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Transgenic Ly49A As An Inhibitory T Cell Receptor Against Autoimmune Diabetes In Mice and Extracellular Receptor-Activated Kinase As A Marker Of T Cell Activation

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This I dedicate
to Carla, Nicolas (you little peanut),
and Julianna (you little monkey)...those
making the real sacrifice
and whose support is making
this dream more of a reality every day of our lives.

I wish to thank Dr. Mary Pauza
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when there is too little time
and too much left to be learned.

Abstract

Type 1 diabetes mellitus is an autoimmune disease characterized by insulin deficiency that is a result of the progressive destruction of the β cells within the islets of Langerhans of the pancreas. The course may be variable, but as the quantity of viable insulin secreting cells becomes fewer, the signs and symptoms of hyperglycemia become manifest and exogenous insulin therapy is required. The disease begins in an individual, usually with a genetic predisposition, as tolerance to self antigens is lost. Several autoantibodies have been discovered and include anti-islet T cell antibodies (ICAs), anti-glutamic acid decarboxylase antibodies (GAD), anti-insulin antibodies, and antibodies directed at insulinoma-associated protein 2 (IA-2). The principal immunologic effector cells are $CD4^+$ T cells, among others.

Currently, one aim of research is dedicated to uncovering the role of inhibitory receptors in immune effector cells. In particular, Ly49A (one of nine inhibitory receptors found on murine Natural Killer (NK) cells, $NK1.1^+$ cells, and a small subset of $CD8^+\alpha\beta$-TCR^+ T cells) and its function in maintaining NK tolerance to self antigens, may shed light on why T cells fail to keep their own self-tolerance. We used transgenic mice predisposed to autoimmune diabetes and expressing Ly49A on $CD4^+$ T cells to observe its effect on the onset of hyperglycemia and to learn more about the intracellular signaling events involved in the inhibitory function of the receptor. Principally, Extracellular Receptor-Activated Kinase (Erk) was used as a marker of T cell receptor (TCR) activation. Without proper ligation, Ly49A failed to offer any protective function against the development of diabetes as diagnosed by serum glucose measurements. However, because of difficulties with intracellular staining techniques, remarkable differences in Erk activation that may explain these findings were not observed.

Resumen

La Diabetes Mellitus de tipo 1 es una enfermedad del sistema inmunológico que se caracteriza por una deficiencia de insulina como resultado de la destrucción progresiva de las células β de los islotes de Langerhan en el páncreas. El curso de la enfermedad puede ser variable, pero conforme la cantidad de células secretoras de insulina disminuye, los signos y síntomas de la hiperglicemia se manifiestan y la terapia exógena de insulina es necesaria. La enfermedad usualmente empieza debido a una predisposición genética del individuo por la perdida de la tolerancia a antígenos propios. Varios anticuerpos se han descubierto e incluyen anticuerpos contra los islotes de células T (ICAs), anticuerpos contra ácido glutámico descarbolyxase (GAD), anticuerpos anti-insulina y anticuerpos dirigidos contra la proteína asociada con insulinota 2 (IA-2). Las principales células inmunológicas efectoras son células T $CD4^+$, entre otras.

Actualmente una de las metas de la investigación está dedicada a descubrir el rol de los receptores inhibidores de las células efectoras. En particular, Ly49A (una de los nueve receptores inhibidores encontrándose en células Natural Killer (NK) murines, $NK1.1^+$ y un pequeño subgrupo de células T $CD8^+\alpha\beta$-TCR^+) y su función de mantener tolerancia NK a antígenos propios puede alumbrar las razones por las que las células T no pueden mantener la tolerancia propia. Usamos ratones transgénicos predispuestos a la diabetes autoinmune y expresando Ly49A en las células T $CD4^+$ para observar su efecto sobre el inicio de hiperglicemia y para conocer más sobre los eventos intracelulares involucrados en la función inhibidora del receptor. La Kinasa Extracelular Activada por Receptor (Erk) fue principalmente utilizada como marcador de la activación del receptor de la célula T (TCR). Sin la ligación adecuada, Ly49A falló al ofrecer alguna función protectora contra el desarrollo de la diabetes diagnosticada por mediciones de glucosa sanguínea. Sin embargo, por las dificultades debidas a las técnicas de marcación intracelular, no se observaron diferencias importantes de la activación de Erk que pudieran haber explicado estos hallazgos.

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Introduction

Background, etiology, and genetic aspects

Diabetes mellitus (DM) describes a state of abnormal carbohydrate metabolism that is characterized by hyperglycemia, relative or absolute impairment of pancreatic ß-cell insulin secretion, and varying degrees of peripheral resistance to the action of insulin. Potential complications of diabetes include macrovascular, microvascular, and neuropathologic complications that generally occur after a long-term course of uncontrolled hyperglycemia. In the United States, diabetes is the leading cause of end-stage renal disease, lower extremity amputations of a nontraumatic cause, and blindness in adults. The worldwide prevalence of diabetes has dramatically risen over the past two decades. This increase is expected to continue especially for type 2 DM (T2D) secondary to increasing obesity and reduced physical activity (1).

In 1998, approximately 16 million individuals in the United States met the diagnostic criteria for DM, representing approximately six percent of the population, while 800,000 individuals develop DM each year (more than 90% T2D). On a worldwide basis, considerable geographic variation exists between type 1 DM (T1D) and T2D mainly at the expense of T1D. This is believed to reflect the frequency of high-risk HLA alleles within ethnic groups (1).

