represents Cells per Lobe generated. Graph C represents an expanded version of the B6 data in graph B above, emphasizing the large decrease in B6 $CD1d^{tet+}$ cells that is not

apparent in the compressed version. Table D shows the number of $CD1d^{tet+}$ C/L generated in B6 and NOD FTOC. Values were normalized to the untreated control for direct comparison. Actual percentages for the untreated control are listed in Table I. Actual C/L can be seen in Table D. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control within the mouse strain. One open circle (°) indicates $p \leq 0.10$ and two open circles (°°) indicate a $p \leq 0.05$, when comparing the NOD vs. B6 strain. For Table D, underlined text indicates $p \leq 0.10$, versus the untreated control within the mouse strain, while bold text indicates $p \leq 0.10$, when comparing the NOD vs. B6 strain. (n=6)

Addition of 10μg/mL of anti-CD1d leads to increased expression of αβTCR and CD3ε in developing DP cell populations.

Additional experiments were performed using 10-14 NOD or B6 fetal thymus lobes, 14-15dg, cultured for 12 days in the presence of 10 μg/mL of anti-CD1d antibody, or no antibody at all, to more closely examine the developmental impact of the higher levels of anti-CD1d. Cell yields from these cultures showed that high level anti-CD1d addition leads to a significant, 32% decrease, in B6 FTOC cell generation, while NOD FTOC treated using the same level of antibody remained unaffected (Fig. 8). The impact of 10 μg/mL of anti-CD1d on B6 mice is also seen in the developmental phenotype distribution. Most notable is the significant decreases in percentage and C/L generated in the B6 DP population, which is a key transitional state in the development of T cells and NKT cells (Fig. 9). The DP percentage and C/L generated in B6 FTOC decrease by 71% and 80% respectively, indicating a substantial impact in normal development. These CD1d induced changes also lead to an increase in the percentage of the SP8 cells. Cell yield and phenotype changes of this type are indicative of additional signaling during development (see Discussion). Conversely, NOD development was less affected, with no significant decrease in the DP population. However, the NOD FTOC did show minor reductions in the mature T cell phenotype populations, SP8 and SP4 cells, coupled with and increase in the DN population.

Anti-CD1d treatment, at 10 μg/mL, also leads to enhanced expression of αβTCR and CD3ε of the developing DP population, particularly in B6 FTOC. An example of flow cytometric analysis of these expression changes can be seen in Figure 10. Also

noticeable in Figure 10 is the much higher inherent expression of αβTCR and CD3ε in the NOD DP populations, as compared to the B6 DP population. This enhanced expression could be a compensatory mechanism to overcome the documented defects in the TCR signaling cascade of NOD mice. Although not apparent in Figure 10, enhancements in DP αβTCR and CD3ε expression occur to some extent in both strains in the immature developmental groups (For αβTCR Immature MFI increase 23 ± 7% in B6 and 10 ± 3% in NOD; For CD3ε MFI increase 34 ± 2% in B6 and no significant change in NOD (Fig. 11)). However, these increases are much less dramatic in the NOD FTOC, perhaps due to an inability to substantially increase expression beyond their inherent high expression levels. Interestingly, cells that pass through selection (mature SP8, SP4, and DN cells) must adjust these enhanced levels of αβTCR and CD3ε expression back to normal levels of expression, perhaps to avoid negative selection. In fact, CD3ε expression is slightly decreased, significantly in some cases (SP8 and DN groups), in both strains in the mature cell groups, possibly indicative of the residual impact from CD1d, which was still present at the time of selection.

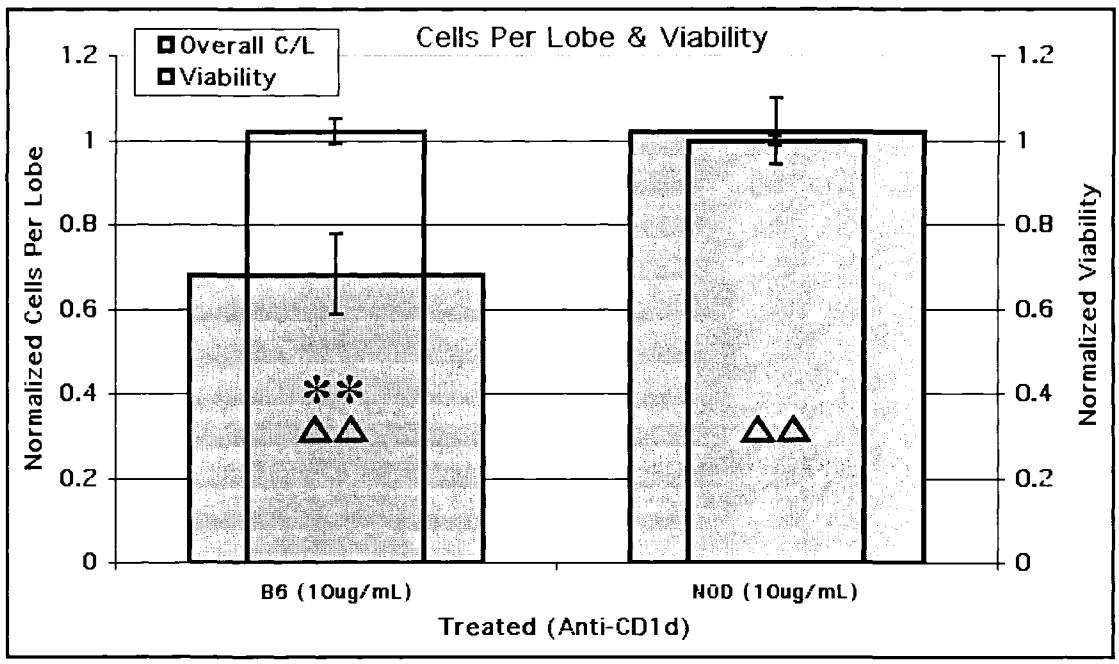
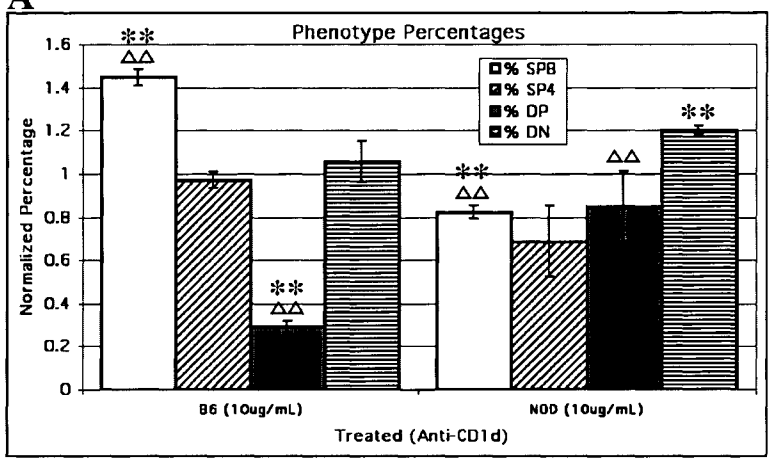


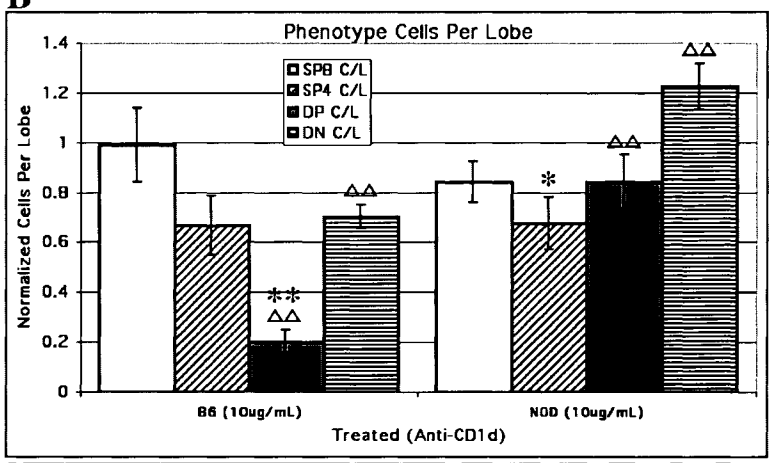
Figure 8. Cell Yields from 10 μg/mL treated FTOC. Cells from a 12-day FTOC, derived from 14-15 day gestation thymi, were analyzed for yield and viability through trypan-blue exclusion. FTOC were untreated or treated with 10 μg/mL of anti-CD1d. Values were normalized to the untreated control for direct comparison. Actual ranges for the C/L generated in the untreated control were 49.2 ± 4.2 x 10^4 for B6 FTOC and 31.0 ± 3.0 x 10^4 for NOD FTOC. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control within the mouse strain. One open triangle (△) indicates $p \leq 0.10$ and two open circles (△△) indicate $p \leq 0.05$, when comparing the treated NOD and B6 FTOC. (n=3)

A



STRAIN	% SP8	% SP4	% DP	% DN
C57BL/6	30.6 ± 0.4	24.0 ± 1.0	20.0 ± 1.7	25.3 ± 0.3
NOD	36.2 ± 0.5	26.5 ± 1.0	9.3 ± 0.2	28.0 ± 1.1

B



STRAIN	C/L SP8	C/L SP4	C/L DP	C/L DN
C57BL/6	15.1 ± 1.4	11.9 ± 1.4	9.8 ± 0.6	12.5 ± 1.1
NOD	11.2 ± 1.0	8.2 ± 1.0	2.9 ± 0.3	8.6 ± 0.7

Figure 9. Phenotype Generation from 10 μg/mL Treated FTOC. Cells from a 12-day B6 and NOD FTOC, derived from 14-15 day gestation thymi, were analyzed for percentage and C/L generated in each developmental group (SP8, SP4, DP, and DN). FTOC were untreated or treated with 10 μg/mL of anti-CD1d. Values were normalized to the untreated control for direct comparison. Actual ranges for the percentage and C/L generated in each developmental group are listed in the table below the graph (C/L values

are x 10^4). One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control within the mouse strain. One open triangle (△) indicates $p \leq 0.10$ and two open circles (△△) indicate $p \leq 0.05$, when comparing the treated NOD and B6 FTOC. (n=3)

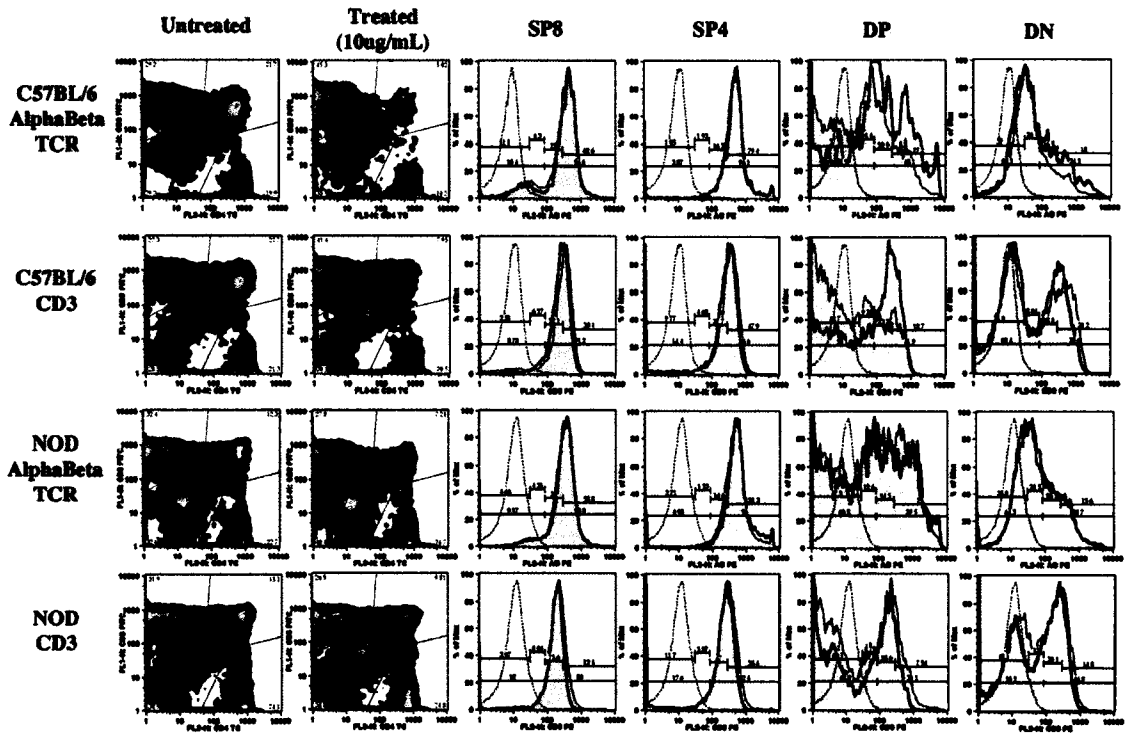
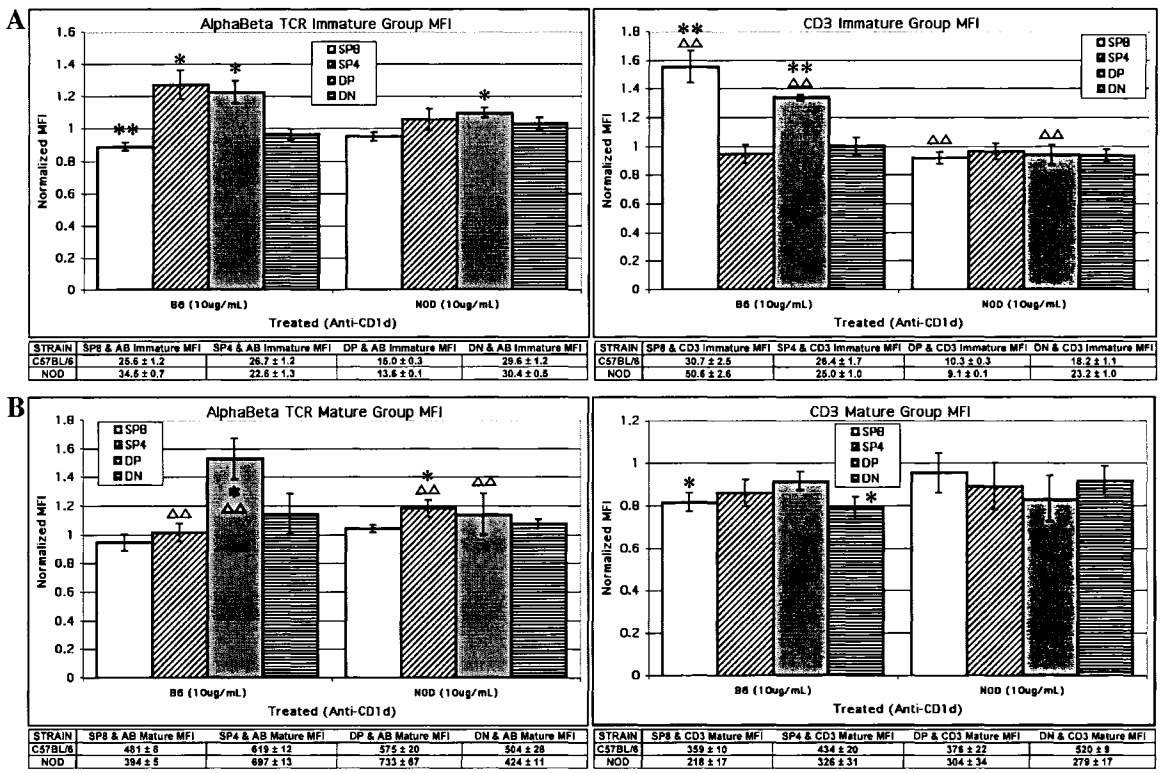


Figure 10. Flow Cytometric Analysis of αβTCR and CD3ε Expression on Developmental Groups. Cells from a 12-day B6 and NOD FTOC, derived from 14-15 day gestation thymi, were analyzed for expression of αβTCR and CD3ε in each developmental group (SP8, SP4, DP, and DN). FTOC were untreated (shaded) or treated (bold line) with 10 μg/mL of anti-CD1d. Gating was established based on the isotype control (dashed line) and the gating of negative, dull, intermediate, and bright staining groups (upper gates). Cells considered negative or dull are defined as immature cell types, while intermediate and bright cells are considered mature (lower gates). All experiments were performed under identical conditions and run at the same settings on the same day.



STRAIN	SP8 & AB Immature MFI	SP4 & AB Immature MFI	DP & AB Immature MFI	DN & AB Immature MFI
C57BL/6	25.6 ± 1.2	26.7 ± 1.2	16.0 ± 0.3	29.6 ± 1.2
NOD	34.6 ± 0.7	22.8 ± 1.3	13.6 ± 0.1	30.4 ± 0.5

STRAIN	SP8 & CD3 Immature MFI	SP4 & CD3 Immature MFI	DP & CD3 Immature MFI	DN & CD3 Immature MFI
C57BL/6	30.7 ± 2.5	26.4 ± 1.7	10.3 ± 0.3	18.2 ± 1.1
NOD	50.6 ± 2.6	28.0 ± 1.0	9.1 ± 0.1	23.2 ± 1.0

STRAIN	SP8 & AB Mature MFI	SP4 & AB Mature MFI	DP & AB Mature MFI	DN & AB Mature MFI
C57BL/6	481 ± 8	619 ± 12	575 ± 20	504 ± 28
NOD	394 ± 5	697 ± 13	733 ± 67	424 ± 11

STRAIN	SP8 & CD3 Mature MFI	SP4 & CD3 Mature MFI	DP & CD3 Mature MFI	DN & CD3 Mature MFI
C57BL/6	359 ± 10	434 ± 20	376 ± 22	520 ± 9
NOD	218 ± 17	326 ± 31	304 ± 34	279 ± 17

Figure 11. Changes in αβTCR and CD3ε Expression in Developmental Groups. Cells from a 12-day B6 and NOD FTOC, derived from 14-15 day gestation thymi, were analyzed for expression of αβ TCR and CD3ε in each developmental group (SP8, SP4, DP, and DN). FTOC were untreated or treated with 10 μg/mL of anti-CD1d. Values were normalized to the untreated control for direct comparison. Actual ranges for the immature (A) and mature (B) group mean fluorescent intensity (MFI) in each developmental group are listed in the table below the graph. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control within the mouse strain. One open triangle (△) indicates $p \leq 0.10$ and two open circles (△△) indicate $p \leq 0.05$, when comparing the treated NOD and B6 FTOC. (n=3)

Addition of 10μg/mL of anti-CD1d leads to increased expression of α-GalCer CD1d tetramer specific TCR in developing NOD DP cell populations.

Additional experiments performed at the 10 μg/mL anti-CD1d level were analyzed for NKT cell development using several different staining techniques (Fig. 12). The most accurate of these staining techniques for defining NKT cells, based on the work of Kronenberg et al. (33), is the analysis of DN/$\alpha\beta^{TCR+}$/$CD1d^{tet+}$ cells, however DN/$CD3\varepsilon^{+}$/$CD1d^{tet+}$ is also frequently used. The impact of 10 μg/mL of anti-CD1d on the NKT cell production using these staining techniques can be seen in Figure 13. The normalized DN/$\alpha\beta^{TCR+}$/$CD1d^{tet+}$ percentage decreases (43 ± 12% in B6) or increases (240 ± 120% in NOD) in $CD1d^{tet+}$ cells, using this alternate staining technique, are similar to those presented in the titration data (Fig. 7).

Also, increases similar to those seen in the general αβTCR and CD3ε expression in the developmental DP B6 FTOC cells (Fig. 11) were also seen in $CD1d^{tet+}$ staining in the DP groups of both experimental strains. However, in contrast to the αβTCR and CD3ε expression, the largest increases in expression were seen in the NOD DP group instead of the B6 DP group ($CD1d^{tet}$ MFI increases of 55 ± 20% in B6 and 611 ± 198% in NOD (Fig 14)). These changes in expression are much larger than those seen in the αβTCR data and show different strain specific increases than the αβTCR changes, due perhaps to a more specific impact by anti-CD1d on this receptor. As seen in the αβTCR data the NOD DN and SP4 groups that are associated with NKT cells show a higher inherent level of TCR expression, again this may be a compensation for poor internal signaling.

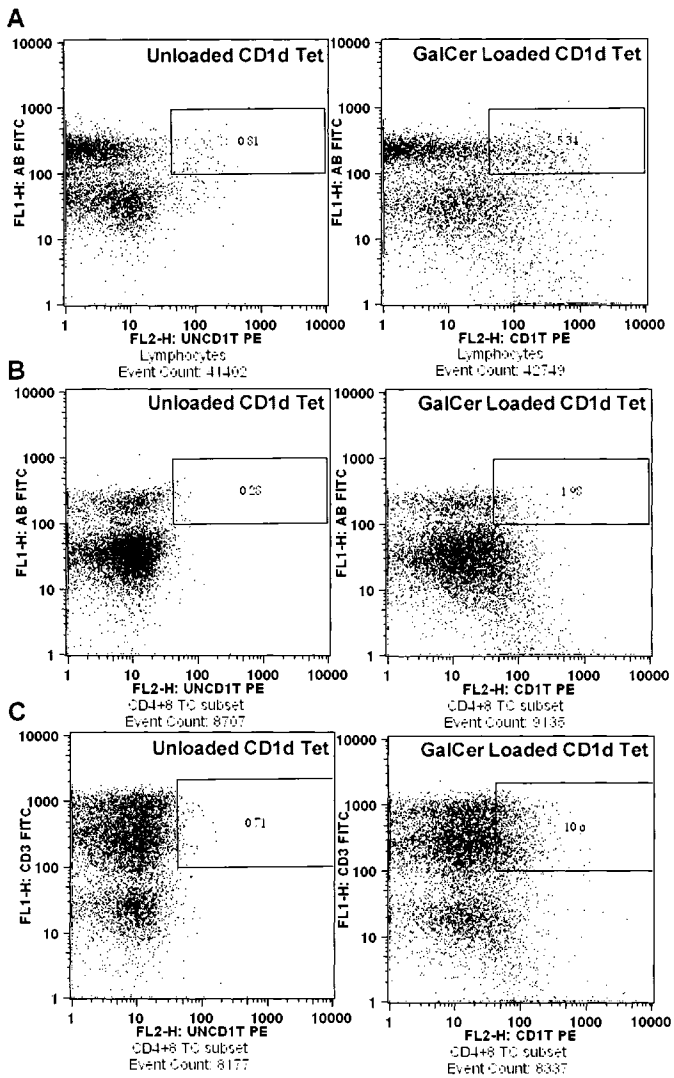
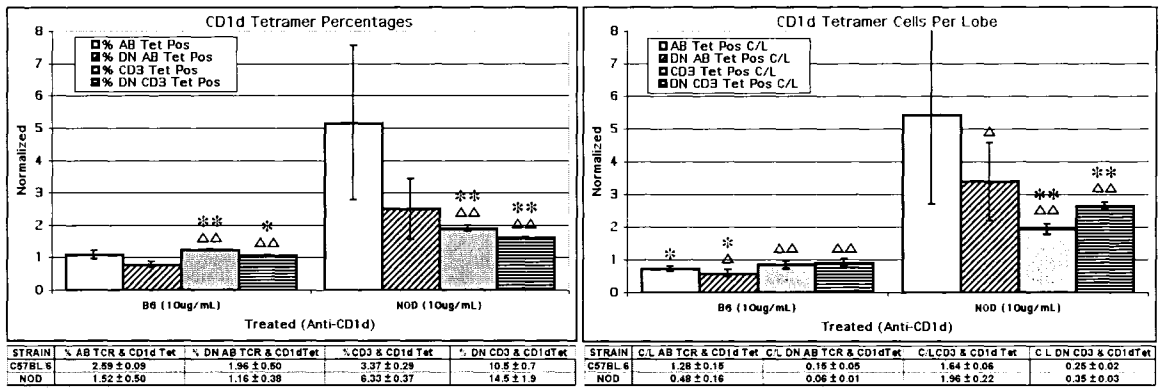


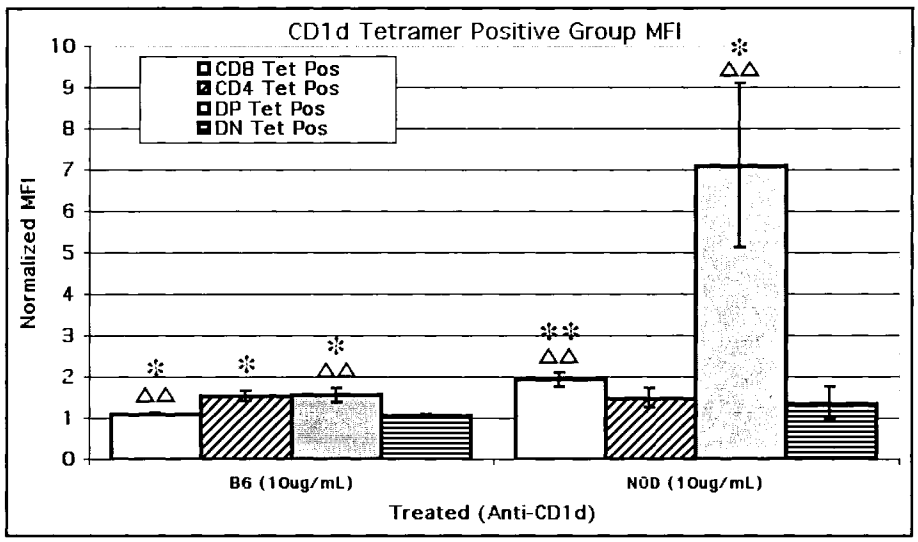
Figure 12. Analysis of α-GalCer CD1d^{tet+} Cells Using αβTCR or CD3ε Staining. A comparison of unloaded and α-GalCer loaded CD1d tetramer staining techniques using cells from the 10 μg/mL treated NOD FTOC is shown. Analysis of αβTCR^{+}/CD1d^{tet+} (A), DN/αβTCR^{+}/CD1d^{tet+} (B), and DN/CD3ε^{+}/CD1d^{tet+} staining is shown. A comparative example of treated versus untreated DN/αβTCR^{+}/CD1d^{tet+} culture staining can be seen in Figure 6D & E.



STRAIN	% AB TCR & CD1d Tet	% DN AB TCR & CD1dTet	%CD3 & CD1d Tet	% DN CD3 & CD1dTet
C57BL 6	2.59 ± 0.09	1.96 ± 0.50	3.37 ± 0.29	10.5 ± 0.7
NOD	1.52 ± 0.50	1.16 ± 0.38	6.33 ± 0.37	14.5 ± 1.9

STRAIN	C/L AB TCR & CD1d Tet	C/L DN AB TCR & CD1dTet	C/LCD3 & CD1d Tet	C L DN CD3 & CD1dTet
C57BL 6	1.28 ± 0.15	0.15 ± 0.05	1.64 ± 0.06	0.25 ± 0.02
NOD	0.48 ± 0.16	0.06 ± 0.01	1.96 ± 0.22	0.35 ± 0.03

Figure 13. Changes in αβTCR or CD3ε α-GalCer CD1d^{tet+} Cells. Cells from a 12-day B6 and NOD FTOC, derived from 14-15 day gestation thymi, were analyzed for αβTCR^{+}/CD1d^{tet+}, DN/αβTCR^{+}/CD1d^{tet+}, CD3ε^{+}/CD1d^{tet+}, and DN/CD3ε^{+}/CD1d^{tet+} cells (A). FTOC were untreated or treated with 10 μg/mL of anti-CD1d. Values were normalized to the untreated control for direct comparison. Actual ranges for the appropriate untreated control are listed in the table below the graph. CD3 groups include αβ and γδ TCR^{+} T cells. Groups not excluding non-DN cell types include all T cell phenotypes. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control within the mouse strain. One open triangle (△) indicates $p \leq 0.10$ and two open circles (△△) indicate $p \leq 0.05$, when comparing the treated NOD and B6 FTOC. (n=3)



STRAIN	SP8 & CD1d Tet MFI	SP4 & CD1d Tet MFI	DP & CD1d Tet MFI	DN & CD1d Tet MFI
C57BL/6	55.0 ± 1.0	145 ± 4	85.6 ± 7.3	65.9 ± 2.4
NOD	63.4 ± 1.2	665 ± 59	75.2 ± 16.7	313 ± 63

Figure 14. Changes in α-GalCer CD1d Binding TCR Expression in Developmental Groups. Cells from a 12-day B6 and NOD FTOC, derived from 14-15 day gestation thymi, were analyzed for expression of α-GalCer CD1d binding TCR in each developmental group (SP8, SP4, DP, and DN). FTOC were untreated or treated with 10 μg/mL of anti-CD1d. Values were normalized to the untreated control for direct comparison. Actual ranges for the α-GalCer CD1d binding TCR MFI in each developmental group are listed in the table below the graph. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control within the mouse strain. One open triangle (△) indicates $p \leq 0.10$ and two open circles (△△) indicate $p \leq 0.05$, when comparing the treated NOD and B6 FTOC. (n=3)

Expansion of the NKT cell population in NOD FTOC results in an increase in the insulin production in the ivT1DM system.

The data presented above suggest that the presence of anti-CD1d antibodies during T cell development may facilitate the expansion of a mixed compartment of cells that are both SP4 $CD1d^{tet+}$ and DN $CD1d^{tet+}$. This phenotype satisfies one of several NKT sub-types as defined by Kronenberg et al (33). We wished to assess the functionality of this NKT cell population in terms of autoimmune regulation in our ivT1DM model. Towards that end, 6-10 NOD fetal thymus lobes, 14-15dg, were treated with logarithmically increasing doses of anti-CD1d. For these experiments, the following titration levels were examined: 0.01 μg/ml, 0.1 μg/ml, 1.0 μg/ml, or 10 μg/ml. As before, antibodies were initially added to the thymus lobes on day 0 of culture and remained in the media until the end of culture. On day 14 of culture, the lobes were transferred to fresh culture dishes to remove any residual anti-CD1d, and an equal number of syngeneic fetal pancreas fragments were placed in direct contact with each of the thymus lobes. (See *Materials and Methods* for details). 500μl of culture supernatant were removed from each dish on days 0, 7, 14, and 21. These samples were then analyzed for insulin content via RIA. The inherent variability between experiments in insulin production is due to pancreas fragment size, maturity of tissue available, and growth over the course of the 21-day co-culture period. Therefore, insulin levels were normalized in each individual experiment to the amount of insulin produced by pancreas alone (i.e. no thymus lobes were present).

The untreated pancreas/thymus co-culture showed an average of a 52 ± 6% reduction in insulin production over the days that supernatant was collected and measured for insulin content via RIA (Fig. 15; days 7, 14 and 21; there is no insulin production at the day 0 control, not shown). This reduction or "*in vitro* T1DM" effect proved to be statistically significant when compared to pancreas cultured alone ($p \leq 0.05$) and is indicative of the presence of mature, functional, immune competent diabetogenic T cells in the co-culture. The quantification of reduced insulin production is an indirect indicator of the destruction of insulin producing islets mediated by these cells. Previous work by our laboratory has shown this effect to be strain specific as similar co-cultures performed with BALB/c derived thymi and pancreata failed to register a statistically significant reduction in insulin production over the course of the 21-day co-culture period. Furthermore, this diabetogenic effect was demonstrated to be T cell-mediated, as it could be completely inhibited by the administration of $F(ab')_2$ anti-CD3 antibodies, which presumably blocks the TCR recognition of pancreas specific antigens and thus prevents T cell activation. Previous work has also indicated that IgG control antibody has no effect upon the severity of *iv*T1DM (25).

The addition of anti-CD1d to FTOC prior to the co-culture period proved to have a statistically minimal impact on the "*iv*T1DM" effect at the lower concentrations (0.01 μg/ml & 0.1 μg/ml). However, anti-CD1d additions at the 1.0 μg/ml concentration proved to rescue insulin production to a degree that was statistically indistinguishable from pancreas cultured alone. The 1.0 μg/ml anti-CD1d treated co-culture dish produced 89 ± 13% of the amount of insulin produced by pancreas alone (Fig. 15). This amount

proved to be statistically distinct upon comparison to the untreated co-culture dish (ivT1DM positive dish) indicating that the addition of anti-CD1d was able to rescue insulin production at this particular concentration.

Surprisingly, the ability of anti-CD1d additions to rescue insulin production in the *iv*T1DM model was lost when the highest concentration of antibody was used (10 μg/ml). These results contrast with experiments that were discussed above in which $CD1d^{tet}$ NKT cell populations were assessed in anti-CD1d treated FTOC. In these experiments, the 10 μg/ml concentration yielded the most dramatic results in terms of expanding the NKT cell populations as measured via $CD1d^{tet}$ (Fig. 7). However, the *iv*T1DM results seemed to equate better with the production of $DX5^{+}$ NKT cells, which were also showed reduction at the highest dose of anti-CD1d (Fig. 5).

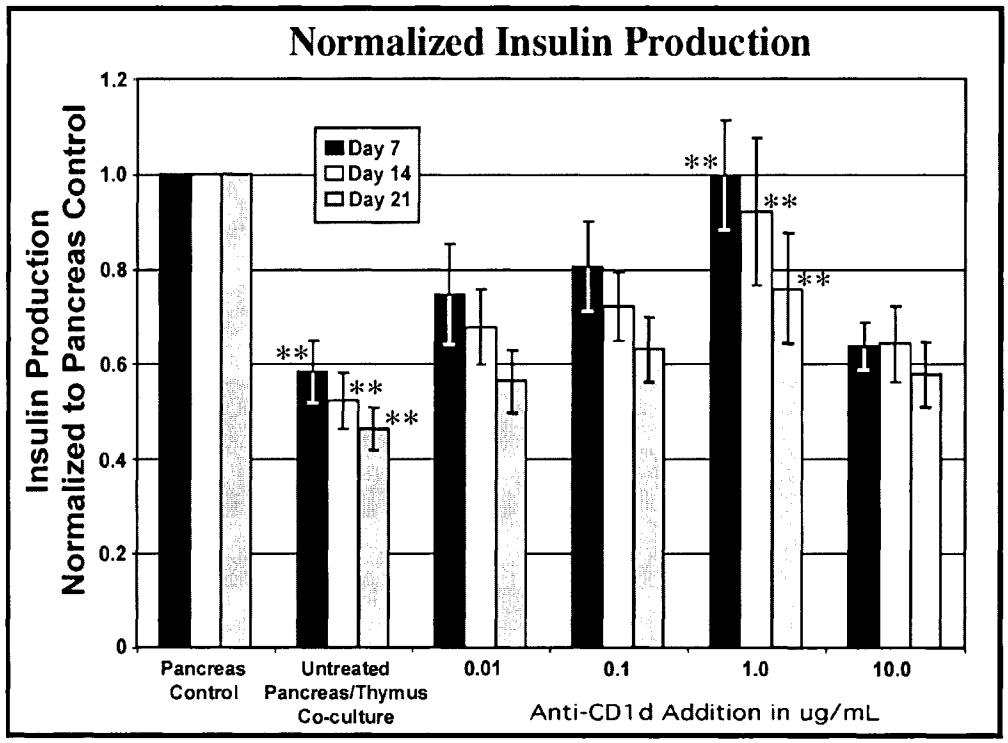


Figure 15. Anti-CD1d Treatment of NOD FTOC Reverses Diabetes Using the *In Vitro* Type 1 Diabetes Mellitus Model. 6-10 NOD fetal thymus lobes, 14-15dg, were placed on the surface of Millipore filters, supported on blocks of surgical Gelfoam, in 3 ml of media and treated with logarithmically increasing doses of anti-CD1d. For these experiments, the following titration levels were examined: 0.01 μg/ml, 0.1 μg/ml, 1.0 μg/ml, or 10 μg/ml. Antibodies were initially added to the thymus lobes on day 0 of culture and remained in the media until the end of culture. On day 14 of culture, the lobes were transferred to fresh dishes to remove antibody, and an equal number of syngeneic fetal pancreas segments were placed in direct contact with each of the thymus lobes. 500 μl of culture supernatant were removed from each dish on days 0, 7, 14, and 21. These samples were then analyzed for insulin content via RIA. Insulin levels were normalized to

pancreas cultured alone in each individual experiment for direct comparison (actual range for untreated control varied from 500-900 IU/ml). Two asterisks (**) on the untreated pancreas/thymus co-culture indicate a statistically significant difference between this dish and the pancreas cultured alone ($p \leq 0.05$). Two asterisks (**) on the 1 μg/ml anti-CD1d treated co-culture indicate a statistically significant difference between this dish and the untreated pancreas/thymus co-culture ($p \leq 0.05$). There was no statistical difference between the 1 μg/ml anti-CD1d treated co-culture and pancreas cultured alone. (n=6)

DISCUSSION

In this study we show that antibodies to CD1d can divergently effect the generation of potential NKT cells in FTOC from different mouse strains. In the case of the non-diabetic B6 FTOC, increasing doses of anti-CD1d caused a decrease in the generation of $DX5^+$ and $CD1d^{tet}$ NKT cells. Conversely, NOD mice, which are known to have cell-signaling abnormalities, showed increases in these NKT cells when exposed to increasing doses of anti-CD1d (Fig. 5 & 7), possibly through the anti-CD1d driven upregulation of the TCR on NKT cells. This increase in NKT cells causes diabetes susceptible NOD co-cultures to become resistant to diabetes when tested using the *iv*T1DM model (Fig. 15). We interpret this change in disease susceptibility to be due to an enhancement in the NKT populations of the "NKT deficient" NOD mice.

NKT cells are thought to play a crucial role in several types of immune processes. Several of the loosely defined NKT types, including the invariant $CD1d^{tet+}$ and the $DX5^+$ subtypes, have shown the capacity to regulate autoimmune responses. The majority of work performed on $CD1d^{tet+}$ NKT cells characterizes them as capable of producing T_H1 (IL-2, IFN-γ and others) and T_H2 (IL-4, IL-10 and others) cytokines, making them capable of skewing the phenotype of developing T cells and modulating the responses of mature T cells in the periphery (37-39). Similarly, $DX5^+$ NKT cells may also control autoimmunity through an immune diversionary process that involves T_H2 cytokines ("damage control") that limits the severity of autoreactive responses in the periphery (31). It should be noted, in regards to the data presented here, that expansion of NKT

cells during development would affect both developmental and peripheral NKT mechanism of regulation.

NKT cells are present very early in thymic development and are thought to play a role in T cell differentiation by controlling cytokine levels during thymic education. "Normal" NKT cells produce high levels of IL-4 and IFN-γ in adult life but preferentially produce IL-4 during early stages of neonatal development, influencing development in a manner that helps to control T_H1 autoimmune responses from occurring (40). Imbalances in production of the T_H1 cytokine IFN-γ by NKT cells, similar to those seen in NOD mice, have been correlated with T1DM (41, 42) and may play a major role in T1DM disease induction. Further work is still needed to define the exact roles of NKT cells during development but it has clearly been proven that their regulatory capacity extends beyond the mature responses in the periphery. Although similar NKT defects have been hypothesized to play a role in human T1DM, conflicting results have left their exact role in human diabetes unclear (37, 43).

Currently, little is known about thymic NKT development. Thymus derived NKT cells are thought to undergo a process of positive selection similar to traditional T cell types, since they both use the same TCR complex. Not surprisingly, Baur et al have shown that NKT cells require a functional CD3 signaling complex to fully mature (44). However, it is still unknown whether these cells are undergoing the process of β selection, similar to that seen in traditional αβ thymocytes. Another difficulty in analyzing NKT development is the lack of clearly defined pre-NKT cells. For example, invariant Vα14-Jα18 NKT cells are initially $NK1.1^-$, implying that before TCRβ and the

subsequent TCRα chain rearrangement occurs, these cells may be indistinguishable from traditional T cell precursors (33). Thus, the current theory of thymic derived NKT cell development suggests that random α and β rearrangement during the $CD4^-/CD8^-/CD3^-$ (triple negative, (TN)) phase leads to the generation of cells with a Vα14-Jα18 α chain and a β chain composed of Vβ8, Vβ7, or Vβ2. These pre-NKT cells are then positively selected on self-glycolipid loaded CD1d molecules on developing DP thymocytes or perhaps on other developing DP pre-NKT cells (33). More recent experiments have shown that the later stages of NKT development are distinct from T cell development since both developmental pathways are affected differently by mutations in certain signaling pathway molecules (e.g. Ras/Mek, Fyn, Ets1 and others); however there are still common components shared by both cell types (reviewed in (33)).