The two principle forms of diabetes mellitus traditionally have been termed either insulin-dependant or non-insulin-dependent. Other terminology used for these conditions may include: juvenile-onset, maturity-onset, adult-onset, and maturity-onset diabetes of the young (1). However, as more was learned about the pathogenesis of the disease it became necessary to revise this nomenclature according to the underlying pathology rather than its etiology. For instance, juvenile-onset diabetes traditionally referred to a young, peripubertal individual that was dependent on exogenous insulin administration. However, since the discovery of autoantibodies directed against pancreatic ß-cell antigens, it is now acknowledged that some adults presumed to have T2D most likely have T1D. One study found that 7.5 to 10 percent of adults with apparent T2D actually had T1D as defined by the detection of autoantibodies toward pancreatic β cells (1). Similarly, T2D generally was

regarded as non-insulin-dependent (NIDDM), because there is no destruction of pancreatic cells, but rather various organ and tissue insulin resistance. Unlike with T1D, patients are not prone to ketoacidosis. However, it is not uncommon for these patients to require insulin during the stress of infection or other illnesses that cause an increase in insulin resistance, and ketoacidotic states have been reported (2). As found in one retrospective study, 15 of 62 adults presenting with diabetic ketoacidosis as their initial manifestation of diabetes were not taking insulin one year later and actually had T2D (12).

For these and other reasons, in 1997 new classifications were to be used that put type 1 and type 2 disease at the extremes of insulin deficiency and insulin resistance, respectively, while also acknowledging that there exists manifestations of varying deficiency and resistance in between. Diseases such as Atypical DM, Pancreatic DM, Type 1.5 DM, and Type 3 DM are examples of those manifestations. Now, terms such as non-insulin-dependent and insulin-dependent are obsolete and their use is discouraged (1).

T2D has generally been associated with a strong genetic influence that, along with environmental and lifestyle factors, leads to insulin resistance, however, less is known about the genetic loci responsible for the predisposition to disease (1). On the other hand, the genetic susceptibility to T1D has been localized, thus far, to at least four different genes located in the major histocompatibility complex (MHC) and elsewhere in the human genome. The lifelong risk of T1D is markedly increased in close relatives of a patient with T1D, averaging about 6% in offspring, 5% in siblings, and 30% in identical twins in comparison to only 0.4% in individuals with no family history. Furthermore, a monozygotic twin of a patient with T1D has a higher risk than a dizygotic twin whose risk is similar to that in non-twin siblings (1).

Accounting for approximately 40% of familial clustering of T1D, the major susceptibility gene, termed IDDM1, is located in the HLA region on chromosome 6p amongst the genes that code for MHC class II proteins. Furthermore, greater than 90% of patients carry either HLA-DR3,DQB1*0201 or HLA-DR4,DQB1*0302, and 30% carry both. Possessing both of these alleles carries the greatest susceptibility. These genes are thought to code for an amino acid other than aspartate at position 57 on the beta chain of the HLA molecule (4); however, several exceptions have been documented where Asp57 fails to provide complete

protection from disease evolution. In addition to susceptibility genes, HLA alleles offering protection to the disease have also been elucidated. One such gene is located at HLA-DQB1*0602. A prospective study evaluated 72 relatives with islet T cell antibodies present and with one or more of the major susceptibility genes. Diabetes developed in 28 of the subjects without the protective gene while all eight with the protective gene remained free of disease (13).

Non-MHC genes located in other chromosomes have also been identified to play roles in the autoimmune process, although to a lesser and more obscure role. Among some of these are genes that regulate insulin transcription (non-coding sequences in regulatory regions), insulin-like growth-factor binding protein, interleukin-2, and interferon gamma-inducing factor (IGIF or IL-18), however less is known about the exact role each play in the pathogenesis (4).

Pathogenesis and autoantibodies

Like insulin resistance in T2D, ß cell destruction and insulin deficiency in T1D may be a gradual process over several years, or it may take on a more abrupt course. Patients remain symptomatically silent as pancreatic islet T cell destruction proceeds steadily until a critical magnitude has occurred (usually when 30-40% functional cells remain), when at that time, signs and symptoms of insulin insufficiency manifest (excessive urination, increased appetite and thirst, and weight loss over several days).

As previously mentioned, T1D is an autoimmune process. Autoimmunity can be defined as an immunologic reaction against self-molecules that has resulted from a failure to establish, or maintain, tolerance to self antigens. Tolerance is defined as unresponsiveness to an antigen and is an acquired rather than an innate property of the immune system (7). There are two main types of tolerance: central tolerance and peripheral tolerance. Central tolerance occurs during T cell development in the thymus as CD4/CD8 double positive thymocytes progress toward the single positive ($CD4^+$ or $CD8^+$) stage of development prior to exiting the thymus. During this time, thymocytes are exposed to self MHC molecules via thymus epithelial cells and dendritic cells where, by means of positive and negative selection, only cells that recognize self antigens with a mild to moderate affinity will

remain viable. This process is very effective at removing self-reactive T cells from the repertoire. However, some self antigens are not expressed in the thymus so self-reactive T cells may still enter the periphery. These potentially autoreactive T cells are kept in check by peripheral tolerance mechanisms (1–4) (similar events occur for B cells).