In this vein, the use of anti-CD1d treatment on FTOC, during the process of T cell and NKT cell development, illustrates the importance of CD1d on NKT selection. As seen in Figures 1 and 2, low-level anti-CD1d addition shows little or no effect on the general number and ratio of the DN and SP4 developmental T cell types generated in B6 or NOD FTOC (DP and SP8 were similarly unaffected). However, higher levels (10 μg/mL) of anti-CD1d treatment can generate significant changes in T cell and NKT development that are strain specific. An examination of the B6 DN population for $\alpha\beta TCR^{mature+}$, $DX5^+$, and $CD1d^{tet+}$ cells revealed that anti-CD1d treatment, with levels as low as 0.1 μg/mL, led to decreases in the production of all of these possible NKT cell types. The most significant of these decreases can be seen in Figure 7C, where at the 10 μg/mL level of treatment there is a 60% reduction in the number of $CD1d^{tet+}$ cells. B6

$CD1d^{tet+}$ cells are highly sensitive to anti-CD1d treatment, showing decreases in cell number and cell percentage at even the low 0.001 μg/ml level. These results would be expected based on the current theory of NKT development and the use of anti-CD1d, which should block CD1d/TCR interaction. If the development of these cells involves a CD1d dependent positive selection step, an inhibition of this interaction via anti-CD1d administration would lead to a lack of positive selection, arresting pre-NKT cells at this stage of development.

Although this theory of interference with NKT positive selection seems viable, work with NOD mice seems to argue against a strict blocking mechanism by anti-CD1d. It has been shown that NOD mice have deficiencies in TCR signaling pathway, leading to decreases in IL-2 and IL-4 production. This defect is thought to not only have effects on the positive and negative selection of developing thymocytes, but also on peripheral tolerance (45-47). Since the TCR of NKT cells use this same signaling complex, it can be inferred that NKT cells will have some similar signaling deficiencies. Therefore, since anti-CD1d treatment on NOD FTOC causes an increase, and not a decrease, in the production of $CD1d^{tet+}$ cells (see Fig. 7A&B and 13) and the expression of $CD1d^{tet}$ binding TCR (Fig. 14), this argues against the concept that anti-CD1d is strictly blocking positive selection by denying access to CD1d molecules. Although there is still the possibility that an anti-CD1d induced block of CD1d could lead to a reciprocal increase in TCR or CD1d, which then could lead to enhanced signaling during selection. Regardless, the effect of anti-CD1d on NOD FTOC leads to an increase in the percentage and number of $CD1d^{tet+}$ NKT cells, leading to the generation of NKT cells at the same or

higher levels than those found in the regulatory competent B6 control mice (Fig. 7D). Another effect of this anti-CD1d treatment is that it is capable of inhibiting NOD T1DM in our *in vitro* model (Fig. 15).

The ability of anti-CD1d to have divergent effects on these different strains of mice is likely due to an impact on both positive and negative selection. While positive selection is thought to take place on DP developing thymocytes or DP pre-NKT cells, more recent work has shown that negative selection of NKT cells takes place on thymic dendritic cells (48, 49). Work by Chun et al, and others, has shown that adding exogenous α-GalCer or increasing CD1d expression can effect NKT cell production in a manner that was consistent with negative selection. In a similar fashion, anti-CD1d treatment may by inducing increased negative selection in the developing pre-NKT cells of B6 mice by enhancing signals to thymic dendritic cells. In other words, cells that would have normally received adequate signal to pass positive selection without being subject to negative selection would, in the presence of anti-CD1d, receive an enhanced reciprocal signal from the thymic dendritic cells which are now engaged with both the pre-NKT cell and CD1d antibodies. This enhanced reciprocal signal may then lead to negative selection of these pre-NKT cells. Suzuki et al have shown that FcR induced cross-linking of antibodies to TCR can influence $CD4^+$ T cell selection in a fashion that is signal strength dependent, with intermediate levels of cross-linking leading to positive selection without negative selection and high levels of cross-linking leading to negative selection (50). A similar mechanism may be affecting the selecting APCs (which could include DP T cells in the case of CD1d), leading to a stronger than normal response when

engaged with developing NKT cells and anti-CD1d. If additional signaling is occurring, then it would also lead to an additional signal to normal developing T cells as well, which would lead to the negative impact we see on normal T cell development in B6 FTOC (Fig. 9) and the enhanced expression of αβTCR and CD3ε in the B6 DP population (Fig. 10 & 11). However, other research in our laboratory using the signal blocking agent hydrocortisone shows similar TCR upregulation, which is thought to compensate for signal loss during development. In other words, anti-CD1d may initially block CD1d and force T cells and NKT cells to respond to the antibody induced signal loss. If the blocking by anti-CD1d dissipates over time or the TCR are upregulated too much in response to CD1d blocking this would also generate an increase in signal.

These higher levels of negative selection induced by increased signaling would occur when using a "normal" mouse model, like the C57Bl/6, which has few or no cell signaling defects. In the case of the poor signaling NOD mouse, it is possible that anti-CD1d induced signal enhancement would increase positive selection at certain levels of treatment while not affecting negative selection. In essence, anti-CD1d treatment of this type may enhance the engagement signal to the cells responsible for NKT positive selection through TCR upregulation. By enhancing this signal, the selecting cells may then send a stronger than normal reciprocal signal to NOD NKT cells, allowing these NKT cells to pass positive selection. This idea is supported by the preexisting high TCR expression on NOD DP T cells (Fig. 10), which does not increase as dramatically, in response to anti-CD1d, as the TCR expression on B6 mice (Fig. 11).

The ability of CD1d to effect both positive and negative selection may be the cause of the changes seen in NOD DN and SP4 $DX5^+$ cells in Figure 5. At the lower doses of anti-CD1d the number of $DX5^+$ cells increased, but at the 10 μg/ml level the $DX5^+$ cells decreased back to basal levels. This decrease may be due to the same negative selection signal experienced by B6 NKT cells when treated with anti-CD1d at lower levels. Also in Figure 5, B6 DN $DX5^+$ cells may have experienced enhanced positive selection at the 0.001 μg/ml treatment level, similar to the enhanced positive selection seen in NOD mice at higher levels of treatment. Contradictory to these statements is the fact that negative selection effects were not seen in the $CD1d^{tet+}$ cells, but these are thought to be a distinct NKT subset that may require a different signal strength during selection as compared to $DX5^+$ cells. Signal strength requirements can also explain the ability of NOD NKT TCR to upregulate expression significantly when normal NOD $\alpha\beta TCR^+$ T cells can only moderately upregulate expression (Fig. 11 and 14). It has been suggested that NKT cells typically require a much stronger signal for activation then normal $\alpha\beta TCR^+$ T cells (33). Although T cells and NKT use slightly different signaling systems, it is also possible that NKT cells have inherently lower levels of TCR expression in general. This lower level expression would allow for the higher levels of TCR upregulation seen in NOD NKT cells, however currently there is sparse information on the comparative levels of TCR expression between T cells and NKT cells.

T1DM has become a focal point for investigating the role of NKT cells in autoimmune diseases. Particularly, the NOD mouse model of T1DM has been used because the model shares several key characteristics with the human disease, including T

cell associated signaling defects. As discussed in the introduction, NOD mice have functional and numerical deficiencies in their NK and NKT cell populations. Work performed in our lab has also shown that the adoptive transfer of cells derived from FTOC, into NOD mice, can inhibit the development of diabetes (25), which is confirmed by the work of others using *in vivo* models (13). Presumably, it is the enhanced number of NKT immune regulators found in FTOC that aids in the inhibition of T1DM upon adoptive transfer.

Based on our work, it is apparent that the addition of anti-CD1d has the capacity to generate increased numbers of potential NKT cells in the NKT deficient NOD mouse (Fig. 3,5, & 7). This enhancement of NKT cells is also the likely reason that this treatment is capable of inhibiting diabetes *in vitro* (Fig. 15). The *iv*T1DM model was implemented as a means of testing whether or not experimentally induced changes in T cell development phenotype profiles would translate into changes in diabetes onset. In the case of NKT cells, the *iv*TIDM model allowed us to determine if the expanded population of NKT cells was functional and truly capable of regulating diabetes onset. The results suggest that at lower concentrations of anti-CD1d additions (0.01 μg/ml & 0.1 μg/ml) the NKT cell compartment expansion was not significant enough to completely mitigate islet cell infiltration and destruction. Although not statistically significant, these concentrations did show a slight improvement in insulin production over the untreated co-cultures. This result suggests a dampening of the "*iv*T1DM effect" and may be evidence that the expanded NKT cell populations were indeed functional. At the 1.0 μg/ml concentration, it appears that the NKT cell population was adequately expanded

mitigate *iv*T1DM. However, at the 10 μg/ml concentration, the concentration that showed the most dramatic expansion of $CD1d^{tet+}$ NKT cells, the addition of anti-CD1d antibodies appears to have little effect on *iv*T1DM onset. It is possible that the excessive amounts of anti-CD1d at this concentration not only provided the supplementary signal necessary to induce the expansion of $CD1d^{tet}$ NKT cells, but it might also have anergized the cells, rendering them non-functional. Alternatively, the $CD1d^{tet+}$ NKT cells may not be the primary inhibitors of diabetes in this instance since the ivT1DM data seems to more closely correlate with the changes in $DX5^{+}$ population.

Our work has focused the ability of anti-CD1d to divergently affect NKT development in NOD and B6 mice. This work is distinct from recent α-GalCer work, in that, it appears that anti-CD1d has the capacity to expand more than just the $CD1d^{tet+}$ NKT pool. Based on this study, it may be possible to use anti-CD1d to manipulate the production of different NKT subtypes in diabetic individuals. If performed early in disease onset, this may lead to control or mitigation of the disease. Work still needs to be performed, and is planned, to assess the viability of this treatment *in vivo*.

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APPENDIX C

Modulation of T cell Development in Fetal Thymus Organ Culture through Exogenous Glucocorticoids[1]

Todd C. White* and Dominick DeLuca[2]*

*Department of Microbiology and Immunology

University of Arizona, Tucson, AZ 85724

Keyword List: T Lymphocytes, Repertoire Development, Thymus, T cell Receptors

ABSTRACT

T cell development requires positive and negative selection signals to ensure that the T cells produced by thymus are not capable of autoreactive responses when they reach the periphery. Recently, steroid hormones (such as glucocorticoids (GCs) have been shown to play a role in T cell development by affecting positive and negative selection signals, possibly through the up-regulation or down-regulation of several immune associated genes. GCs are thought to affect the perceived avidity of cell-to-cell interactions by dampening cytokine production, internal signaling, and/or receptor expression during selection. To more closely examine the effects of GCs on T cell development, we have used fetal thymus organ culture to analyze the impact of exogenous hydrocortisone (HC) on the developing T cell repertoire. Addition of 10^{-9} M exogenous HC caused T cell expansion and showed influence at two distinct control points of development. These HC-sensitive stages coincide with the time of β selection and positive/negative selection, respectively. HC treatment at these time points lead to increased expression of several receptors, including the TCR β chain and CD3ε. Interestingly, HC exposure either during β selection or positive/negative selection led to the generation of different mature SP4/SP8 T cell ratios and distinct immune populations as determined by T cell phenotype and TCR usage. These changes are thought to be due to the inhibitory nature of HC and the subsequent tuning of TCR expression levels.

INTRODUCTION

Glucocorticoids have long been known as modulators of immune inflammatory responses. The anti-inflammatory effects of GCs are produced through the up-regulation or down-regulation of several immune-associated genes. GCs have shown the capacity to directly or indirectly induce several genes including: the Type II IL-1 receptor ("IL-1 decoy receptor"), the secretory leukocyte proteinase inhibitor (an inhibitor of neutrophils), as well as the IL-7R α chain, which plays a crucial role in T cell development and T cell survival in the periphery (1-3). Conversely, GCs also play a role in the inhibition of several immune related genes, including: IL-1 through IL-6, IL-8, IL-11, IL-13, IL-16, GM-CSF, TNFα, matrix matalloproteinase 9, RANTES, and others. GCs are thought to effect these genes through the inhibition of the transcription factors NF-κB and AP-1 (reviewed in (4, 5)). Based on the breadth of their impact on cytokine production it is not surprising that GCs are thought to be the major regulatory molecule in controlling peripheral immune responses.

Although the role of GCs in peripheral immune responses is still being thoroughly investigated, in recent years, the role of GCs in T cell development has begun to garner much more attention. Initially, the role of GCs in T cell development was thought to be limited to GC-induced apoptosis, during the diurnal stress cycle, of those cells that failed during selection (6). In 1994, work by Vacchio et al. showed that the thymus was capable of producing GCs based on the presence of all of the enzymes needed for steroidogenesis (7). This was unusual since a very limited group of cells were known to produce GCs. Based on this information it was likely that GCs were playing a role in T cell

development. This concept was heavily supported by data from fetal thymic organ cultures (FTOC) where thymi are isolated from non-thymic sources of GCs. The inhibition of GCs in FTOC, using steroidogenisis blocking agents such as metyrapone, led to disruptions between the double negative ($CD4^-CD8^-$ (DN)) and double positive ($CD4^+CD8^+$ (DP)) phases of T cell Development (7, 8). Currently, GCs are thought to impact development through signal dampening effects, which partially inhibit cell-cell signaling and the perceived overall avidity of the developing T cells (8-10). Through these dampening effects, GCs are thought to change the necessary signal strength that determines whether positive and/or negative selection occurs.

To better understand the impact that GCs can have on T cell development we investigated the effects of exogenous HC on C57BL/6 (B6) FTOC. T cells undergoing selection appear to compensate for HC induced signal dampening by increasing the expression of several cell surface receptors, including αβ TCR and CD3. These effects are shown to be dose dependent. TCR Vβ usage also appears to be impacted by HC. Experiments investigating timed administration of HC during T cell development indicate that there are two distinct developmental time points that are influenced by HC. These time points are those related to TCR β selection and positive/negative selection. Treatment during these time points leads to the generation of distinct SP4 and SP8 mature T cell populations, indicating that the use of HC or HC blocking agents has the potential to modulate immune system development in several ways.

MATERIALS AND METHODS

Mice

Timed-pregnant C57BL/6 (B6) mice were obtained from the National Cancer Institute (Frederick, MD) at 13-days gestation (dg). They were maintained under specific pathogen-free conditions and sacrificed between 13-dg and 15-dg. The fetuses were removed from pregnant females at the indicated time points (plug date = day 0). We consistently found that animals arriving at our facility were variable with regard to their stage of development, even though they had been vaginally plugged on the same day. We therefore re-staged the pups that we obtained based on their developmental characteristics (such as digit separation on the paws) as given in "The Mouse, its Reproduction and Development" (11).

Fetal Thymus Organ Culture

The organ culture methods used have been described in detail by our laboratory and others (12). Briefly, at least 6 thymus lobes, dissected from 13-15 dg fetal mice were placed on the surface of Millipore filters (25 μm thick, 0.45 μm pore size; Millipore, San Francisco, CA), which were supported on blocks of surgical Gelfoam (Upjohn Co, Kalamazoo, MI) in 10 x 35 mm plastic Petri dishes with 3 ml of organ culture medium. Organ culture media (standard FTOC media) consisted of Dulbecco's Modification of Eagle's Medium:(DMEM plus 4.5 g/L D-glucose with L-glutamine; Mediatech, Inc., Herndon, VA) supplemented with 20% heat inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). The medium also contained 1 ml per 500 ml DMEM

of an Antibiotic-Antimycotic Solution (containing streptomycin (10,000 mg/ml), penicillin (10,000 U/ml) and amphotericin (25 mg/ml); Mediatech, Inc., Herndon, VA), 0.25 ml per 500 ml DMEM gentamycin (50 mg/ml; Mediatech, Inc., Herndon, VA), 10 ml per 500 ml DMEM 20X non-essential amino acids (Mediatech, Inc., Herndon, VA), 2-mercaptoethanol (4.6 x 10-5 M) as well as an additional 10 ml per 500 ml DMEM of L-glutamine (200mM; Mediatech, Inc., Herndon, VA). The cultures were grown in a humidified incubator in 5% CO_2 at 37°C. Cells were harvested as previously described (13, 14). The thymus lobes were placed into a solution of Accutase (ISC BioExpress, Kaysville, Utah). The tissue was incubated at 37° C for 15 minutes. The lobes were then dispersed into a single cell suspension by gentle aspiration with a Pasteur pipette. This treatment disaggregates most of the lymphoid and non-lymphoid stromal cells from the tissue. After washing once in Hanks' balanced salt solution (HBSS) plus 5% FBS to prevent further enzyme action, cell viability in Accutase-extracted samples was determined by 0.4% trypan blue exclusion. Viability was always greater than 85%. The results are expressed as the total cells recovered x 10^4 per thymus lobe. Unless otherwise noted, FTOC was carried out for 12 days.

Reagents

Initial experiments were performed using hydrocortisone (Sigma-Aldrich, St. Louis, MO) prepared by placing 36 mg of HC into 10 ml of a 40% ethanol solution in PBS creating a 10^{-2} M solution. Dilutions from that point on were made in our standard FTOC OCDME media. Ethanol controls were used for comparison. Later experiments, including all of

those addressed in this paper were performed using water-soluble HC (100mg HC/g packaged in cyclodextrin) to eliminate solubility problems and the use of ethanol. No significant difference was seen between ethanol diluted and water soluble HC. Experiments using metyrapone (Sigma-Aldrich, St. Louis, MO) were performed using 225 μg/mL diluted in our standard OCDME media. Experiments using anti-CD3 NA/LE (BD Pharmingen, San Diego, CA) were performed through serial dilution and addition to developing fetal thymus lobes.

Antibodies and Flow Cytometry

Cell suspensions were stained with directly conjugated monoclonal antibodies (Caltag Laboratories, Burlingame, CA or BD Pharmingen, San Diego, CA) specific for CD4-Tri-color, CD8-FITC, α/β TCR-PE, γ/δ TCR-PE, CD3ε-PE, CD25-PE, CD45RA-PE, CD69-PE, CD95-PE, CD95L-PE, CD122-PE, CD132-PE, DX5-PE, NK1.1-PE, TCR Vβ7-PE, and TCR Vβ8-PE at a concentration of 1 μg per $1x10^6$ cells. After staining the cells were fixed in 1% ρ-formaldehyde before flow cytometric analysis. Three-color FC analysis was performed using a FACScan (BDIS, San Jose, CA) equipped with photomultiplier tubes and optical fibers as recommended by the manufacturer. FITC, PE and TC were excited by a 488-nm Argon laser. Fluorescence data were collected using 3-decade logarithmic amplification on 10,000-30,000 viable lymphoid cells as determined by forward and 90° light scatter intensity to exclude stromal and other non-lymphoid elements. Data was collected with CellQuest (Santa Rosa, CA) and analyzed using

FlowJO (Treestar) software. Examples of flow cytometric results and how analysis is performed in our laboratory can be found in the following reference (15).

Statistical Analysis

There is some variability in total cell production across FTOC preparations (i.e. across distinct experiments). However, total cell productions from replicate samples within a given experiment are typically in much closer agreement. Concordantly, proportions and/or features of HC's effects did not change markedly across experiments. However, because of variations in the end culture cell production percentages and numbers, based on slight differences in the developmental/gestational states of tissue used in separate experiments, the values were normalized within each experiment to a percentage of the untreated control (Value of normalized untreated control is always equal to 1). Statistical analyses (mean and standard error of the mean) were then assessed using these normalized values, eliminating developmental/gestational variance. This technique allows for a more accurate comparisons when dealing with FTOC, which is constantly undergoing percentage changes throughout the course of development, but this may lead to discrepancies between the normalized and raw data. Paired and/or Unpaired Student's T tests were performed on all data shown based on what was most appropriate for the experiments being compared.

RESULTS

Addition of High Dose Exogenous GCs to Murine FTOC inhibits T cell Development

To study the effect of GCs on T cell development we investigated various levels of HC addition to B6 FTOC. Initial studies examined a wide range of HC concentrations (as high as 10^{-5} M). Through continued titrations we found that HC could effect T cell development negatively at high levels and, unexpectedly, that it could also effect T cell development positively (in a non-apoptotic fashion) at much lower levels. Once we established that HC concentrations above 10^{-6} M had toxic effects and those below 10^{-11} M produced no significant effect (not shown), the experimental titration range was narrowed. 6-10 B6 fetal thymus lobes, 13-15dg, were cultured for 12 days in the presence of logarithmically increasing doses of HC. The following titration levels were examined: $1x10^{-10}$ M, 5 x 10^{-10} M, $1x10^{-9}$ M, 5 x 10^{-9} M, $1x10^{-8}$ M, 5 x 10^{-8} M, or $1x10^{-7}$ M. Thymus lobes were cultured, starting day 0 of culture, in the presence of HC. HC remained in the media until the end of culture (See *Materials and Methods* for details).

Titration experiments showed high dose HC (10^{-8} M and higher) to generate significant decreases in overall cell yield and cell viability in FTOC (Fig. 1A). Cell yields decreased from 34.7 ± 2.2 cells per thymus lobe (C/L) in our control cultures to 28.5 ± 1.1 (decrease of (↓) 18%), 19.0 ± 5.4 (↓46%), and 11.2 ± 3.4 C/L (↓67%) in the $1x10^{-8}$ M, 5 x 10^{-8} M, and $1x10^{-7}$ M treated cultures, respectively. Similarly, viability of cells developing in the cultures dropped from 90.1 ± 1.0% in our control cultures to 85.5 ± 1.8% in the $1x10^{-8}$ M treated cultures, 82.6 ± 2.3% in the 5 x 10^{-8} M treated cultures, and 82.4 ± 4.2% in the $1x10^{-7}$ M treated cultures. These experiments fall in line with the

standard apoptotic effects caused by HC during T cell development but could also be caused by decreases in proliferation during T cell expansion (16, 17). Concordantly, cultures treated with high doses of HC show significant decreases in the number and percentage of $CD4^+CD8^+$ (DP) thymocytes generated (Fig. 1B, Table I), shifting from 20.7 ± 5.7% of the total cell population in the untreated controls to 7.9 ± 2.0% (↓62%) at the highest treatment dose ($1x10^{-7}$ M). These decreases in the DP group are coupled with significant increases in the percentage of $CD4^-CD8^-$ (DN) cells (33.5 ± 1.7% vs. 56.9 ± 7.5% at $1x10^{-7}$ M; increase of (↑) 70%). $CD4^+CD8^-$ (SP4) and $CD8^+CD4^-$ (SP8) groups showed moderate increases in their percentage of the overall cell population, but as in the case of the DP and DN cell types, the number of cells per lobe generated goes down at HC levels of $1x10^{-8}$ M and above.

Developmental receptor analysis of FTOC cultured in higher doses of HC indicate that the vast majority of DP thymocytes which survive treatment show a more "mature" T cell phenotype, based on αβTCR expression (28.1 ± 7.3% vs. 19.5 ± 4.0% in the untreated control (↑44%), Table I). This is in contrast to the surviving DN, SP4, and SP8 cell populations in which cells tend to show decreased maturity (in terms of αβTCR expression). In general, cultures treated with high dose HC generated DP populations that contained higher percentages of cells positive for several T cell associated receptors, including γδ TCR, CD45RA (a + and – regulator of the TCR signaling complex), CD69 (a activator/inducer molecule), CD122 (the IL-2 and IL-15 receptor β chain), and CD132 (the common γ chain for IL-2, 4, 7, 13, and 15 receptors). The lone exceptions to this are CD25 (IL-2R α chain), which shows decreased representation in all developing

thymocytes groups (DP, DN, SP4, and SP8) and CD95/CD95L (Fas/Fas Ligand), which showed no significant change. General trends for developmental receptors in each subpopulation can be seen in Table 1, but an extensive analysis of these receptors is beyond the scope of this paper. It is important to note that, despite increases in the percent of cells positive for these receptors, that the number of C/L generated at these higher HC levels is always drastically reduced versus the untreated control. It may be that at these higher doses of HC, developing T cells are beginning to enter apoptosis or death by neglect due to HC signal inhibition, leading the DP developmental group to modify receptor expression in an attempt to survive.

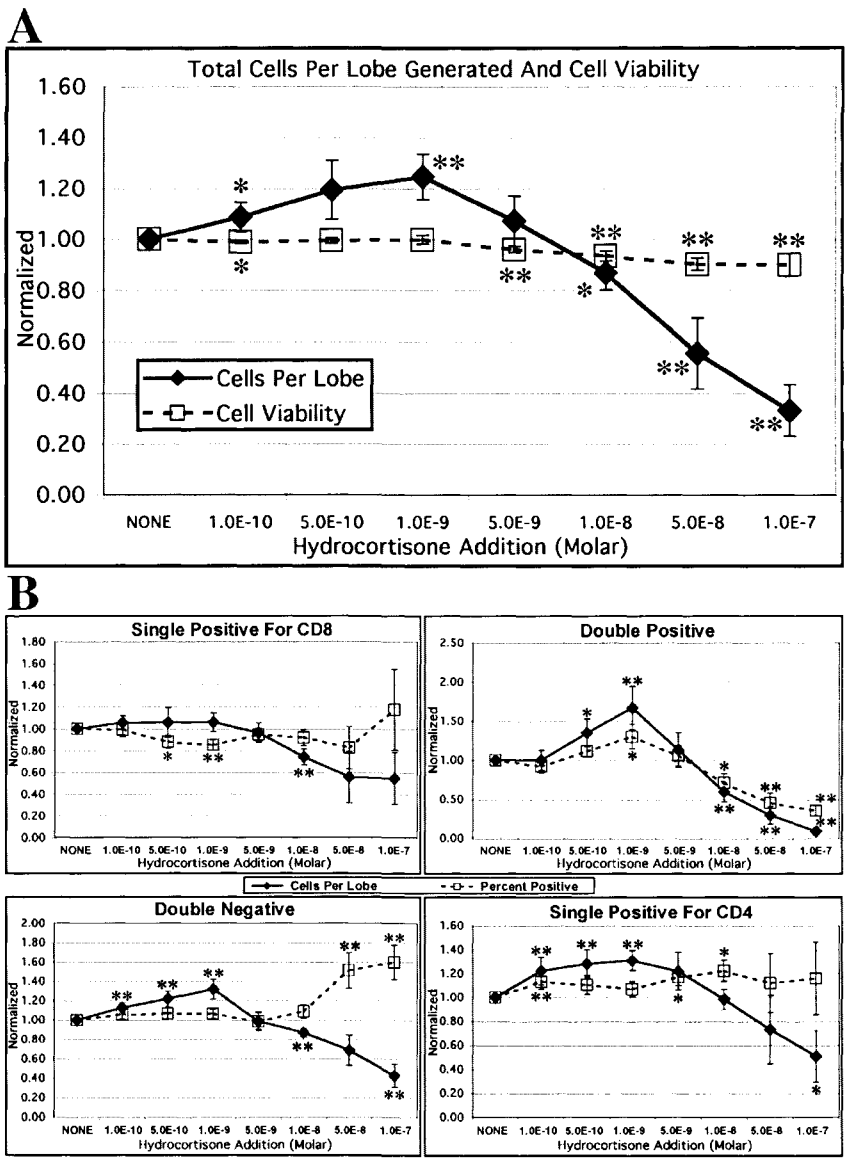


Figure 1. Cell Yields and Phenotype Analysis from FTOC. Cells from a 12-day B6 FTOC, derived from 13-15 dg thymi, were analyzed for yield and viability through trypan-blue exclusion (A) and for general T cell phenotype distribution (B). FTOC were treated with a titration of HC ranging from 10^{-10} to 10^{-7} M. The open squares (□) represent the viability in (A) and percent of general T cell phenotype in (B). The closed

diamonds (◆) represent the total C/L yield in (A) and the phenotype C/L yield in (B). Values were normalized to the untreated control for direct comparison. Actual ranges for the C/L generated in the untreated control for (A) was $30.9 \pm 2.3 \times 10^4$. Cell viability for the untreated control in (A) was 91.5 ± 0.5%. Untreated controls for (B) phenotype C/L were 6.68 ± 1.16 for SP8, 5.11 ± 0.87 for SP4, 8.31 ± 2.85 for DP, and 10.8 ± 0.9 for DN (all values x 10^4). Untreated controls for (B) phenotype % were 21.9 ± 3.5 for SP8, 16.7 ± 2.7 for SP4, 25.9 ± 7.9 for DP, and 35.5 ± 2.1 for DN. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control (n=9).

Table I. Changes in Receptor Expression for Developing T cells Treated with **High Doses** of Hydrocortisone										
Cell Population	Hydrocortisone Level	αβ TCR Mature	γδ TCR Mature	CD25	CD45RA	CD69	CD95	CD95L	CD122	CD132
DN ($CD4^-CD8^-$)	Untreated Control	11.0 ± 1.8	52.0 ± 4.4	74.2 ± 5.1	61.2 ± 8.4	80.6 ± 3.6	77.9 ± 3.2	36.1 ± 3.9	35.5 ± 4.2	89.5 ± 2.0
	$1x10^{-8}$ M	**8.5 ± 2.1**	**42.4 ± 8.5**	68.9 ± 5.2	64.3 ± 9.2	78.5 ± 2.9	70.5 ± 4.4	32.0 ± 8.2	31.1 ± 2.8	85.5 ± 2.2
	$5x10^{-8}$ M	5.3 ± 1.3	**33.4 ± 7.0**	61.7 ± 4.9	65.9 ± 6.2	74.3 ± 4.4	**62.5 ± 5.8**	25.9 ± 4.9	47.6 ± 6.4	**83.8 ± 2.9**
	$1x10^{-7}$ M	5.4 ± 1.7	**33.0 ± 9.5**	**53.2 ± 4.3**	71.7 ± 5.3	**68.8 ± 3.8**	**51.1 ± 8.9**	**15.4 ± 2.4**	**51.9 ± 4.4**	77.8 ± 3.0
DP ($CD4^+CD8^+$)	Untreated Control	19.5 ± 4.0	7.0 ± 0.7	83.1 ± 4.6	29.6 ± 5.9	25.2 ± 2.4	79.5 ± 6.5	44.3 ± 9.3	17.4 ± 1.7	36.2 ± 2.5
	$1x10^{-8}$ M	**25.2 ± 3.8**	**10.3 ± 1.4**	77.4 ± 6.6	44.1 ± 10.0	**41.7 ± 7.1**	80.0 ± 5.8	36.3 ± 4.4	21.6 ± 3.6	46.4 ± 4.5
	$5x10^{-8}$ M	**25.1 ± 1.2**	**22.0 ± 6.6**	**64.3 ± 3.9**	**61.1 ± 14.2**	**52.0 ± 10.6**	85.5 ± 3.4	60.1 ± 10.3	**47.2 ± 13.0**	**64.9 ± 10.6**
	$1x10^{-7}$ M	**28.1 ± 7.3**	**24.3 ± 7.0**	**64.3 ± 5.1**	**63.9 ± 8.3**	**54.3 ± 7.0**	67.6 ± 7.4	47.7 ± 6.7	**48.9 ± 10.2**	**63.9 ± 4.7**
SP4 ($CD4^+CD8^-$)	Untreated Control	86.0 ± 2.6	10.4 ± 2.5	71.3 ± 12.6	50.1 ± 10.7	72.0 ± 3.0	84.3 ± 4.6	49.8 ± 5.9	34.7 ± 3.5	69.2 ± 3.6
	$1x10^{-8}$ M	72.1 ± 4.8	10.6 ± 2.8	65.8 ± 14.7	55.4 ± 12.2	73.0 ± 4.0	**76.9 ± 6.9**	38.4 ± 4.6	28.3 ± 2.2	57.7 ± 4.3
	$5x10^{-8}$ M	59.5 ± 10.2	**16.9 ± 0.4**	60.7 ± 13.5	58.2 ± 5.6	76.4 ± 2.2	**79.3 ± 5.8**	47.1 ± 8.1	43.0 ± 4.9	69.8 ± 4.6
	$1x10^{-7}$ M	**48.8 ± 9.8**	**17.3 ± 5.3**	**54.8 ± 11.9**	53.9 ± 6.6	76.3 ± 2.1	76.9 ± 5.2	42.9 ± 6.6	**49.6 ± 4.8**	70.5 ± 5.3
SP8 ($CD4^-CD8^+$)	Untreated Control	60.8 ± 8.0	21.1 ± 2.6	61.7 ± 6.7	63.3 ± 5.8	70.7 ± 3.0	78.5 ± 5.8	40.0 ± 6.6	38.2 ± 2.6	55.7 ± 4.9
	$1x10^{-8}$ M	41.9 ± 7.4	24.0 ± 2.1	62.5 ± 8.1	65.9 ± 9.0	69.0 ± 5.2	73.7 ± 6.6	31.8 ± 3.1	38.6 ± 2.8	53.9 ± 4.5
	$5x10^{-8}$ M	37.0 ± 1.9	28.3 ± 4.2	**42.8 ± 4.1**	69.4 ± 7.4	66.9 ± 3.0	71.7 ± 6.8	33.8 ± 2.5	**56.8 ± 2.9**	53.2 ± 2.8
	$1x10^{-7}$ M	33.3 ± 7.7	38.1 ± 6.7	42.9 ± 5.0	**76.0 ± 5.8**	71.0 ± 4.4	62.5 ± 9.8	37.9 ± 2.8	**69.2 ± 4.2**	61.0 ± 3.4

Table I. Developmental Receptor Analysis from FTOC. Cells from a 12-day B6 FTOC, derived from 13-15 dg thymi, were analyzed for cells considered positive for each receptor type. FTOC were treated with a titration of HC ranging from 5 x 10^{-8} to $1x10^{-7}$ M in half molar increments. Actual values are presented. Bolded text indicates $p \leq 0.10$ and Bolded/Underlined text indicate $p \leq 0.05$, versus the untreated control (n=9).

Addition of Low Dose Exogenous GCs to Murine FTOC Generates Increased T cell Yields

As stated previously, titration experiments using exogenous HC were performed across a wide range of molar concentrations, from $1x10^{-7}$ M to $1x10^{-10}$ M. Interestingly, unlike the higher doses of HC ($1x10^{-8}$ M to $1x10^{-5}$ M), lower doses of HC ($1x10^{-10}$ M to 5 x 10^{-9} M) led to significant increases in cell yield versus the control group. Cell yields increased 19% in the 5 x 10^{-10} M treated cultures and 25% in the $1x10^{-9}$ M treated cultures (Fig. 1A). These cell yield increases occurred with no significant change in cell viability.

Basic SP4, SP8, DP, DN phenotyping performed on these low dose HC treated cultures showed that cell yield increases also carry over into all phenotype groups, although not always in a significant fashion (Fig. 1B). Aside from overall cell yield increases, low doses of HC also showed the ability to modify the CD4/CD8 profile generated by the thymus. The most dramatic changes are seen at the $1x10^{-9}$ M level where the overall T cell profile shifts, showing a 15% decrease in the percentage of SP8 cells generated, coupled with a moderate 7% increase in the percentage of SP4 cells (Fig. 1B). The most prevalent phenotype increases were seen in the DP cell population, which showed a 31% increase at the $1x10^{-9}$ M level, leading to a 67% increase in the number of DP cells produced per thymus lobe (due to the overall increase in cell yield). Mean values of the actual data for the untreated controls are listed in the figure legend for reference.

Developmental receptor analysis, as described above, was also performed on cultures treated with low dose HC. Unlike the higher doses, lower doses of HC had little

impact on receptor expression. The only consistent and significant change was seen in the cells considered to be $\alpha\beta TCR^{mature}$ (expressing TCR at mid to high levels) at the $1x10^{-9}$ M level. At this level of treatment, the DN, SP4, and SP8 groups, which contain mostly mature cells at the end of the 12 day culture period, showed significantly reduced levels of $\alpha\beta TCR^{mature}$ cells (Table II).

Table II. Changes in Receptor Expression for Developing T cells Treated with **Low Doses** of Hydrocortisone										
Cell Population	Hydrocortisone Level	αβ TCR Mature	γδ TCR Mature	CD25	CD45RA	CD69	CD95	CD95L	CD122	CD132
DN ($CD4^-CD8^-$)	Untreated Control	11.0 + 1.8	52.0 + 4.4	74.2 + 5.1	61.2 + 8.4	80.6 + 3.6	77.9 + 3.2	36.1 + 3.9	35.5 + 4.2	89.5 + 2.0
	$1x10^{-10}$ M	10.3 ± 2.2	53.0 ± 6.0	74.0 ± 3.9	60.2 ± 9.2	87.4 ± 1.8	81.1 ± 2.9	28.0 ± 8.6	33.0 ± 3.6	86.0 ± 2.9
	$5x10^{-10}$ M	10.0 ± 1.2	51.4 ± 4.9	67.0 ± 5.1	64.8 ± 8.4	85.8 ± 1.8	82.3 ± 1.2	33.6 ± 6.0	31.7 ± 1.3	88.5 ± 1.2
	$1x10^{-9}$ M	**7.9 + 1.2**	52.5 ± 4.5	71.2 ± 3.1	64.0 ± 6.0	79.5 ± 2.3	77.1 ± 2.4	32.8 ± 6.2	35.8 ± 4.5	88.1 ± 1.5
DP ($CD4^+CD8^+$)	Untreated Control	19.5 + 4.0	7.0 + 0.7	83.1 + 4.6	29.6 + 5.9	25.2 + 2.4	79.5 + 6.5	44.3 + 9.3	17.4 + 1.7	36.2 + 2.5
	$1x10^{-10}$ M	15.7 ± 2.0	7.2 ± 0.8	81.1 ± 4.1	34.3 ± 6.0	30.5 ± 1.6	81.3 ± 5.7	29.1 ± 5.0	16.1 ± 1.4	35.5 ± 1.5
	$5x10^{-10}$ M	15.5 ± 2.3	6.9 ± 0.6	73.0 ± 5.8	**36.5 ± 7.6**	**36.6 + 5.2**	85.7 ± 2.6	41.2 ± 7.1	16.3 ± 1.9	36.4 ± 2.3
	$1x10^{-9}$ M	15.0 ± 2.0	7.1 ± 0.5	76.0 ± 2.9	31.2 ± 4.5	26.3 ± 1.2	79.0 ± 5.5	42.1 ± 5.5	17.9 ± 2.4	38.5 ± 0.7
SP4 ($CD4^+CD8^-$)	Untreated Control	86.0 + 2.6	10.4 + 2.5	71.3 + 12.6	50.1 + 10.7	72.0 + 3.0	84.3 + 4.6	49.8 + 5.9	34.7 + 3.5	69.2 + 3.6
	$1x10^{-10}$ M	82.3 ± 2.2	9.7 ± 2.2	62.9 ± 15.9	53.6 ± 11.3	75.7 ± 2.3	84.1 ± 5.7	34.7 ± 7.4	30.8 ± 2.4	62.4 ± 4.6
	$5x10^{-10}$ M	82.9 ± 2.5	12.5 ± 2.9	70.4 ± 12.0	52.1 ± 9.6	79.1 ± 4.8	91.1 ± 3.1	51.0 ± 7.1	39.0 ± 5.8	67.4 ± 3.6
	$1x10^{-9}$ M	**76.2 ± 2.9**	11.0 ± 2.0	64.2 ± 12.1	49.7 ± 9.4	70.4 ± 2.8	84.6 ± 3.8	45.8 ± 5.5	39.2 ± 4.9	66.6 ± 1.7
SP8 ($CD4^-CD8^+$)	Untreated Control	60.8 + 8.0	21.1 + 2.6	61.7 + 6.7	63.3 + 5.8	70.7 + 3.0	78.5 + 5.8	40.0 + 6.6	38.2 + 2.6	55.7 + 4.9
	$1x10^{-10}$ M	51.2 ± 8.6	20.9 ± 3.5	57.1 ± 6.0	68.9 ± 5.2	73.0 ± 2.5	76.4 ± 7.1	29.7 ± 3.0	39.4 ± 4.1	50.9 ± 7.6
	$5x10^{-10}$ M	48.5 ± 6.8	21.6 ± 3.3	55.0 ± 8.4	66.4 ± 5.4	73.7 ± 4.2	82.8 ± 2.4	40.2 ± 3.6	36.4 ± 1.9	52.8 ± 5.0
	$1x10^{-9}$ M	**43.6 + 6.5**	22.3 ± 2.7	54.7 ± 4.1	63.5 ± 4.9	61.0 ± 5.1	76.1 ± 5.0	37.1 ± 2.7	37.7 ± 2.0	53.2 ± 3.6

Table II. Developmental Receptor Analysis from FTOC. Cells from a 12-day B6 FTOC, derived from 13-15 dg thymi, were analyzed for cells considered positive for each receptor type. FTOC were treated with a titration of HC ranging from $1x10^{-10}$ to $1x10^{-9}$ M in half molar increments. Actual values are presented. Bolded text indicates $p \leq 0.10$ and Bolded/Underlined text indicate $p \leq 0.05$, versus the untreated control (n=9).