While genetic markers for diabetes are present at birth, immunologic markers, or autoantigens, are only detectable after the onset of the autoimmune process but long before the onset of symptomatic hyperglycemia. The principle autoantigens isolated in T1D patients include: islet T cell autoantibodies (ICAs), anti-glutamic acid decarboxylase antibodies (GAD), anti-insulin antibodies, and antibodies directed at insulinoma-associated protein 2 (IA-2) (1,4,5). These three autoantibodies have been used in combination to identify patients who are genetically susceptible to disease and several studies have already documented their usefulness (1,4,9). Several other autoantigens exist suggesting that the immune response likely does not involve only one antigen but rather a role exists for many.

While the role for autoreactive T cells is solid, several mechanisms not mediated directly by the T cells are likely involved. For example, pancreatic islet T cell autoantibodies are not thought to be involved in the on-going destructive process. Generally, they have been found unreactive with the pancreatic islet beta cell surface and incapable of transferring DM to non-genetically predisposed animals. Instead, it is thought that they are involved chiefly in the initiating event (1). Insulitis results from this initial insult, which describes an early process of spreading cellular necrosis and lymphocytic infiltrations mediated by B-cells, T cells, macrophages, natural killer cells, with production of damaging cytokines such as tumor necrosis factor and interleukin-1, IFN-γ (1). These events likely release additional intercellular autoantigens that are not recognized by macrophages and normal clearing of cellular debris is absent, compounding the pathologic process.

While type 1, autoimmune diabetes is by far less common than T2D, accounting for less than 10% of the cases of DM in the U.S. and affecting only 0.2% of the population, large amount of research is dedicated to it. Elucidation of its exact pathogenic process may also give insight into other autoimmune diseases such as rheumatoid arthritis, vasculitis, Goodpasture´s syndrome, and systemic lupus erythematosus, among others. In one report, it was estimated that 1 in 31 individuals in the U.S. are afflicted with an autoimmune

disorder, and the existence of more than one disorder in the same individual is not uncommon. Approximately 93% of the cases of autoimmune diseases are due to Grave's disease, T1D, pernicious anemia, rheumatoid arthritis, thyroiditis, and vitiligo (7).

The use of transgenic mice has greatly changed the ability to study self-tolerance by giving the investigator the ability of inserting manipulated genes into the genome of the animal. For example, antigen receptors on mouse T cells specific for desired autoantigens, such as those that might be found on pancreatic islet cells, can be expressed using this technology. Briefly, to create transgenic mice, foreign DNA sequences (transgenes) engineered by recombinant DNA technology are introduced into the male pronuclei (before fusion) of fertilized mouse eggs, which are then implanted into the oviducts of female mice. This creates transgenic offspring with the desired gene inserted into the genome of every cell, including germ cells, which, after the mating of heterozygote offspring, conveniently facilitates homozygous breeding. In addition, tissue-specific transgenes under control of hormones or drugs can be inserted such that their expression can be turned on or off upon administration of the inducing agent.

Regulatory T cells, Helper T cells, chemokine and cytokine environments, and lymphocyte transmigration

As previously mentioned, in human disease a long period of several years of subclinical disease typically occurs before the clinical manifestations of autoimmune diabetes becomes manifest as hyperglycemia. A perpetual destructive process towards the β cells of the pancreas continues until less than half of the β cell population remains viable at which time endogenous insulin production becomes inadequate. The non-obese diabetic (NOD) mouse exhibits a pathology that is in many ways comparable to human disease, but in a shorter timeframe. Shortly after weaning, infiltration of the pancreatic islets by T cells ensues and diabetes is evident typically at 12 weeks when 85-90% of insulin-secreting β cells have been destroyed (17).

During this twelve-week period it is now hypothesized that there is an ongoing balance between effector and regulatory T cell populations until gradually the balance is lost and effector cells succeed in the destructive process. Of particular importance are regulatory

cells found in the thymus of young healthy mice belonging to two subpopulations, mature $CD4^+CD62L^+$ thymocytes (14) and T cell receptor (TCR) $\alpha\beta^+CD4^-CD8^-$ double negative thymocytes (15). The presence of $CD62L^-$ in the T cell population of diabetic mice has been found to rapidly transfer diabetes into immunoincompetent recipients while $CD62L^+$ are poorly capable of inducing the disease (16). The regulatory function of these cells appears to be independent of the actual CD62L addressin; instead it serves more as a marker discriminating between Ag-experienced diabetogenic T cells and naïve T cells.

Lepault and Gagnerault (17) have confirmed this finding and have further characterized the regulatory T cells by using cotransfer experiments in which diabetes induced in immunodificient hosts given spleen T cells from diabetic mice is prevented by cells from prediabetic mice. Briefly, it was found that regulatory $CD4^+$ T cells were different from effector T cells in that they expressed high levels of CD62L, they homed to the periphery of the islets instead of invading the islets, and upon stimulation in vitro they produced low levels of IL-2 and IFN-γ and no IL-4 or IL-10, as opposed to $CD62L^-$ T cells that produced a wide range of cytokines.

Perhaps one of the most promising regulatory T cells contributing to immunologic tolerance not only to innate autoreactive cells in the periphery and in autoimmune disease, but also in transplant recipients and tumor cell research is the $CD4^+CD25^+$ T cell. Found naturally during the course of inflammatory conditions, it is hypothesized that $CD4^+CD25^+$ cells are able to actively constrain inflammation by arriving shortly after effector cells and subsequently "tuning-down" the effector response. Actually, depletion of $CD4^+CD25^+$ T cells leads to the spontaneous development of various autoimmune diseases in susceptible animals, enhances immune response to non-self antigens such as allogenic tissue grafts, and it provokes effective tumor immunity in otherwise non-responsive animals (32). Indeed, expansion of $CD4^+CD25^+$ T cells or augmentation of their activity can suppress allograft rejection and even induce allograft tolerance (33).

Martin *et. al*(34) shows how in models of Inflammatory Bowel Disease in mice, coinjection of $CD4^+CD25^+$ T cells protected the recipient mice from the disease, not by deterring the initial activation/proliferation of injected naïve T cells or by affecting their differentiation in Th1 effector cells, but rather by suppressing the T lymphocyte effector

functions. The naïve T cells did not become anergic after the coinjection, as they were still able to respond in vitro to antigenic stimulation when the regulatory cells were removed. The cells regained the capacity to produce preinjection IFN-γ quantities and where still capable of causing IBD upon reinjection into to lymphopenic recipients.

On a molecular level, as a possible mechanism of the inhibition by $CD4^+CD25^+$ T cells it was found that CD45 up-regulation on expanding naïve T cells was suppressed (34). CD45 is known to augment activated TCR signaling by positively regulating the activity of both Lck ($p56^{lck}$) and Fyn ($p59^{fyn}$) so that suppression of this signaling event hinders the T cell proliferation by depressing initial signaling events. Similarly, it was found that naïve T cells expressed more CD5, an established TCR signal transduction inhibitor, on the surface when in the presence of the $CD4^+CD25^+$ T cells thus raising the activation threshold and limiting their expansion.

Current evidence also includes a role for cytotoxic lymphocyte-associated antigen-4 (CTLA-4) as it is constitutively expressed on the $CD4^+CD25^+$ T cells (35) and that any T cell can express it upon activation. Studies in autoimmune disease and tumor immunity have shown that it's blockade results in hindered $CD4^+CD25^+$ T cell inhibition suggesting that CTLA-4 may be a costimulatory molecule for $CD4^+CD25^+$ T cell activation (32).

Guntermann and Alexander (36) detail on a molecular level the events in CTLA-4 concomitant binding with its B7-1 (CD80) or B7-2 (CD86) ligand and T cell activation. Upon CTLA-4 ligation and parallel CD3 stimulation in resting human $CD4^+$ T cells there was a decrease in downstream protein tyrosine phosphorylation of signaling effectors and marked inhibition of extracellular signal-regulated kinase 1/2 activation (Erk1/2). The TCR ζ-chain phosphorylation and subsequent ZAP70 recruitment was not affected, however, the association of $p56^{lck}$ with ZAP70 was inhibited correlating with reduced actions of $p56^{lck}$ in the ZAP70 immunocomplex by inhibition of phosphorylation at the Y319 positive regulatory site. It is this site on the ZAP70 complex where, upon phosphorylation, the SH2 (SLP-76) domain of $p56^{lck}$ amplifies and continues the downstream signal. As expected in an inhibitory signal, tyrosine phosphatase activity was associated with the CTLA-4 cytoplasmic tail. The major phosphatase activity was attributed to Src homology domain 2-bearing protein tyrosine phosphatase 1 (SHP-1).

Investigation continues about the role of T helper 1 (Th1) and T helper 2 (Th2) T cell subsets in autoimmune diabetes and most findings concur that a Th1 environment is pathogenic while a Th2 environment may be protective. Th1 and Th2 cells are most easily distinguished by the profile of cytokines that they secrete (18). Th1 cells secrete principally IFN-γ while Th2 cells secrete mainly IL-4 and IL-5. In light of these cytokines secreted by each of these subsets, it would seem logical that the Th1 cells are responsible for much of the destructive process through the pro-inflammatory cytokines that they release. Indeed, Th1 cells that secrete IFN-γ and lymphotoxin induce disease in both NOD and immunodificient NOD.*scid* mice (19) while, in contrast, Th2 cells that secrete IL-4, IL-5, IL-6, and IL-10 fail to transfer diabetes unless recipients are immunocompromised (19), in part due to IL-4 (20).

Among the first effector cells to home in on the pancreatic cells in NOD mice are macrophages and dendritic cells, followed later by B and T lymphocytes (18). These macrophages and dendritic cells are potent secretors of TNFα and IL-12, initiating a differentiation of uncommitted T cells toward a Th1 phenotype. This is characterized predominantly by IFN-γ and IL-2 secretion (18), which further contributes to activation of macrophages and proliferation of T cells in an autocrine fashion. In contrast, IL-10, produced in large amounts by Th2 cells, inhibits macrophage functions such as the secretion of IL-12 and antagonizes the up regulation of MHC cell surface molecules induced by IFNα (22).