Comparison of GC based signal inhibition and CD3 antibody based signal enhancement on T cell development

To examine the general impact of GCs on T cell development, in terms of changes in signal strength, we investigated the effect of HC and CD3 antibody (anti-CD3) on T cell development. Currently, HC is thought to be a selection signal inhibitor through transcription level inhibition of several genes (4, 5), while experiments by Suzuki et al have shown that anti-CD3 can generate increases in T cell selection signals through the cross-linking of TCR on the surface of developing thymocytes (18). In these experiments we treated 12 day B6 FTOC with nothing, HC ($1x10^{-7}$, $1x10^{-8}$, $1 x10^{-9}$ or $1 x10^{-10}$ M), or anti-CD3 (0.0005 (~3.33×10^{-9} M), 0.005 (~3.33×10^{-7} M), or 5.0 μg/mL (~3.33×10^{-5} M)). Treatments were added to cultures on day 0 and remained for the duration of the culture.

Cell yield and culture viability analysis shows the normalized impact of HC and anti-CD3 induced changes in signal strength (Fig. 2). High levels of signal inhibitor (HC) and signal enhancer (anti-CD3) lead to decreased FTOC cell yields (↓67 ± 10% at $1x10^{-7}$ M HC, ↓13 ± 7% at $1x10^{-8}$ M HC, and ↓34 ± 1% at 5.0 μg/mL anti-CD3), while low levels HC and anti-CD3 lead to either increased cell yields or no change versus the untreated control. Culture viability was unaffected except at the higher levels of HC ($1x10^{-8}$ M and $1x10^{-7}$ M). FTOC viability is typically unaffected by treatment, despite decreases in cell yield, due to the high rate of cell clearance in the thymus. High levels of signal inhibitor (HC at $1x10^{-7}$ M) and signal enhancer (anti-CD3 at 5.0 μg/mL) lead to the generation of increased percentages of DN and SP8 cells (% increase versus untreated

control DN: ↑60 ± 18% at $1x10^{-7}$ M HC and ↑77 ± 26% at 5.0 μg/mL. For SP8: ↑18 ± 37% at $1x10^{-7}$ M HC and ↑115 ± 14% at 5.0 μg/mL) coupled with decreases in the DP and SP4 cells (% decrease versus untreated control for DP: ↓64 ± 7% at $1x10^{-7}$ M HC and ↓61 ± 7% at 5.0 μg/mL. For SP4 ↓78 ± 2% at 5.0 μg/mL. Fig. 2B).

The profiles generated at these high treatment levels are indicative of a block at the DP phase of development, likely due to an inability of most cells to pass either positive or negative selection (positive selection in the case of HC and negative in the case of anti-CD3). This notion is supported be the accumulation of immature transitional cell types (DN and Immature SP8 cells), which is shown in Figure 2B as decreases in the percent of αβ TCR maturity (% decrease in maturity versus untreated control for DN: ↓25 ± 26% at $1x10^{-7}$ M HC and ↓81 ± 2% at 5.0 μg/mL. For SP8: ↓34 ± 24% at $1x10^{-7}$ M HC and ↓52 ± 3% at 5.0 μg/mL). Also of note is the dose dependent high level of "maturity" induced in the DP group when HC is used (% increase in maturity versus untreated control for DP: ↑107 ± 48% at $1x10^{-7}$ M HC and ↑158 ± 72% at 5.0 at $1x10^{-8}$ M). Because DP cells are by definition immature, the increases in "maturity" seen in the DP group are actually due to compensatory increases in αβTCR expression (determined through mean fluorescence intensity (MFI), discussed below), which further supports reports indicating that HC as a signal inhibitor. In similar fashion the lowest levels of anti-CD3 treatment used show modest increases in T cell maturity (% increase in maturity versus untreated control for DP: ↑10 ± 11% at 0.0005 μg/mL), however whether these increases are generated through low level signal enhancement or inhibition is not currently known.

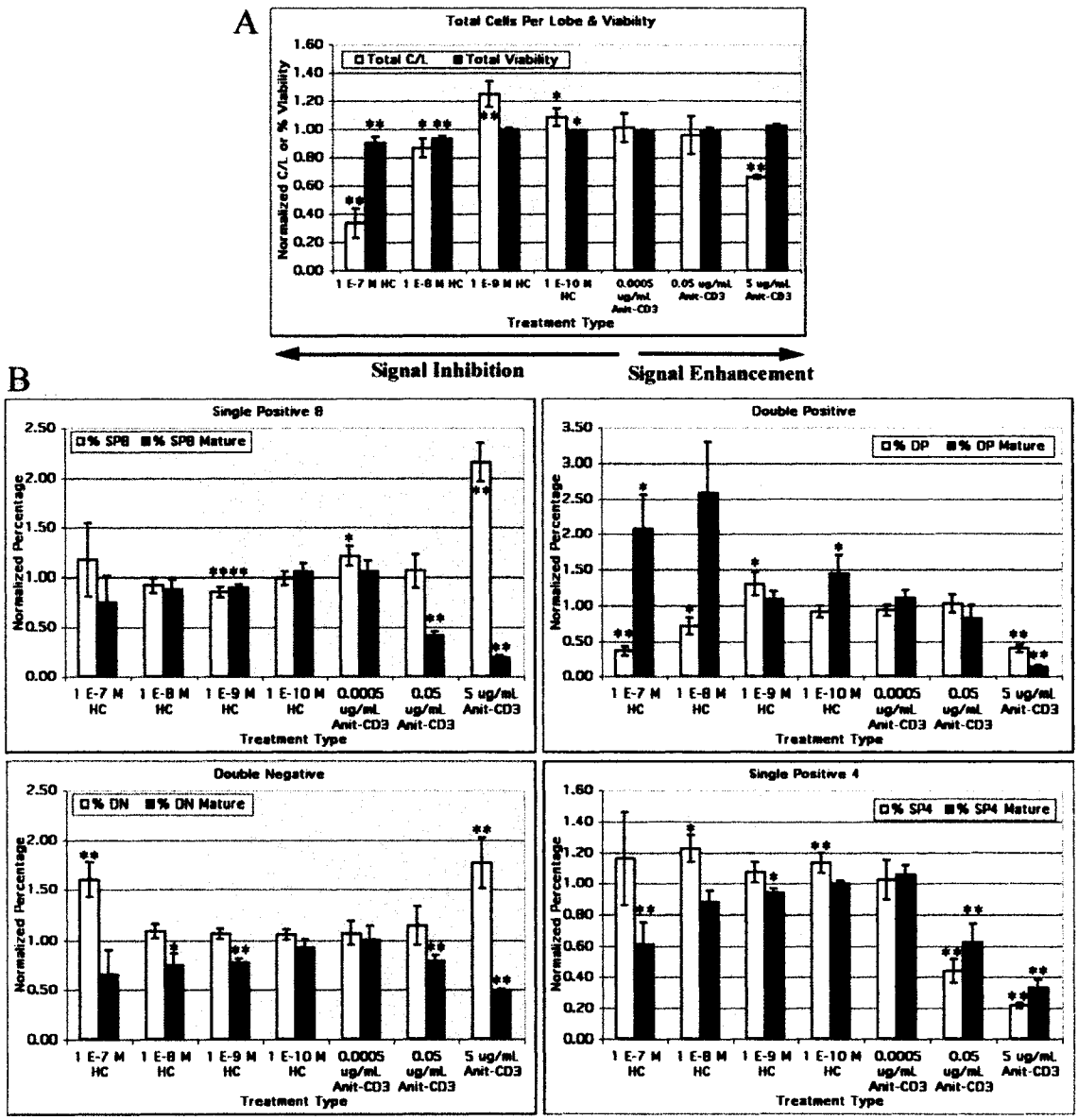


Figure 2. Impact of a signal inhibitor (HC) and a signal enhancer (anit-CD3) on T cell Development. Cells from a 12-day B6 FTOC, derived from 13-15 dg thymi, were analyzed for yield and viability through trypan-blue exclusion (A) and for general T cell phenotype and mature phenotype distribution (B). FTOC were treated with a titration of

HC ranging from 10^{-7} to 10^{-10} M or anti-CD3ε ranging from 0.0005 μg/mL to 5.0 μg/mL. The open columns represent the changes in total C/L yield in (A) and the percent of general T cell phenotype changes in (B). The shaded columns represent the changes in culture viability in (A) and changes in phenotype maturity, based on αβTCR expression, in (B). Values were normalized to the untreated control for direct comparison. Actual ranges for the C/L generated in the untreated control for (A) was 30.9 ± 2.3 x 10^4. Cell viability for the untreated control in (A) was 91.5 ± 0.5%. Untreated controls for (B) phenotype % were 21.9 ± 3.5 for SP8, 16.7 ± 2.7 for SP4, 25.9 ± 7.9 for DP, and 35.5 ± 2.1 for DN. Untreated controls for (B) phenotype maturity within the group were 82.3 ± 2.7% for SP8, 49.5 ± 8.7% for SP4, 13.2 ± 3.0% for DP, and 11.6 ± 2.8% for DN. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the untreated control (n=9).

Addition of HC Generates Changes in the Percentages of Mature T cells using TCR Vβ7 and Vβ8.

To further investigate if HC was causing shifts in the T cell selection window, TCR Vβ usage was investigated. To do this B6 FTOC were treated with nothing, HC ($1x10^{-9}$ M), anti-CD3 (0.0005 μg/mL), or both HC and anti-CD3. FTOC was harvested after 12 days and analyzed through flow cytometry for the percentage of T cells using Vβ7 or Vβ8 in their TCR.

The addition of $1x10^{-9}$ M HC generated an increase in the percentage of mature group T cells using Vβ7 (↑32 ± 12% in SP8 and ↑35 ± 23% in SP4) and a slight decrease or no change in the percentage of mature group T cells using Vβ8 (↓27 ± 21% in SP8 and ↓11 ± 24% in SP4, Fig 3A&B). The addition of 0.0005 μg/mL anti-CD3 generated the opposite effect, causing a slight decrease or no change in the percentage of mature group T cells using Vβ7 (↓2 ± 19 % in SP8 and ↓27 ± 2% in SP4) and increases in the percentage of mature group T cells using Vβ8 (↑68 ± 45% in SP8 and ↑26 ± 7% in SP4). The addition of both HC and anti-CD3 showed a corrective profile for Vβ8 when compared to cultures treated with HC or anti-CD3 alone. However, there was no correction in Vβ7 percentages when both HC and anti-CD3 were used, so the ability of HC to completely correct for this level of anti-CD3 is unclear.

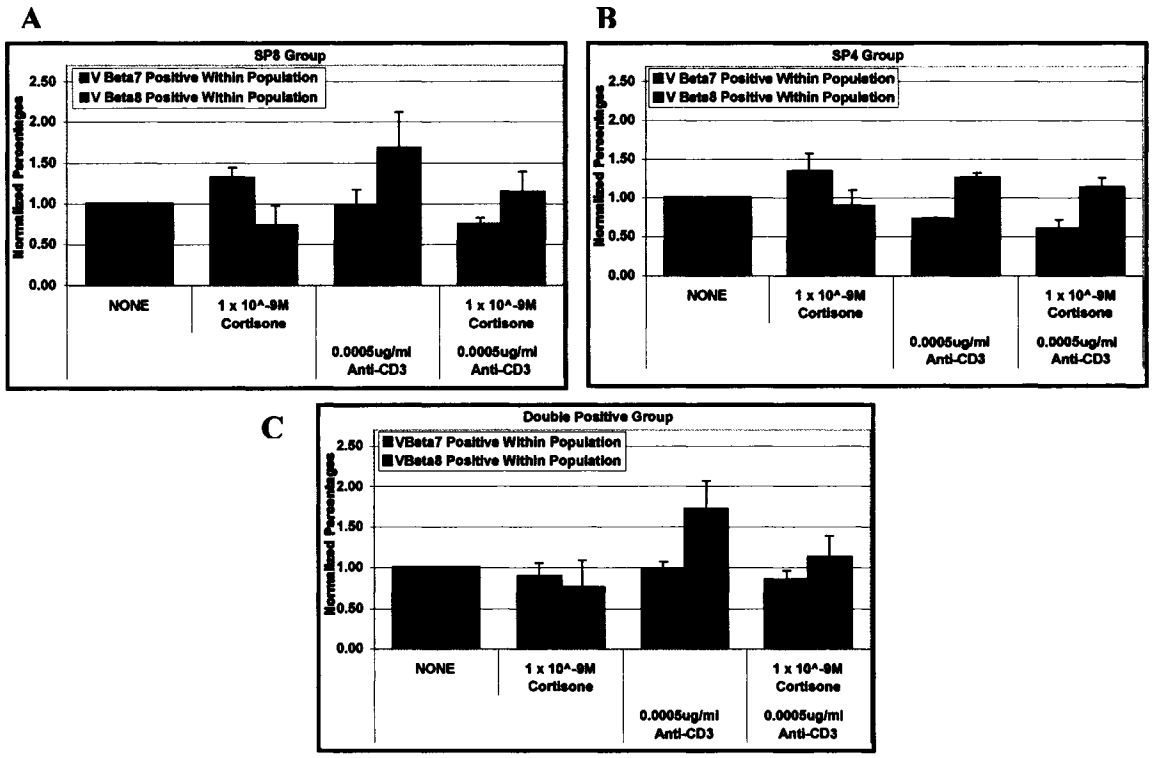


Figure 3. TCR V Beta Segment Analysis. Cells from a 12-day B6 FTOC, derived from 13-15 dg thymi, were analyzed using flow cytometry for TCR Vβ chain usage in the SP8 (A), SP4 (B), and DP (C) populations. FTOC were treated with nothing, $1x10^{-9}$ M HC, 0.0005 μg/mL of anti-CD3, or both. The red columns represent the % Vβ7 usage and the blue columns represent the % Vβ8 usage. Values were normalized to the untreated control for direct comparison. Actual Vβ7 percentages were: 17.6 ± 5.1 for SP8, 11.7 ± 1.7 for SP4, and 24.3 ± 3.8 for DP. Actual Vβ8 percentages were: 4.2 ± 1.6 for SP8, 11.1 ± 2.2 for SP4, and 6.2 ± 1.6 for DP .One asterisk (*) indicates $p \leq 0.10$ and two asterisks

(**) indicate $p \leq 0.05$, versus the untreated control. One diamond (◆) indicates $p \leq 0.10$ and two diamonds (◆◆) indicate $p \leq 0.05$, when comparing HC and anti-CD3 treated cultures. For two circles (OO) $p \leq 0.05$, indicates a significant correction by HC in HC and anti-CD3 cultures (n=3).

Addition of HC at Different Stages in T cell Development Generates Distinct Mature Phenotypic Profiles

Addition of HC at $1x10^{-9}$ M was further investigated, based on cell expansion effects seen in the titration data. To accomplish this, Pulse and Analysis (P&A) and Pulse and Chase (P&C) experiments were performed. For P&A experiments 6-10 B6 fetal thymus lobes, 13-15dg, were cultured from days 0-3, 3-6, 6-9, or 9-12 in the presence of $1x10^{-9}$ M of HC. FTOC was then harvested after the treatment period on day 3 (for 0-3), 6 (for 3-6), 9 (for 6-9), or 12 (for 9-12). P&C experiments were similarly cultured from days 0-3, 3-6, 6-9, or 9-12 in the presence of $1x10^{-9}$ M of HC. However, in contrast to P&A experiments, P&C FTOC were allowed to develop to maturity (culture day 12) in the absence of exogenous HC and were then harvested. P&A experiments are representative of the more immediate impact of HC treatment, while P&C experiments are representative of the long-term developmental effects of HC treatment.

Cell yield analysis of P&C FTOC revealed that addition of $1x10^{-9}$ M of HC at any point during culture (0-3, 3-6, 6-9, or 9-12) led to larger cell yields when compared to the untreated control. However, cell yields from P&A FTOC only generated cell yield increases in the 6-9 and 9-12 treatment points (Fig. 4). In fact, 0-3 and 3-6 P&A FTOC generated reduced cell yields when compared to the untreated control, indicating the possibility of distinct impacts of HC in early and late T cell development.

Similarly, phenotype analysis of P&A and P&C FTOC showed that treatment with HC led to distinct phenotype changes that were associated with the time of treatment (Fig. 5). P&A data indicates that addition of $1x10^{-9}$ M of HC leads to a developmental

delay in phenotype production. This delay can be seen at the P&A 0-3 time point as an accumulation of early developmental phenotypes (immature SP8 and DN cells, as determined by αβTCR maturity; Fig. 5C) and the small but significant decrease in the transitional DP population when compared to the untreated control (Fig. 5A, left columns). This delay can also be seen in the P&A 6-9 time point where there are minor decreases in mature SP8 and mature SP4 phenotypes and an accumulation of immature DP cells when compared to the untreated control (Fig. 5A, right columns).

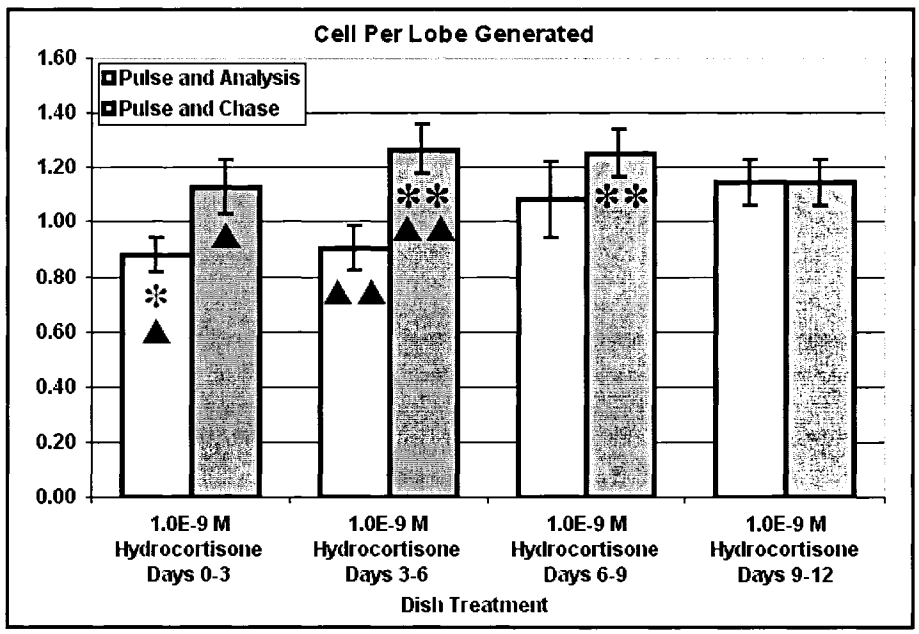
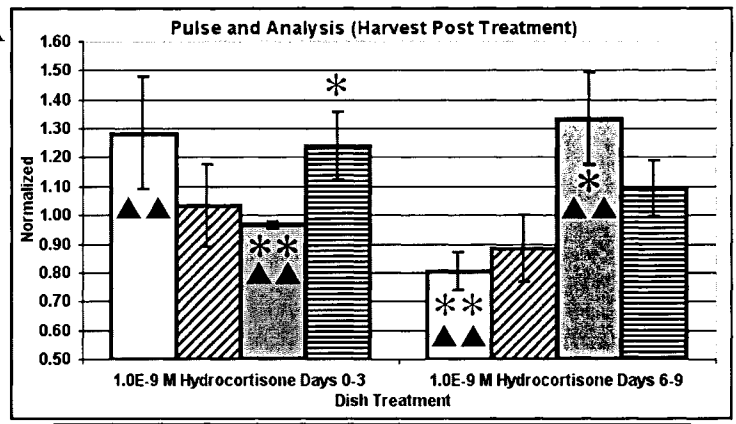
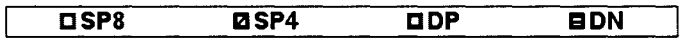


Figure 4. Cell Yields from Pulse and Analysis and Pulse and Chase FTOC. Cells from a P&A and P&C B6 FTOC, derived from 13-15 dg thymi, were analyzed for yield and viability through trypan-blue exclusion. FTOC were treated with $1x10^{-9}$ M HC. See text for explanation of experimental setups. Values were normalized to the appropriate untreated control for direct comparison. Actual ranges for the C/L generated in P&A untreated controls were 96.6 ± 17.2 for day 0-3, 85.5 ± 7.2 for day 3-6, 52.4 ± 5.3 for day 6-9, 38.9 ± 3.2 for day 9-12. Actual range for the C/L generated in P&C was 31.0 ± 2.6. Culture viability was always between 87 to 96%. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the appropriate untreated control. One triangle (▲) indicates $p \leq 0.10$ and two triangles (▲▲) indicate $p \leq 0.05$, when comparing P&A to P&C FTOC (n=6-12).