With the use of Linomide, an immunosuppressive agent known to prevent autoimmune insulitis and DM in NOD mice (21), Weiss *et al.*(18) have shown that NOD mice receiving Linomide from the age of 5-6 weeks do not develop severe insulitis or show any clinical evidence of the disease. By characterizing the cytokine production profile of lymphocytes under Linomide administration, they showed that elevated IL-4, IL-6, and IL-10 levels with concomitant reduction in IL-1β and IFN-γ prevented the development of diabetes in NOD mice, further suggesting that alteration of T cell subsets, particularly Th1 to Th2, may play a role in immune regulation of autoimmunity in NOD mice.

In addition to the cytokine secretions in the pathogenesis of, or protection against autoimmunity unique to Th1 and Th2 respectively are the different properties between the

two T cell subsets with respect to their capacity to infiltrate the pancreatic β cells. The extravasation of lymphocytes from blood into tissue is regulated by multiple adhesion receptor/counter-receptor pairs and chemokines that mediate primary adhesion, activation-dependent adhesion, and finally transmigration (23).

Lymphocyte transmigration is regulated by β_1 or β_7 integrins followed by LFA-1, and the adhesiveness of these integrins is regulated by chemokines that are subdivided into four families, C, CC, CXC, and CX_3C (24,25). Just as Th1 and Th2 T cell subsets can be distinguished by the cytokine profile they secrete, their responsiveness to different chemokines further differentiates their pathogenic potential via differential expression of chemokine receptors CCR5 and CXCR3 vs. CCR3, CCR4, and CCR8, respectively (26).

Bradley *et al.*(23) demonstrated that both Th1 and Th2 cells transferred into NOD.*scid* recipients infiltrated the pancreas and expressed multiple adhesion receptors (peripheral lymph node addressin, mucosal addressin cell adhesion molecule-1, LFA-1, ICAM-1, and VCAM-1) on vascular endothelium, but that the infiltration of Th1 cells was more rapid than that of Th2 cells, and only Th1 cells induced diabetes. In addition, they detected the RNA responsible for the synthesis of the respective chemokines. More specifically, Th1 cells produced RNA for lymphotactin, MCP-1, MIP-1α, RANTES, and CRG-2/IP-10, and MIP-1β was produced by both CD4 subsets and was the only chemokine RNA detected in Th2 cells, but in less abundance. Their data also suggested that Th1 cells were able to infiltrate the pancreata at a quicker rate allowing them to outpace the Th2 cells and undergo a greater, earlier expansion.

With all data taken together it is suggested that Th1 cells have a better innate ability of homing to the pancreas and infiltrating the tissue. At that time MCP-1 is secreted and additional macrophages producing CRG-2/IP-10 are attracted in response to Th1-derived IFN-γ with additional Th1 cell recruitment via response to macrophage CRG-2/IP-10 secretion (23). Thus, there is a harmful communication between macrophages and Th1 cells via both chemokines and cytokines that overwhelm and eventually negate any protective effect of Th2 cells.

Inhibitory NK receptors, their structure, and the intracellular events associated with inhibitory function

Currently, numerous research studies are being applied to the physiologic mechanisms by which the immune system's natural killer cells maintain their self-tolerance in hopes to shed light on the failure of T cells to keep their self-tolerance. In the case of NK cells, tolerance to self is sustained by ligation of inhibitory cell-surface receptors for self-MHC class I molecules. These receptors include killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LIRs) in humans, the Ly49 molecules in mice, and CD94/NKG2A in both species (27). In contrast, one mechanism of NK activation termed "missing self recognition" is by recognizing target cells that lack class I MHC molecules, such as virus infected cells, that by inhibiting expression of class I molecules and evading lysis by $CD8^+$ T cells, become susceptible to lysis by NK cells.

Ly49 receptors are C-type lectin-like molecules (28) in which currently eleven have been described; nine are inhibitory in function (Ly49A, Ly49B, Ly49C, Ly49E, Ly49F, Ly49G, Ly49I, Ly49J, and Ly49Q) while only two are activating (Ly49D and Ly49H) (27). These activating and inhibitory isoforms are highly similar in respect to their extracytoplasmic domains with the ligand affinity for the inhibitory receptor reported as being stronger than the activating receptor (27). The nature of the intracytoplasmic domain of the receptor is the determining factor in whether it is inhibitory or activating. More specifically, the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) will make the receptor inhibitory while an immunoreceptor tyrosine-based motif (ITAM) will make the receptor activating.

The activating Ly-49 receptors are expressed only on NK cells while the inhibitory receptors are found on NK cells and a small subset of T cells. These T cells include natural killer T cells (NKT or $NK1.1^+$ cells) and $CD8^+\alpha\beta$-TCR^+ T cells bearing markers of the memory phenotype, $CD44^{hi}CD122^+Ly6C^+$ (27,28). In the murine genome, the family of Ly49 receptors are located on chromosome 6 and most of the genes are clustered in a region spanning ~600 kb. (29). Genes of the receptors are expressed in a variegated pattern such that each receptor is expressed by 10–50% of NK cells and the average NK cell expresses three to four different receptors (29)

Activation of the Ly49 family of receptors, whether it is inhibitory or activating, occurs upon ligation with specific MHC class I molecules. Moreover, each receptor has specificity for only certain class I MHC complexes; for example, Ly49A has a high affinity for H-2D^d and H-2D^k, but a low or negligible binding affinity for H-2D^b or H-2K^b (30). There exists two possible interaction sites in the case of H-2D^d molecule, one uses N-terminal residues of the α1 helix and C-terminal residues of the α2 helix while the second possible binding site is with the α2 and α3 domain together with β_2-microglobulin. Sundback, *et. al.*(31) found that the α2 domain alone is integral in the specificity and function of the H-2D^d molecule in YB2/0 cells. Later, Matsumoto *et. al.*(31) demonstrates specific determinants on the β_2-microglobulin chain necessary for ligation as well as three residues on α1, α2, and α3 that are also critical for H-2D^d recognition by Ly49A.

Less is known about the molecular mechanisms involved in Ly49A inhibition, both on NK cells and on transgenic T cell models. Current studies, however, all point to a role for SHP-1 recruitment during proximal dephosphorylation events in the presence of an ITIM. The cytoplasmic region of the Ly49A contains the amino acid sequence VxYxxV, which upon ligand recognition and subsequent phosphorylation of a key tyrosine (Tyr) residue, recruits SHP-1. SHP-1 is believed to dephosphorylate unknown critical substrates to shut down positive signaling (27,39).

Data from Nakamura *et. al.*(38) supporting the role of SHP-1 in Ly-49A inhibition show that SHP-1 deficient, viable motheaten and motheaten, mice exhibit markedly impaired and absent Ly49A inhibition, respectively. The fact that Ly49A retained partial activity in NK cells from viable motheaten mice suggests that there are other important cytoplasmic mediators capable of inhibition by Ly49A, such as SHP-2 (38,40). Early (30-60s) decreases in tyrosine phosphorylation and complete elimination of Ly49A inhibition via disruption of the ITIM phosphorylation site by substituting tyrosine for phenylalanine also strengthen the study's findings.

Adoptive transfer models and intracellular staining

As T cells are clearly implicated in autoimmune diabetes and β cell destruction, methods of curbing T cell activation, proliferation, and subsequent effector functions would likely

provide a beginning to controlling disease onset in genetically susceptible individuals. One current approach to achieving this is by transgenically expressing the NK inhibitory receptor, Ly49A, on $CD4^+$ T cells, which by nature, are highly mobile and interact extensively with antigen presenting cells (APCs). In doing so it is hypothesized that, upon arrival to the pancreas, the inhibitory T cells will compete with autoreactive T cells for ligation with APCs (expressing self antigens) as well as competing for space and nutrients while remaining unresponsive to other immune processes.

In previous adoptive transfers models (Mary Pauza and Sherry Smith, unpublished data), onset of hyperglycemia was significantly delayed in transgenic mice expressing the Ly49A inhibitory receptor. Briefly, when $Ly49A^+$ diabetogenic $CD4^+$ T cells were injected into recipients expressing the $H\text{-}2D^d$ MHC class I ligand (the high affinity ligand for Ly49A), hyperglycemia was delayed more than 75 days post-transfer while in control mice, hyperglycemia was rapid and occurred within ten days.

In in vitro studies of T cell activation and proliferation, as measured by IL-2 secretion (important interleukin in the autocrine proliferation and expansion of T cells), Ly49A was shown to suppress its secretion as compared to $H\text{-}2D^b$ APC controls. Moreover, T cell activation and up regulation of surface expression of CD25 (receptor for IL-2), CD40L, and CD69 (expressed on activates T cells) as well as activation-induced down regulation of CD62L, were all attenuated upon Ly49A ligation with the $H\text{-}2D^d$ ligand. These results further implicate the inhibitory function of Ly49A in vitro while in the presence of $H\text{-}2D^d$, but not $H\text{-}2D^b$ (Pauza and Smith, unpublished data).

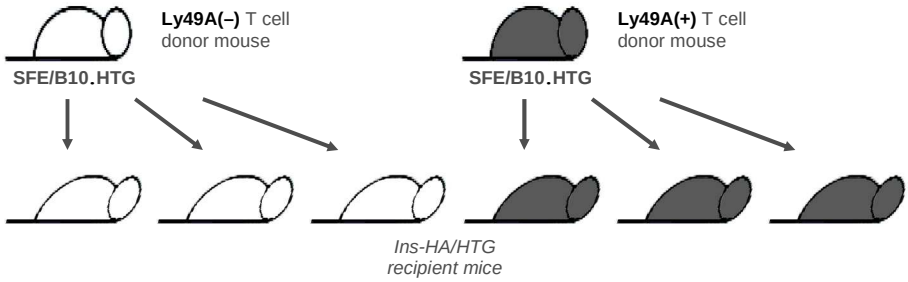

Figure 1. Diagram depicting adoptive transfer model. $CD4^+$ T cells from SFE/B10.HTG or Ly49A/SFE/B10.HTG donor mice were injected intravenously (i.v.) into three Ins-HA/B10.HTG recipient mice. All six recipient mice were approximately 3 months old at time of injection and were sublethally irradiated with 700 rads hours before the injection. Recipients of the B10.HTG lineage do not express the high affinity ligand for Ly49A.