A



P & A	% SP8	% SP4	% DP	% DN
0-3	4.15 ± 0.54	4.00 ± 0.39	78.3 ± 3.4	13.5 ± 2.9
6-9	22.3 ± 2.4	19.7 ± 3.8	27.0 ± 2.7	31.3 ± 5.5



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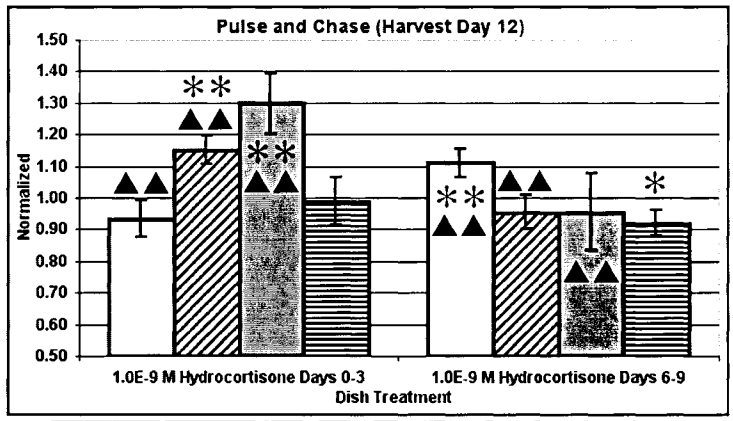

P & C	% SP8	% SP4	% DP	% DN
All	40.4 ± 2.4	20.5 ± 0.7	4.47 ± 0.21	34.6 ± 1.8

C

1 x 10⁻⁹ M HC		% SP8 & AB TCR Mature	% SP4 & AB TCR Mature	% DP & AB TCR Mature	% DN & AB TCR Mature
P & A	0-3	7.74 ± 1.5	52.6 ± 4.3	3.05 ± 0.79	18.5 ± 5.7
	3-6	37.9 ± 7.7	78.6 ± 5.5	7.81 ± 2.29	17.5 ± 3.5
	6-9	57.0 ± 8.7	79.6 ± 6.2	11.7 ± 3.4	18.2 ± 3.0
	9-12	61.0 ± 6.4	82.5 ± 3.8	16.0 ± 5.5	20.6 ± 3.9

Figure 5. Phenotype Percentages from Pulse and Analysis and Pulse and Chase FTOC. Cells from a P&A (A) and P&C (B) B6 FTOC, derived from 13-15 dg thymi, were analyzed for T cell developmental phenotypes. FTOC were treated with $1x10^{-9}$ M HC. See text for explanation of experimental setups. Values were normalized to the appropriate untreated control for direct comparison. Actual ranges for the percentages of

the appropriate untreated control are listed in the table below the graph. (C) Indicates the average percentage of mature T cells in each phenotype group (based on medium to high levels of αβTCR expression) at each P&A time point. Maturity at the P&A 9-12 time point (harvested on day 12) is typical for end stage organ culture. P&C FTOC (all harvested on day 12) showed a similar high level of maturity, with 80-92% of SP4 cells and 63-83% of SP8 cells considered αβTCR mature. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the appropriate untreated control. One triangle (▲) indicates $p \leq 0.10$ and two triangles (▲▲) indicate $p \leq 0.05$, when comparing the same phenotype between day 0-3 treated and day 6-9 treated within each experimental type (n=6).

Addition of HC at Different Stages of T cell Development Generates Distinct T cell Developmental Receptor Changes

To determine how $1x10^{-9}$ M HC might be affecting T cell avidity during selection we investigated several receptors associated with T cell development. To accomplish this, data generated from P&A experiments and 12 day FTOC were analyzed for changes in receptor expression to determine why distinct T cell development profiles were being generated at the 0-3 and 6-9 treatment points.

Analysis of several receptors associated with T cell development indicated that HC had the capacity to, either directly or indirectly, effect receptor expression. One of the more noticeable changes is found in the expression of αβTCR. Figure 6 shows representative changes in αβTCR expression in the SP8, SP4, DP, and DN developmental groups over the course of a P&A experiment. The stage of T cell development that the majority of the cells were in can be determined through the progression of cells in each developmental phase (Fig 6, left). FTOC allows for this progression-based analysis because of the capacity to analyze the first wave of T cell development (13). Typically, during time points 0-3 the majority of cells are progressing

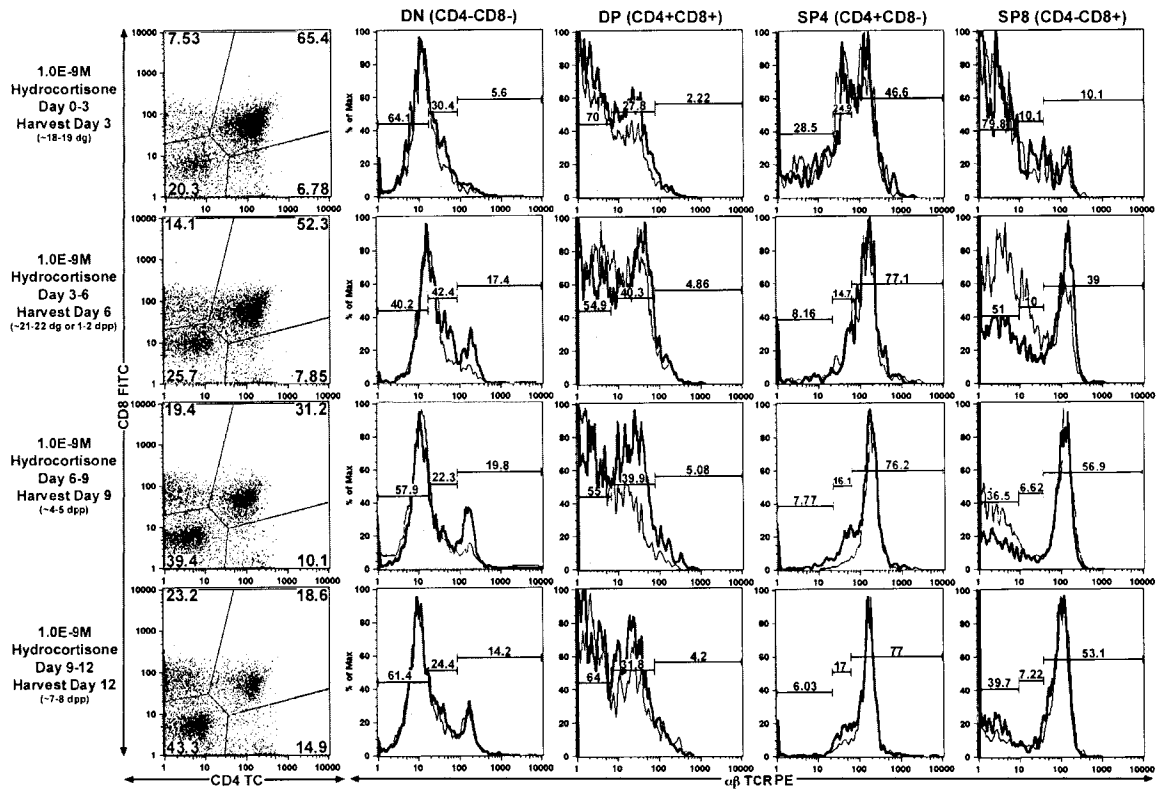

Figure 6. Flow Cytometric Analysis of αβTCR Expression During P&A Experiments. Cells from a P&A (i.e. harvested after treatment period (day 3, 6, 9, or 12)) B6 FTOCs, derived from 13-15 dg thymi, were analyzed for expression of αβTCR in each developmental group (SP8, SP4, DP, and DN). FTOC were untreated (shaded) or treated (bold line) with $1x10^{-9}$ M HC. Gating was established based on the isotype control (not shown) and the gating of negative, dull, and mature staining groups (based on population features). Cells considered negative or dull were defined as immature cell types, while cells considered intermediate or bright were considered mature. All experiments were performed under identical conditions and run at the same settings on the same day.

from DN→ISP→DP, from 3-6 the majority are in the DP phase, from 6-9 the majority are progressing from DP→SP(4 or 8), and by day 12 the majority of cells have reached naïve maturity (i.e. SP8, SP4, and DN).

Examination of the early developmental groups (DN, DP and SP8 (early ISP group)) in figure 6 indicates that treatment with HC leads to increased levels of αβTCR expression in both number (also seen in Fig 2B, DP group) and intensity (also see Fig. 7A). This trend is best seen in the DP group where TCR expression is most noticeably higher during this selection-associated stage of development. HC induced changes in αβ TCR and CD3ε expression, determined by MFI, occur most significantly at the 6-9 treatment period, when the majority of cells are transitioning from DP to SP (4 or 8) during the final stages of selection (Fig 7). However, $1x10^{-9}$ M HC is only mildly inhibitory when compared to the higher levels of HC treatment, therefore Figure 8 shows the receptor expression changes for αβTCR and CD3ε across the 12 day HC titration. These 12 day cultures allow for the examination of major mature T cell groups (SP4, SP8, and DN) and the major immature T cell group (DP). Analysis of SP4, SP8, and DN mature cells shows that increasing concentration of HC leads to significant increasing inhibition of CD3ε expression and, to some extent, αβTCR as well. A similar analysis of DP immature or mature cell groups indicates that cells in the DP phase of development do not show these drastic decreases in expression. Instead, cells considered DP mature (i.e. with intermediate/bright TCR expression) are able to maintain expression, until the highest levels of HC treatment, and the DP immature cells (i.e. with negative/dull

expression) increase expression versus the untreated control as the amount of HC increases.

These effects are not limited to αβTCR and CD3ε. Table III shows changes in the percentage of cells considered positive for γδTCR, CD25, CD45RA, CD69, CD95, CD95L, CD122, CD132, and DX5 over the course of a P&A experiment. Table IV shows changes in receptor expression (based on MFI) for these same receptors. Similar to αβTCR and CD3ε, some of these receptors (CD25, CD69, CD95L, CD122, CD132, and DX5) show increases during early HC treatment points, when the majority of cells are still immature, and decreases late in culture when the majority of the cells are mature. The primary exception to these late stage decreases in receptor expression is the DP group, which still holds the remaining immature cells in the culture. However, not all receptor types are affected by HC in a similar fashion.

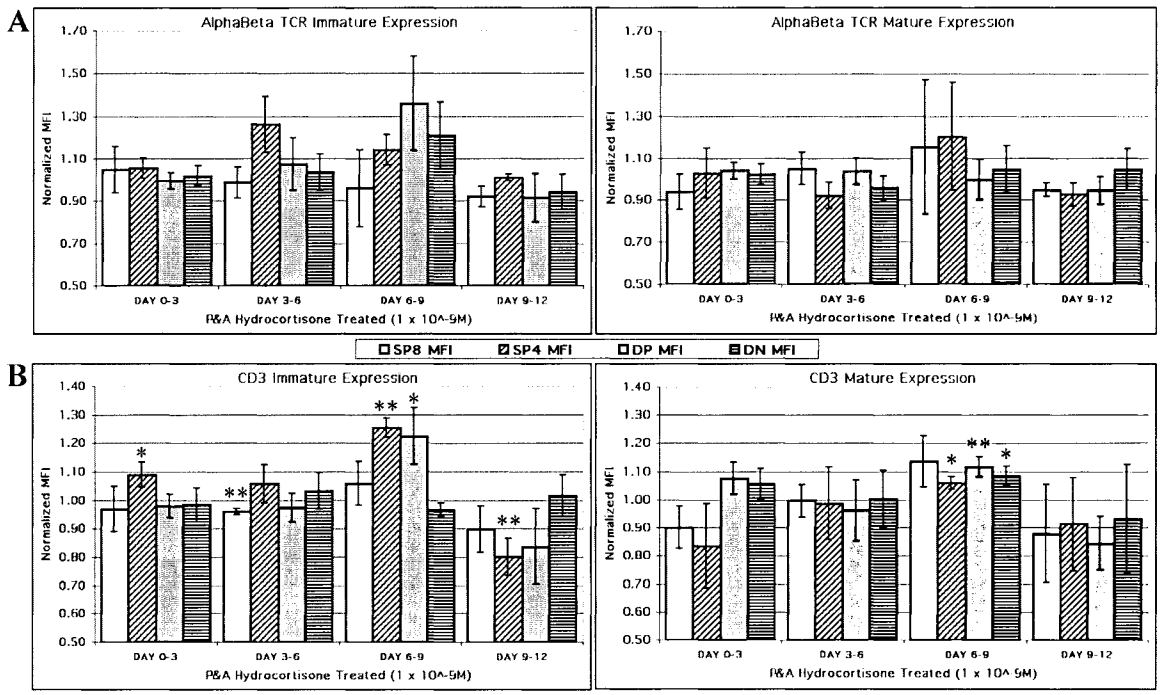

Figure 7. Mean Fluorescence Intensity Analysis of αβTCR and CD3ε Expression During P&A Experiments. Cells from a P&A B6 FTOCs, derived from 13-15 dg thymi, were analyzed for expression of αβTCR and CD3ε in each developmental group (SP8, SP4, DP, and DN) through MFI. FTOC were untreated (value=1) or treated with $1x10^{-9}$ M HC. Values were normalized to the appropriate untreated control for direct comparison. One asterisk (*) indicates $p \leq 0.10$ and two asterisks (**) indicate $p \leq 0.05$, versus the appropriate untreated control (n=3-6).

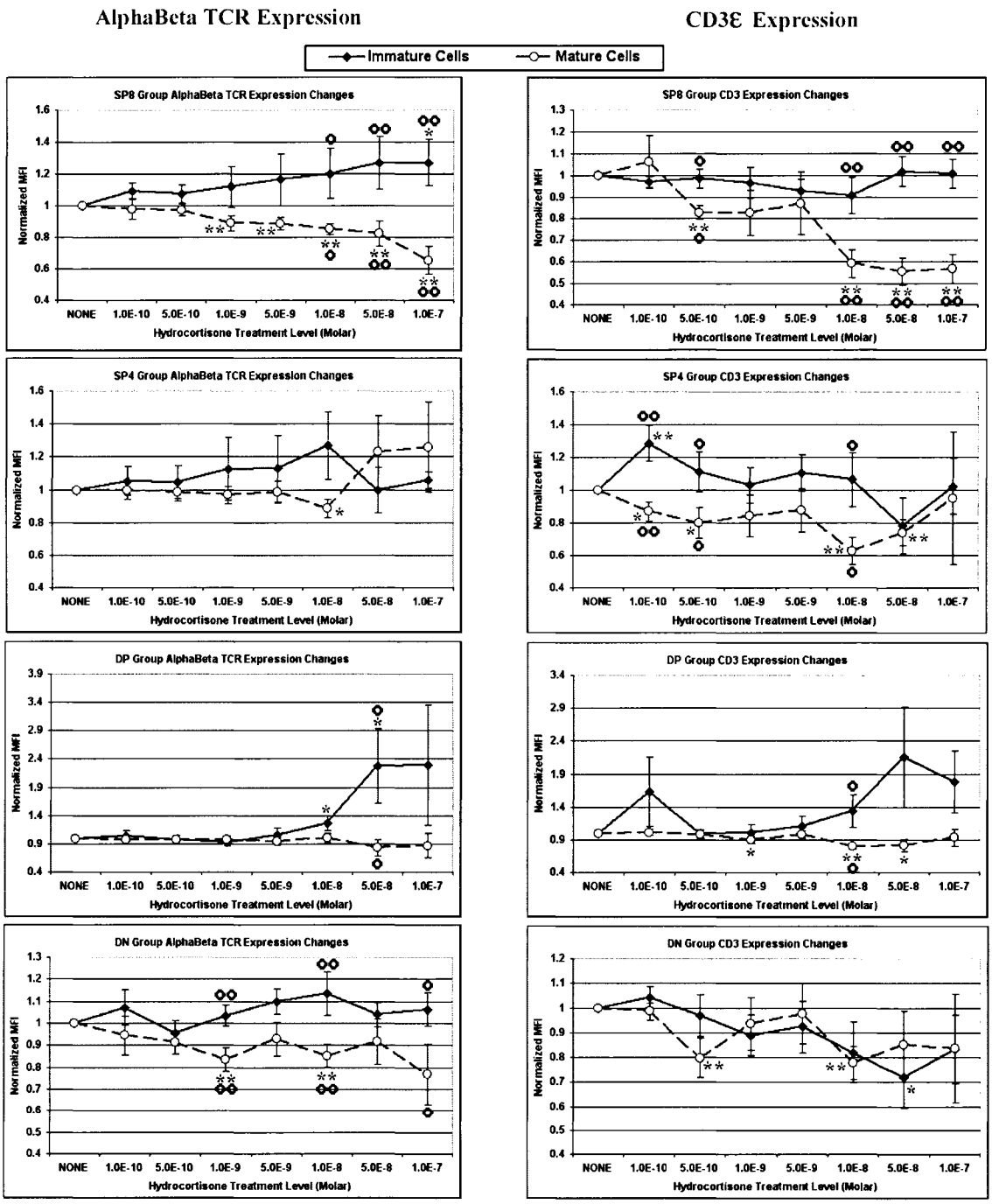

Figure 8. Mean Fluorescence Intensity Analysis of αβTCR and CD3ε Expression During HC Titration Experiments. Cells from B6 FTOCs, derived from 13-15 dg thymi, were

analyzed for expression of αβTCR and CD3ε in each developmental group (SP8, SP4, DP, and DN) through MFI. FTOC were treated with nothing or a titration of HC ranging from 10^{-10} to 10^{-7} M. The open circles (○) represent the MFI of the mature αβTCR or CD3ε phenotype. The closed diamonds (◆) represent the MFI of the immature αβTCR or CD3ε phenotype. Values were normalized to the untreated control for direct comparison. One asterisk (*) indicates p≤0.10 and two asterisks (**) indicate p≤0.05, versus the untreated control. One circle (**O**) indicates p≤0.10 and two circles (**OO**) indicate p≤ 0.0 5, when comparing mature and immature changes in expression (n=6).

Table III. Percentage of cells positve for receptor expression in each developmental phenotype group during P & A experiments. The **actual percentage of cells considered positive for each receptor type** in the untreated control is represented followed by the (normalized change) that occurred when treated with 1 x 10^{-4} M HC. Percentage of each major phenotype (SP8, SP4, DP, DN) at the time of harvest is listed next to the pheontype. Underlined normalized values indicate $p \leq 0.05$ (n=3-6).