In the current experiment, an in vivo, adoptive transfer model was needed as a negative control for the previously successful models mentioned above that conferred protection from hyperglycemia via Ly49A. In other words, it was set out to prove that the simple expression of Ly49A by autoreactive T cells does not confer protection against T cell effector function, and instead, it requires active binding with the MHC class I ligand H-$2D^d$. To do this requires a system with appropriate antigen presentation, Ly49A ligation (or in this instance, lack thereof), and diabetogenic T cell populations. More specifically, T cells were isolated from $Ly49A^+$ and $Ly49A^-$ donor mice (Ly49A/SFE/B10.HTG double-transgenic and SFE/B10.HTG single-transgenic mice, respectively) and injected intravenously into six sublethally irradiated (700 rads) recipient mice of the Ins-HA/B10.HTG lineage (Figure 1). The B10.HTG mouse strain expresses MHC class I H-$2D^b$ as compared to B10.D2 mice (not used in these models) that express H-$2D^d$, the ligand for Ly49A (11). B10.HTG and B10.D2 mice are congenic strains; they contain identical genomes except at the MHC class I locus. SFE mice express a T cell receptor (TCR) transgene. This SFE TCR recognizes a peptide from the influenza hemagglutinin (HA) protein (amino acids 110–119; SFERFEIFPK) and is restricted to I-E^d (MHC class II), as described previously (11,41). As a result of the MHC class II restriction of the TCR, peripheral T cells in these mice are nearly all $CD4^+$. Ins-HA mice are also transgenic, Islet beta cells in these mice express HA since transgene expression is controlled by an insulin promoter. When SFE specific T cells are transferred into Ins-HA recipients, T cells home to the pancreas and destroy HA-expressing beta cells resulting in hyperglycemia (41). Finally, transgenic mice that are $Ly49A^+$ have the NK cell inhibitory receptor expressed on their $CD4^+$ T cell population.

Other experiments involved flow cytometry and intracellular staining for the extracellular receptor-activated kinase (Erk), which is part of the mitogen-activated protein (MAP) kinase cascade of phosphorylation events occurring after TCR activation. Briefly, this requires adequate TCR stimulation and thus, T cell activation, fixing the cell, or capturing the intracellular phosphorylation state, permeabilizing the cell (facilitating entry of the Erk-specific stain), and finally staining the cell. Many processes are involved in the T cell response to antigen at the TCR. Briefly, upon antigen recognition there is clustering of membrane bound complexes and coreceptors and CD4-associated Lck ($p56^{lck}$) becomes activated (likely by dephosphorylation via CD45). Activated Lck phosphorylates tyrosines in the ITAMs of nearby ζ chains, which is closely associated with the TCR and provides downstream signals following ligation of the TCR. Subsequently, syk family tyrosine kinases, such as ZAP70, are activated by phosphorylation. Activated ZAP70 then phosphorylates adapter proteins such as LAT, SLP-76, and Grb-2. Phosphorylated adapter molecules recruit other signaling molecules such as the GTP/GDP exchange factor Sos that in turn activates the small G protein Ras. Activation of the Ras signaling pathway leads to initiation of the MAP kinase cascade, Erk and Elk activation, and ultimately upregulation of activation protein-1 (AP-1) (46).

Discovering a difference between the levels of activated Erk between Ly49A in B10.HTG ($H\text{-}2D^b$) and B10.D2 ($H\text{-}2D^d$) will be a good starting point in understanding mechanisms involved in the inhibitory process of Ly49A over the TCR signal. As it may be considered a temporally intermediate protein kinase, if indeed it is found to be inactive upon TCR stimulation in the presence of Ly49A, a search further upstream (e.g. Lck, Fyn, ZAP70) could be the next step in elucidating where the Ly49A receptor carries out its effect over the TCR signal.

Mouse	No. of mice used
Ly49A/SFE/B10.HTG	3
SFE/B10.HTG	11
Ins.HA/B10.HTG	18
B10.HTG	1
B10.D2	1

Table 1. Tabulation of mice by strain used in all protocols.

Materials and methods

Mice (Table 1)

Ins-HA transgenic mice expressing hemagglutinin on islet β cells under the control of the rat insulin promoter have been previously described (41,42). The development of the double transgenic Ly49A mice is as follows. Ly49A-transgenic mice were backcrossed to B10.D2 mice until homozygosity was achieved at the MHC as determined by immunophenotyping using flow cytometry. Ly49A/B10.D2 mice were then crossed with TCR-SFE/B10.D2 (43) mice to make double-transgenic Ly49A/SFE/B10.D2 mice (11). These double-transgenic B10.D2 mice were also bred with B10.HTG mice to create Ly49A/SFE/B10.HTG double-transgenic mice as confirmed by flow cytometry using D^d and D^b specific antibodies (11). B10.HTG and B10.D2 mice are maintained on an ongoing basis in the Southern Illinois University animal housing facility and verified using an appropriate genotyping protocol. Finally, the mice used for intracellular staining were single-transgenic mice expressing the TCR-SFE specific for the HA peptide 110–119 (SFERFEIFPK) presented by I-E^d which has been previously characterized (11,44). These were maintained on a B10.D2 and B10.HTG background. All mice were housed in specific pathogen-free barrier at the Southern Illinois University School of Medicine Laboratory Animal Facility.