Treatment Type	Phenotype	GD TCR IMM	GD TCR MAT	CD25	CD45RA	CD69	CD95	CD95L	CD122	CD132	DX5
1 x 10^{-4} M HC P&A 0-3	SP8 (5.1%)	82.7 ± 2.6 (1.01)	17.3 ± 2.6 (0.96)	11.8 ± 3.2 (1.00)	15.3 ± 4.5 (1.17)	15.7 ± 6.0 (1.30)	42.0 ± 11.3 (0.87)	3.2 ± 1.2 (1.48)	6.7 ± 2.5 (1.30)	19.5 ± 8.9 (1.37)	2.9 ± 0.3 (1.26)
	SP4 (5.1%)	94.9 ± 0.3 (0.97)	5.1 ± 0.3 (1.53)	8.5 ± 1.1 (1.35)	31.4 ± 9.2 (0.89)	50.7 ± 15.1 (1.03)	75.4 ± 5.9 (0.89)	15.0 ± 4.7 (1.42)	10.9 ± 0.6 (1.19)	45.9 ± 15.0 (1.61)	8.9 ± 1.2 (1.55)
	DP (70.5%)	99.4 ± 0.1 (1.00)	0.6 ± 0.1 (1.44)	1.3 ± 0.3 (1.03)	4.4 ± 1.7 (0.93)	5.2 ± 2.0 (1.28)	48.4 ± 15.3 (0.74)	1.9 ± 0.6 (1.32)	1.3 ± 0.2 (1.35)	7.6 ± 3.3 (2.78)	1.4 ± 0.1 (1.25)
	DN (19.4%)	76.2 ± 1.1 (0.98)	23.8 ± 1.1 (1.07)	57.4 ± 4.4 (0.96)	66.7 ± 6.8 (0.89)	76.1 ± 2.3 (1.02)	86.7 ± 3.3 (0.96)	55.3 ± 1.6 (0.99)	49.9 ± 8.9 (1.12)	86.0 ± 3.9 (1.01)	57.4 ± 11.2 (1.00)
1 x 10^{-4} M HC P&A 3-6	SP8 (10.2%)	79.9 ± 1.2 (0.99)	20.1 ± 1.2 (1.03)	13.0 ± 1.5 (1.10)	26.9 ± 1.4 (0.93)	37.8 ± 9.1 (0.77)	49.3 ± 20.4 (1.07)	9.2 ± 1.0 (1.07)	20.2 ± 1.2 (0.65)	27.6 ± 3.7 (0.88)	8.3 ± 0.4 (1.07)
	SP4 (6.1%)	92.9 ± 1.3 (0.98)	7.1 ± 1.3 (1.30)	10.5 ± 2.4 (1.30)	28.0 ± 6.4 (1.23)	63.8 ± 12.7 (1.02)	65.3 ± 21.4 (1.43)	17.5 ± 3.8 (1.26)	22.1 ± 1.8 (1.07)	42.3 ± 11.7 (1.15)	9.0 ± 1.0 (1.21)
	DP (64.0%)	98.5 ± 0.2 (1.00)	1.5 ± 0.2 (1.15)	3.2 ± 0.4 (1.21)	7.2 ± 1.2 (0.99)	11.0 ± 3.0 (0.80)	51.1 ± 23.9 (2.28)	4.4 ± 0.5 (1.27)	4.7 ± 0.6 (0.98)	10.4 ± 2.4 (1.03)	3.2 ± 0.4 (0.97)
	DN (19.8%)	62.4 ± 2.5 (0.95)	37.6 ± 2.5 (1.11)	58.2 ± 8.4 (1.07)	83.0 ± 1.4 (0.98)	90.2 ± 1.31 (0.99)	92.3 ± 2.5 (1.03)	71.0 ± 3.7 (0.93)	79.3 ± 1.3 (0.94)	91.1 ± 1.5 (1.00)	69.0 ± 1.7 (0.98)
1 x 10^{-4} M HC P&A 6-9	SP8 (16.4%)	90.5 ± 2.1 (0.99)	9.5 ± 2.1 (1.49)	6.0 ± 0.5 (1.05)	44.6 ± 9.5 (0.81)	38.8 ± 17.6 (2.28)	56.4 ± 24.0 (1.27)	6.2 ± 2.7 (1.28)	15.4 ± 5.5 (0.96)	38.3 ± 12.0 (0.84)	4.3 ± 0.1 (0.83)
	SP4 (10.3%)	89.1 ± 3.6 (0.98)	10.9 ± 3.6 (1.25)	20.1 ± 2.8 (1.17)	38.5 ± 11.2 (1.03)	53.7 ± 9.7 (1.09)	61.5 ± 15.5 (1.23)	20.0 ± 2.6 (1.49)	20.5 ± 6.6 (1.00)	51.1 ± 8.4 (1.18)	8.0 ± 1.7 (1.09)
	DP (28.7%)	97.1 ± 0.6 (1.00)	2.9 ± 0.6 (1.00)	13.4 ± 6.0 (1.34)	11.4 ± 1.0 (0.92)	11.1 ± 3.5 (1.90)	51.1 ± 20.2 (1.10)	7.7 ± 1.2 (1.14)	5.3 ± 0.6 (1.27)	22.8 ± 5.6 (1.20)	3.0 ± 0.6 (1.03)
	DN (44.6%)	35.4 ± 5.6 (1.09)	64.6 ± 5.6 (0.96)	54.5 ± 11.2 (1.14)	84.0 ± 3.9 (0.92)	92.6 ± 0.7 (1.04)	92.6 ± 2.7 (1.01)	68.0 ± 6.7 (0.97)	76.8 ± 4.9 (1.00)	94.6 ± 1.7 (1.02)	61.5 ± 11.3 (0.95)
1 x 10^{-4} M HC P&A 9-12	SP8 (15.9%)	92.6 ± 1.7 (1.01)	7.4 ± 1.7 (0.94)	8.5 ± 2.2 (0.81)	44.1 ± 9.2 (1.06)	31.0 ± 14.0 (0.97)	51.5 ± 23.0 (0.96)	5.1 ± 0.6 (0.61)	11.9 ± 3.1 (0.81)	23.0 ± 10.3 (0.68)	5.1 ± 0.8 (0.66)
	SP4 (13.4%)	90.1 ± 1.7 (1.00)	9.9 ± 1.7 (1.19)	24.3 ± 5.2 (0.90)	46.1 ± 7.1 (0.86)	47.7 ± 18.3 (0.86)	53.5 ± 16.1 (0.80)	18.6 ± 4.1 (0.79)	18.4 ± 4.2 (0.82)	36.1 ± 10.6 (0.87)	11.8 ± 5.1 (0.95)
	DP (29.7%)	97.9 ± 0.4 (1.00)	2.1 ± 0.4 (1.41)	27.8 ± 10.9 (1.01)	12.0 ± 3.5 (1.45)	9.5 ± 3.7 (1.17)	45.8 ± 20.4 (1.15)	9.0 ± 1.3 (1.12)	4.8 ± 0.5 (1.04)	13.5 ± 4.3 (1.13)	4.5 ± 0.2 (1.23)
	DN (41.0%)	36.2 ± 3.6 (0.95)	63.8 ± 3.6 (1.02)	69.1 ± 5.4 (0.84)	89.6 ± 3.7 (1.07)	92.0 ± 3.2 (0.91)	91.1 ± 3.0 (0.77)	75.7 ± 3.7 (0.69)	72.4 ± 11.7 (0.77)	90.2 ± 2.5 (0.82)	72.1 ± 5.9 (0.68)

Table IV. Receptor expression in each developmental phenotype group during P & A experiments. The **relative total group receptor expression value** (determined by MFI) in the untreated control is represented, followed by the (normalized change) that occurred when treated with 1 x 10^{-9} M HC. Underlined normalized values indicate $p<0.05$.

Treatment Type	Phenotype	GD TCR IMM	GD TCR MAT	CD25	CD45RA	CD69	CD95	CD95L	CD122	CD132	DX5
1 x 10^{-9} M HC P&A 0-3	SP8 (5.1%)	12.3 ± 8.7 (1.13)	114 ± 31 (0.86)	6.3 ± 1.8 (1.00)	12.4 ± 3.2 (1.17)	8.7 ± 3.2 (1.30)	17.1 ± 4.6 (0.87)	3.3 ± 0.6 (1.48)	4.6 ± 0.8 (1.30)	7.5 ± 2.6 (5.59)	3.5 ± 0.7 (1.26)
	SP4 (5.1%)	10.4 ± 6.9 (1.45)	230 ± 52 (1.53)	8.7 ± 2.3 (1.35)	24.7 ± 2.0 (0.89)	29.3 ± 10.6 (1.83)	245 ± 123 (0.89)	41.8 ± 28.1 (1.45)	120 ± 35 (1.19)	20.2 ± 6.5 (1.51)	7.7 ± 0.7 (1.55)
	DP (70.5%)	9.2 ± 7.2 (1.53)	127 ± 21 (1.44)	2.2 ± 0.2 (1.03)	3.8 ± 0.7 (0.93)	3.8 ± 0.9 (1.28)	21.0 ± 6.7 (0.74)	2.6 ± 0.3 (1.32)	2.9 ± 0.2 (1.36)	4.5 ± 1.4 (2.78)	2.3 ± 0.2 (1.25)
	DN (19.4%)	14.7 ± 3.0 (1.24)	169 ± 39 (1.87)	99.5 ± 21.0 (0.95)	31.7 ± 0.7 (0.89)	23.8 ± 4.0 (1.82)	53.8 ± 9.9 (0.96)	19.1 ± 2.8 (0.92)	54.3 ± 20.5 (1.12)	25.0 ± 4.3 (1.81)	20.7 ± 0.7 (1.88)
1 x 10^{-9} M HC P&A 3-6	SP8 (10.2%)	9.8 ± 6.5 (1.15)	124 ± 34 (1.83)	6.5 ± 0.9 (1.10)	22.7 ± 2.3 (0.89)	16.6 ± 4.7 (0.77)	21.0 ± 8.8 (1.87)	4.9 ± 0.6 (1.87)	9.6 ± 0.5 (0.69)	9.4 ± 1.4 (0.80)	4.6 ± 0.6 (1.87)
	SP4 (6.1%)	11.2 ± 8.1 (1.58)	259 ± 25 (1.30)	8.1 ± 2.3 (1.30)	83.3 ± 24.4 (1.25)	45.3 ± 15.2 (1.82)	166 ± 12 (1.43)	43.5 ± 6.5 (1.26)	598 ± 65 (1.87)	25.9 ± 1.5 (1.15)	133 ± 14 (1.21)
	DP (64.0%)	9.4 ± 7.3 (1.22)	162 ± 30 (1.15)	3.0 ± 0.3 (1.21)	5.7 ± 1.1 (0.99)	6.1 ± 1.6 (0.86)	25.0 ± 10.7 (2.28)	4.0 ± 0.2 (1.27)	8.2 ± 0.8 (0.79)	5.6 ± 1.0 (1.83)	3.4 ± 0.6 (0.97)
	DN (19.8%)	16.3 ± 2.7 (1.82)	214 ± 49 (1.11)	51.4 ± 16.3 (1.87)	75.6 ± 1.1 (0.86)	43.0 ± 6.3 (0.99)	42.5 ± 4.3 (1.83)	22.4 ± 0.2 (0.93)	129 ± 4 (0.94)	30.7 ± 2.3 (1.86)	73.2 ± 15.0 (0.88)
1 x 10^{-9} M HC P&A 6-9	SP8 (16.4%)	11.5 ± 8.6 (1.41)	107 ± 30 (1.49)	4.9 ± 1.2 (1.85)	36.4 ± 9.0 (0.81)	12.7 ± 4.0 (2.28)	19.8 ± 7.7 (1.27)	3.8 ± 0.4 (1.28)	11.8 ± 5.9 (0.96)	10.1 ± 2.5 (0.90)	7.3 ± 3.8 (0.83)
	SP4 (10.3%)	10.9 ± 7.4 (1.38)	229 ± 21 (1.25)	14.8 ± 4.1 (1.17)	613 ± 536 (1.83)	25.5 ± 5.9 (1.80)	66.8 ± 7.2 (1.23)	20.3 ± 3.4 (1.49)	432 ± 408 (1.88)	28.5 ± 1.7 (1.18)	249 ± 223 (1.89)
	DP (28.7%)	10.0 ± 7.9 (1.69)	136 ± 29 (1.88)	7.3 ± 3.0 (1.34)	28.1 ± 19.7 (0.92)	5.5 ± 1.1 (1.90)	23.8 ± 9.8 (1.10)	5.6 ± 0.2 (1.14)	9.3 ± 5.7 (1.27)	9.5 ± 2.1 (1.28)	5.1 ± 2.1 (1.83)
	DN (44.6%)	17.5 ± 3.9 (1.52)	232 ± 48 (0.96)	33.4 ± 7.4 (1.14)	137 ± 20 (0.92)	48.2 ± 6.6 (1.84)	32.1 ± 4.0 (1.81)	20.4 ± 0.6 (0.97)	101 ± 73 (1.88)	30.4 ± 2.8 (1.82)	136 ± 89 (0.99)
1 x 10^{-9} M HC P&A 9-12	SP8 (15.9%)	11.0 ± 7.8 (1.82)	102 ± 30 (0.94)	4.1 ± 0.6 (0.81)	43.1 ± 7.5 (1.86)	16.9 ± 7.6 (0.97)	25.5 ± 12.4 (0.86)	5.4 ± 0.5 (0.61)	9.58 ± 1.7 (0.81)	10.0 ± 3.8 (0.69)	3.8 ± 0.5 (0.65)
	SP4 (13.4%)	11.2 ± 7.3 (1.34)	220 ± 40 (1.19)	19.5 ± 3.5 (0.98)	1396 ± 355 (0.86)	40.4 ± 7.3 (0.86)	58.9 ± 9.7 (0.88)	27.1 ± 6.1 (0.79)	305 ± 219 (0.82)	21.9 ± 4.6 (0.87)	20.5 ± 10.4 (0.99)
	DP (29.7%)	10.1 ± 7.6 (1.38)	115 ± 18 (1.41)	10.5 ± 4.1 (1.81)	32.2 ± 16.0 (1.43)	6.1 ± 1.7 (1.17)	24.9 ± 11.0 (1.15)	5.7 ± 0.9 (1.12)	5.5 ± 1.9 (1.84)	9.4 ± 2.9 (1.13)	3.4 ± 0.3 (0.93)
	DN (41.0%)	17.2 ± 3.0 (1.11)	206 ± 49 (1.82)	48.6 ± 18.6 (0.94)	315 ± 16 (1.87)	50.5 ± 15.3 (0.91)	37.9 ± 10.4 (0.77)	25.0 ± 4.2 (0.69)	111 ± 46 (0.77)	27.5 ± 5.1 (0.82)	53.2 ± 13.4 (0.68)

DISCUSSION

In this paper we, as others before us (4, 19), have shown that GCs have the capacity to effect T cell development. The addition of the glucocorticoid HC at low levels ($1x10^{-9}$ M), approximately doubling the predicted thymic endogenous levels (7), can have expansive effects on T cell development in terms of cell yield and mature cell generation (Fig. 1). In contrast, the addition of HC at high levels causes decreases in cell yield and a loss of T cell development. These effects appear to be generated through the same inhibitory effects GCs have on T cells in the periphery (5), including the capacity of GCs to cause decreases in receptor expression (Table I; III and IV (Day 9-12)).

The effect of HC on avidity signals perceived by T cells during development appears to be inhibitory as well. HC generates the opposite effect on T cell maturity and receptor expression when compared to anti-CD3, which has been shown to be a signal generator by others (Fig. 2)(18, 20, 21). However, the totality of the effect of HC during development is not just receptor down regulation, but also the capacity of the T cell to respond to this down regulation/signal dampening. To better understand the dynamics of T cell development several theories and models have been proposed. "the tunable-activation-threshold (TAT) model", when applied to development, proposes that slow progressive increases in receptor expression, as experienced by TCR (Fig. 5C), CD3 and other receptor types during development (Table III), take place in order to tune receptor expression and set the activation thresholds for thymocytes as they mature (22, 23). The TAT model essentially proposes the existence of a desired "time-dependent" window of avidity that changes over the course of selection to generate T cells with receptor

expression that is optimal for cell survival, while minimizing the chance of autoreactivity in the periphery.

Data presented in this paper tends to fit well into the TAT theory of development. Two of the major components inhibited by the presence of GCs are the nuclear transcription factors NF-κB and AP-1 (24), which play essential roles in the production of signaling components for the TCR/CD3 pathway (25). This broad-spectrum, transcriptional level, inhibition by GCs can be thought of as having an overall dampening effect on the interaction avidity perceived by T cells during selection or in the periphery. However, it appears responses to these effects during development and in the periphery are distinct. In mature T cells, GCs lead to the inhibition of a multitude of cytokines and receptors, which are thought to play a role in immune system activation. Conversely, immature thymocytes appear to increase the expression of certain receptor types in response to GCs, possibly in order to overcome GC induced signaling inhibition and maintain the desired avidity in the developing cell.

Figure 9 shows a graphic depiction of this GC induced process based on the normal T cell window of selection. In this figure, the green line depicts the percentage of cells passing positive selection and the red line depicts the percentage of cells avoiding negative selection, based on the actual avidity ("normal" perceived avidity) of the interaction between the T cell and the selecting cell. The processes of positive and negative selection then generate the window of selection for maturing T cells based on the desired avidity. The introduction of a signal inhibitor (i.e. decreasing perceived

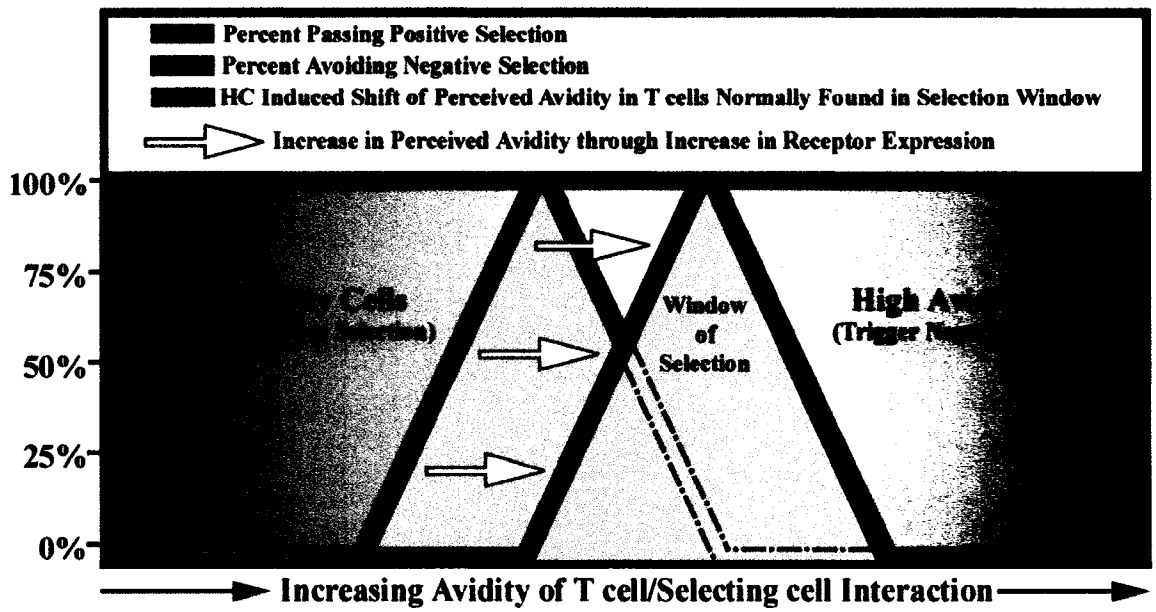

Figure 9. Impact of HC on Avidity Based Window of T cell Selection. Depiction of the impact of HC on T cell avidity and the response by the developing T cell is shown. See text for detailed description.

avidity) or a signal enhancer (i.e. increasing perceived avidity) has the capacity to change the avidity perceived by the cell, despite the actual avidity. In the case of HC treatment, some T cells that were normally in the window of selection may now perceive their avidity to be lower than acceptable (blue lines) and must increase receptor expression (in receptors that play a role in selection and that expression can be increased) to compensate for the lost signal in order to reenter the window of selection. If GCs can impact both surface expression and the internal signaling of those receptors, as proposed by several investigators (25, 26), then the increases produced would have to be larger than strictly returning receptor expression to normal levels. This idea is supported by the increased levels of receptor expression in the developmental DP group (Fig. 7 and Table IV) and the HC based dose dependent increases in expression seen in Figure 8.

A response of this type would also have time dependent ramifications on the composition, number, and overall avidity of the T cells generated. The results from P&C and P&A experiments (described in Results) also support the TAT model in this case. When HC is given early in development, during times associated with the first wave of β selection (FTOC days 0-3), and are then allowed to complete development (until culture day 12) they generate increased levels of SP4 mature cells and decreased levels of SP8 mature cells (Fig. 5B). Using the TAT and avidity models of development, a depiction of the predicted impact of treatment with an inhibitor, such as HC, on early T cell development is shown in Figure 10 (phases are strictly suggestive). Essentially, the

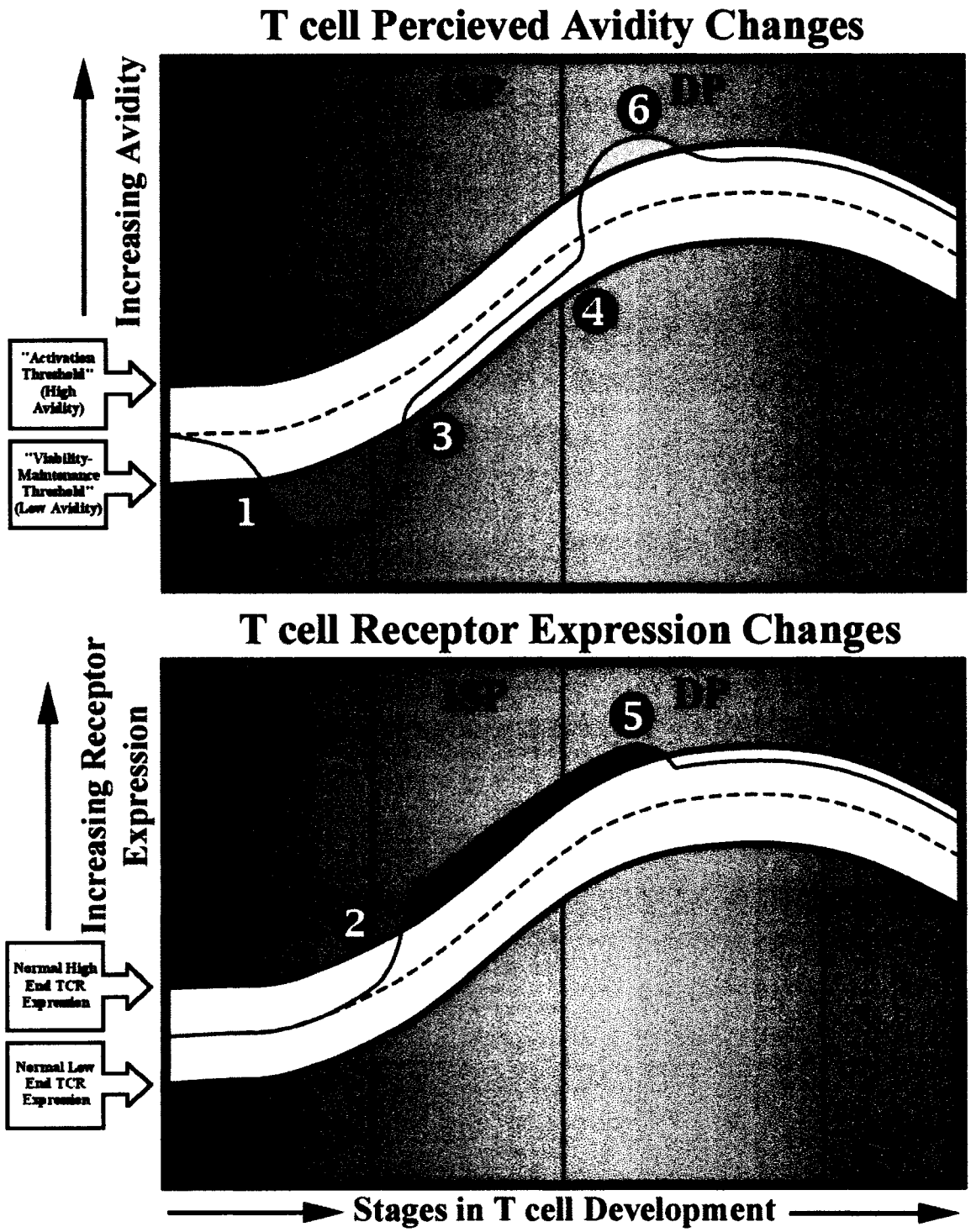
T cell Percieved Avidity Changes
Increasing Avidity
"Activation Threshold" (High Avidity)
"Viability-Maintenance Threshold" (Low Avidity)
DP
6
4
3
1
T cell Receptor Expression Changes
Increasing Receptor Expression
Normal High End TCR Expression
Normal Low End TCR Expression
DP
5
2
Stages in T cell Development

Figure 10. Effect of HC on the early stages of T cell development. A model depicting the impact of signal inhibitors, when given during the early stages T cell development, is proposed. Predictions are based on TAT model of T cell development. When an inhibitor is added (❶) developing T cells experience a decrease in perceived avidity. The T cell the responds by increasing receptor expression to compensate for signal loss (❷). The receptor increase then causes the T cell to reenter the desired window of avidity (❸). When the inhibitor is removed (❹) the perceived avidity then increases again due to the high level of receptor expression. Again, the T cell must adjust receptor expression (❺) to reenter the desired window of avidity (❻).

inhibitor reduces the perceived avidity of the T cell (Fig. 10 #1), causing receptor expression to increase to compensate for lost signal (Fig. 10 #2). The T cell then returns to the desired avidity (Fig. 10 #3). If the inhibitor is then removed, the T cell has a higher than normal level of receptor expression. Thus, the avidity is higher than normal (Fig. 10 #4), so the T cell must reduce receptor expression towards normal levels (Fig 10 #5). T cells reentering the desired avidity window would only need to return to low enough level of avidity to pass negative selection, instead of the normal level of avidity they would have generated if no inhibitor had been used (Fig. 10 #6). This would create T cells that experienced a higher level of avidity (solid line) during the selection process than normal untreated cells (dotted line). Research by some has shown that signal strength experienced during selection can influence the mature phenotypes that T cells become (i.e. SP4 or SP8)(27-29). The majority of this work indicates that SP4 cells require or can tolerate higher avidity signals during selection, while SP8 cells typically require lower avidity signals. These differences between SP4 and SP8 signal requirements are likely due to the 20 times higher affinity of the Lck molecule for the SP4 co-receptor (30, 31) and/or the enhanced role of ZAP70 in SP8 signaling (32).

The impact of the addition of an inhibitor during the later stages of T cell development, when T cells are going through the final stages of selection, is depicted in Figure 11 (equivalent of days 6-9 when the majority of T cells are leaving DP phase into SP mature phase). Similar to early stage addition, the T cell perceives a drop in avidity (Fig. 11 #1) and responds by increasing receptor expression (Fig. 11 #2). These increases then generate an increase in avidity, allowing the T cell to reenter the desired avidity

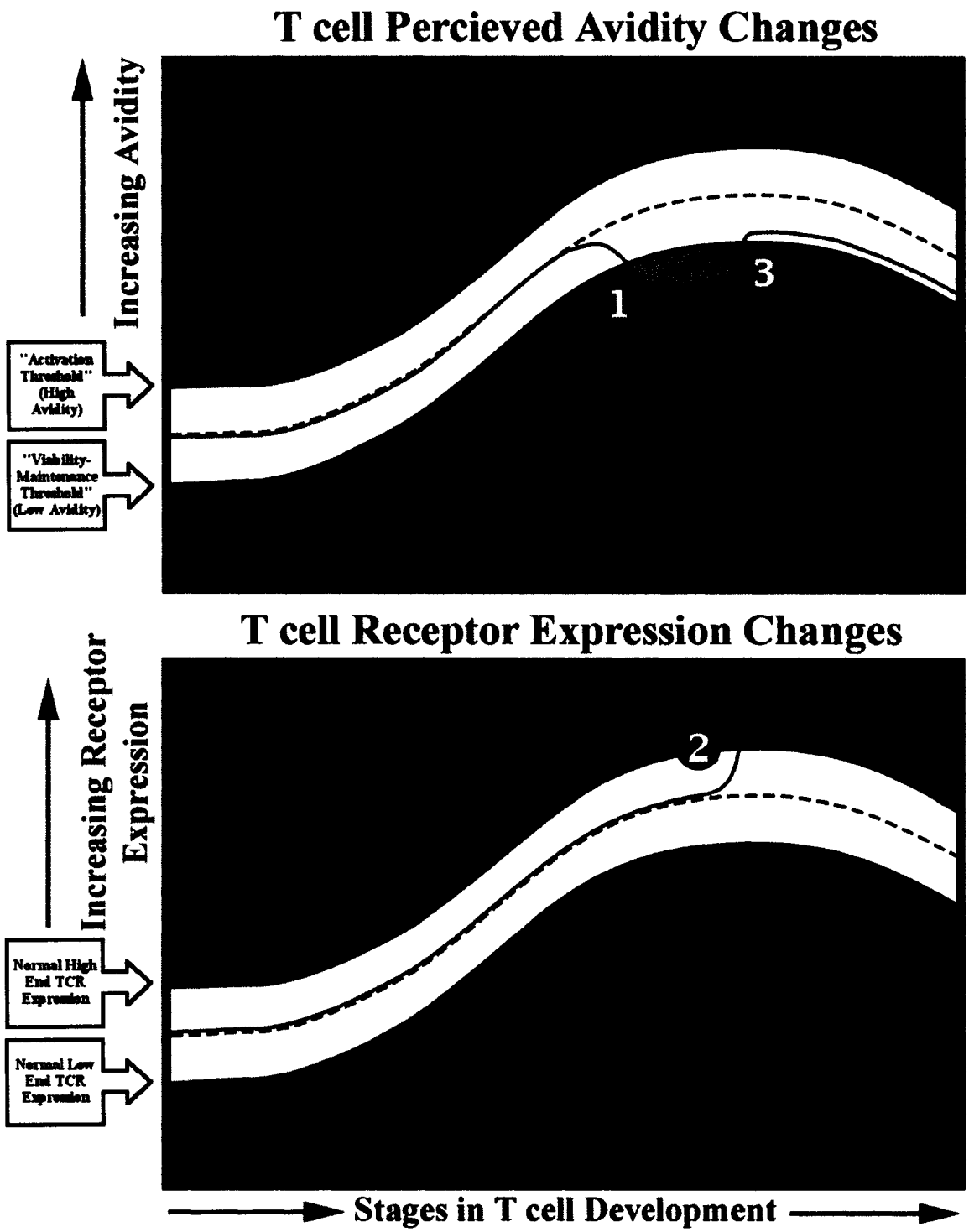
T cell Percieved Avidity Changes
Increasing Avidity
"Activation Threshold" (High Avidity)
"Viability-Maintenance Threshold" (Low Avidity)
1
3
T cell Receptor Expression Changes
Increasing Receptor Expression
Normal High End TCR Expression
Normal Low End TCR Expression
2
Stages in T cell Development

Figure 11. Effect of HC on the late stages of T cell development. A model depicting the impact of signal inhibitors, when given during the late stages T cell development, is proposed. Predictions are based on TAT model of T cell development. When an inhibitor is added (❶) developing T cells experience a decrease in perceived avidity. The T cells the respond by increasing receptor expression to compensate for signal loss (❷). The receptor increase then causes the T cells to reenter the desired window of avidity (❸). See text for details.

window (Fig. 11 #3). However, if the inhibitor is present throughout the remainder of development, receptor expression will remain increased. Despite the increased receptor expression, these T cell would develop under a lower perceived avidity than untreated cells.

In both early and late addition, receptor expression of cells in the DP group would be increased versus normal T cell development. Data from P&A DP 6-9 groups in Fig. 7 and Table IV do show these expected receptor increases. These increases were also present in the DP groups of P&C long-term cultures that were treated with HC during early or late time points (data not shown). Based on this model, inhibition early in development would subject T cells to higher than normal avidity signals, while inhibition later in development would subject T cells to lower than normal avidity signals. This could explain why early inhibition causes a shift in phenotype towards SP4 and late inhibition causes a shift towards SP8 (Fig. 5B).

If these shifts in avidity do indeed occur, they would also have the potential to increase the number of T cells that enter the selection window. Treatment with HC during early stages of development could drive receptor expression up (response to HC) or down (through inhibition by HC) on cells that naturally fall below or above the desired avidity window of selection. Once the inhibitor is removed, cells that would normally fall below the "maintenance threshold" (i.e. would normally fail positive selection) or cells that would normally fall above the "activation threshold" (i.e. would normally be negatively selected) may be able to stay in the window of selection and pass selection by maintaining higher or lower than normal expression levels, as necessary. As follows, the

presence of an inhibitor late in selection, after positive selection occurred, could allow for T cells with avidities above the "activation threshold" to pass negative selection, through the inhibitory effects of HC. Data from P&A and P&C experiments could support this. Long term cultures in P&C experiments that are treated early or late both show increases in T cell yields (Fig. 4 gray bars), but only P&A cultures with cells actively in the DP phase (Day 6-9 P&A open bars) show immediate cell yield increases. P&A cultures, treated early and harvested immediately after treatment (before the majority of selection occurs), show no cell yield increases, instead cell yields appear to be decreased (Day 0-3 P&A open bars).

Changes in mean avidity levels during development, as proposed above, would also have impacts on the types of TCR that pass selection and their distribution in the T cell population. Research has shown that the avidity generated based on the MHC type used, in limited MHC mouse models, can predict the Vβ usage within mouse strains (33). Additionally, work by several investigators has shown that signal strength and GCs can impact TCR Vα and Vβ usage (34-37). Work presented here shows that treatment with inhibitory HC in long-term cultures leads to shifts in the ratio of T cells using Vβ7 or Vβ8 in their TCRs (Fig. 3). Also, data from anti-CD3 treated FTOC indicate that cultures treated with signal enhancers generate the opposite shifts in Vβ usage when compared to those cultures treated with the signal inhibitor HC. These data are by no means conclusive, but do fit in well with the current avidity and TAT models of development. Experimentation using dose and time dependent administration of inhibitors and

enhancers, as well as investigating a larger array of Vβ and Vα types in a single MHC model, would be needed to better understand how signaling effects TCR usage.

Other possibilities exist that could perhaps explain the overall divergent effects of HC on mature and immature cells. Recent investigation indicates that PPARα has the capacity to interfere with the effects of GCs through the increased production of IκBα, thus forming an autoregulatory loop (38). If this feedback is stimulated by the presence of excess HC, it may generate different responses in immature and mature T cells depending on gene accessibility. Also, research has been performed investigating the presence of the glucocorticoid receptor (GR) during different stages in development. GR appears to be expressed at lower levels in DP thymocytes than in SP and DN mature cells, which may play a role in the results presented here (17, 39). However, these DP cells have been shown to still be susceptible to GC and undergo GC induced apoptosis, which tends to indicate that DP cells are not immune to the effects seen other T cell phenotypes (16).

The results presented here also fall in line with GC related work with metyrapone. Treatment with metyrapone, a known GC production inhibitor, disrupts T cell development at the DP phase of development, causing apoptosis, in a manner that resembles treatment with high levels of signal enhancing anti-CD3 (7)(Fig. 2). 12 day FTOC performed in our lab using metyrapone also show inhibition at the DP phase of development. In addition, these cultures generate mature SP8 cells that have bypassed the DP phase (data not shown). The generation of mature SP8 cells in this manner has also been seen in research investigating the pre-TCR. This research suggests that lowering signal strength requirements, as metyrapone theoretically does, allows for the generation

of SP8 cells through stronger than normal signaling to the pre-TCR complex (i.e. T cells develop but bypass the DP stage) (40). Data such as this tends to further support an HC based impact on avidity.

Granted, avidity models discussed/presented here fit well with the data but are still, in some respects, considered theoretical. However, the effects generated through the addition of HC are consistently reproducible and dose dependent. Data presented here (Fig. 2), and other work performed in our lab, has shown that inhibitors and enhancers generate distinct changes in T cell phenotype, maturation, and cell yield. These types of changes can be important for understanding how development in the thymus can be modified to change immunity, fight infectious diseases, or eliminate risk of autoimmunity. For example, significant research is currently being performed to investigate the role of NKT cells in autoimmunity. Enhancement of NKT cells during development has shown to be effective in mitigating diabetes in the NOD mouse model (41) through shifting of T_H1/T_H2 ratios (42, 43). NKT cells are thought to require/tolerate higher avidity signals (44) and have been shown to be resistant to the inhibitory effects of GCs (45). These characteristics of NKT cells would make them less susceptible to HC induced changes in avidity, which could be used in a beneficial manner to promote NKT expansion. HC has other mechanism that alone could enhance protection versus diabetes, such as the reduction of NF-κB, which is elevated in NOD peripheral DCs and contributes to disease severity (46, 47). Also, HC has the capacity to induce increases in GAD67 production (48). Increases in GAD during development have shown the capacity to mitigate diabetes (12).

It is possible that avidity-based treatments using signal modulators could produce long-term beneficial impacts on T cell development that could help reduce autoreactive T cells and enhance regulatory cell production. Our work with HC hints that there may be an impact on the regulatory population because of the effect of HC on the number of potential regulatory cells generated during early thymic development, including $DX5^+$ (Table III)(49) and $NK1.1^+$ cells (data not shown). Essentially, as further research is performed, the capacity to predictably manipulate immune system development, to eliminate autoimmune risk or battle disease, may become an essential part of modern medical science.

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APPENDIX E

Institutional Animal Care
and Use Committee

THE UNIVERSITY OF
ARIZONA.
TUCSON ARIZONA

P.O. Box 210101
Tucson, Arizona 85721-0101

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use.
The following listed proposal has been granted ***Final Approval*** according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:

#99-059 - "Organ Culture Approaches for the Study of Immune Cell Development in Health and Disease"

PRINCIPAL INVESTIGATOR/DEPARTMENT:

Dominick DeLuca - Microbiology & Immunology

GRANTING AGENCY:

ADCRC, American Diabetes Association, Arizona Elks

SUBMISSION DATE: **April 22, 1999**

APPROVAL DATE: **September 8, 1999** APPROVAL VALID THROUGH*: **September 7, 2002**

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: **September 9, 1999**

REVISIONS (if any):

- **Animals will be sacrificed by CO_2 rather than cervical dislocation.**

MINORITY OPINIONS (if any):

Richard C. Powell

Richard C. Powell, PhD, MS
Vice President for Research

DATE: September 9, 1999

Institutional Animal Care
and Use Committee

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P.O. Box 210101
Tucson, AZ 85721-0101

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use.
The following listed proposal has been granted ***Final Approval*** according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:

#02-122 - "Anti-CD1 Effects In Vitro and In Vivo"

PRINCIPAL INVESTIGATOR/DEPARTMENT:

Dominick DeLuca, PhD - Microbiology & Immunology

GRANTING AGENCY:

American Diabetes Association

SUBMISSION DATE: **July 10, 2002**

APPROVAL DATE: **August 7, 2002** APPROVAL VALID THROUGH*: **August 6, 2005**

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: **August 8, 2002**

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell

Richard C. Powell, PhD, MS
Vice President for Research

DATE: August 8, 2002

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.

MEMORANDUM FROM THE IACUC OFFICE

Central Animal Facility - Building #101
P.O. Box #210101, Tucson, AZ 85721-0101
Phone: 621-9305, FAX: 621-3355
E-Mail: musgravl@u.arizona.edu

August 27, 2002

TO: Dr. Dominick DeLuca
MBIM
P.O. Box #245049
LSCN #605

FROM: Linda S. Musgrave, IACUC Coordinator

RE: Verification of Protocol Approval

Enclosed please find the new Verification of Approval Form(s) for the following protocol(s):

#02-142 - "Cortisone Effects in Vitro and In Vivo"

Please remember that it is your responsibility to send a copy of the Verification form to your study section, or granting agency.

Please also remember to use the control number(s) listed above for all animal-use related requests and amendments associated with the above noted protocol(s). The approval is good for three years starting **August 22, 2002 through August 21, 2005.**

Any revisions to this work must be forwarded to my attention for the appropriate IACUC review.

Thank you for your consideration in this matter.

ADDITIONAL NOTES:

/lsm
Enclosure

Please notify University Animal Care facility supervisors of this approval date for you and your participating personnel to assure that you will all have security clearance for the duration of your protocol.