Adoptive transfer model

Three adoptive transfer models were performed according to Protocol 1 (Appendix A). In each case, spleens and lymph nodes were harvested from one Ly49A positive and negative TCR-SFE/HTG donor of >6 weeks of age. These tissues were ground-up between the

rough edges of frosted glass microscope slides and cultured in RPMI 1640 media (Gibco) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L glutamine, 25 mmol/L HEPES, and 5 X 10^{-5} mmol/L β mercaptoethanol (complete medium). Red blood cells were lysed with hypotonic 1.44% NH_4Cl and total cells were counted using a Neubauer slide. The two cell solutions were then adjusted such that 2.0 X 10^7 cells would be transferred to each recipient mouse. In the second trial of this adoptive transfer, after making the two cell solutions at equal concentrations, an aliquot of cells were incubated at 4˚C for 30 minutes in the presence of anti-CD4 mAbs. After washing cells once, flow cytometry was performed. Based on the cytometry data (Figure 6), the two cell solutions were than adjusted so that each recipient group received 3.9 X 10^6 $CD4^+$ T cells from the corresponding donor mouse in equal volume injections. A third adoptive transfer model involved injection of 2.0 X 10^6 $CD4^+$ T cells. The six Ins-HA/B10.HTG recipient mice in each of the three trials (all ~3 months of age) were sublethally irradiated with 700 rads the morning of the adoptive transfer. In each trial, 0.3 ml of the appropriate cell solution was injected retroorbitally into each recipient mouse. The mice were returned to the animal housing facility within the pathogen free barrier.

Diabetes monitoring and statistical analysis

Blood glucose levels were measured using OneTouch Ultra test strips with a OneTouch Ultra (LifeScan) blood glucose monitor and measurements were made approximately every four days with tail blood. These particular strains of mice under normal conditions have a serum glucose ranging from 88.5 to 154.7 mg/dL irregardless of sex. A mouse was considered diabetic if the blood glucose level was ever ≥300 mg/dL or ≥250 in two consecutive readings, at which time the mouse was euthanized. These high serum glucose levels are considered adequate and allow for serum glucose variabilities as a result of unpredictable feeding times and sleep/wake cycles. Diabetes onset was graphed using Kaplan-Meier nonparametric survival curves, and statistical significance was determined by the Mantel-Cox log-rank test with StatView software version 5.0.1.

T cell purification

For the first intracellular staining experiment (Protocol 2, Appendix A) $CD4^+$ T cells were purified by negative selection using anti-rat IgG-conjugated magnetic beads (Qiagen). Cell solution was first incubated for 30 minutes at 4˚C with appropriate dilutions of anti-B220, anti-CD8, F4/80, and M5/114 to bind with B cells, $CD8^+$ T cells, monocytes, and macrophages, respectively. Magnetic beads were washed three times with PBS/2% Horse Serum and were used at a volume of 1 ml of bead suspension for each 10^7 cells. Cell suspensions were washed to remove excess antibody, then mixed with the washed magnetic beads, and incubated for 30 minutes at 4˚C. Cells were separated using a standard magnet, after which, the cells were allowed to rest at 37˚C, 5% CO_2 in RPMI complete medium without FCS for 1-2 hours.

T cell activation and intracellular staining

T cells were harvested from spleen and/or lymph nodes of TCR-SFE/B10.HTG mice. Single cell suspensions were prepared as described previously in *Adoptive transfer model*. In some intracellular staining experiments, elicited peritoneal macrophages were used as a source of APC. APCs were obtained from one B10.HTG and one B10.D2 four days after intraperitoneal injection with 3% thioglycolate. Non-enzymatic cell dissociation solution was injected into peritoneal cavity and lavage fluid was removed by fine needle aspiration after one minute of gentle message. MHC molecules on the APCs were loaded with SFE peptide by incubation with stock SFE peptide (influenza hemagglutinin peptide 110–119; SFERFEIFPK) at 10 μg/ml for 30 minutes at 37°C in 5% CO_2.

In other protocols, stimulation of the T cell population was accomplished using 1 μg/ml phorbol myristate acetate (PMA) or 10 μg/ml anti-CD3 (Pharmingen) plus 20 μg/ml anti-CD28 (Pharmingen). Stimulation with PMA was used as a positive control for these studies. In some experiments, 2 μg/ml anti-Armenian hamster IgG (Jackson Immunoresearch) was used to crosslink bound anti-CD3. In one experiment, T cell stimulation was via anti-CD3 bound to a 96-well plate prepared by incubating the plate with 10 μg/ml anti-CD3 in PBS for one hour at 37˚C. In all cases cells were stimulated for various time points: generally 0, 5, 15, 30, and 60 minutes.

To identify $CD4^+$ T cells, cell suspensions were stained with 0.1 μg/ml anti-CD4 conjugated to R-phycoerythrin (PE; Pharmingen) for 30 minutes at 4˚C. Intracellular staining was made possible by fixation followed by permeabilization of cells. After fixing the cell with BD PhosFlow Fix Buffer I solution (BD Biosciences) for ten minutes at 37˚C, cells were permeabilized using BD PhosFlow Perm/Wash Buffer I (BD Biosciences). Cells were washed twice in Perm/Wash, and then incubated for one hour in the dark at room temperature with intracellular staining antibodies. To detect the level of Erk1,2 activation, phosphorylation-specific anti-p44/p42 Alexa Fluor 488 Conjugate (Cell Signaling Technology) were used at 2.22 μg/ml. Then, cells were washed once with Perm/Wash and resuspended in PermWash for immediate cytometric analysis.

Flow cytometry

All flow cytometric analyses were performed using a FACS calibur flow cytometer with CellQuest software (BD Biosciences).

Results

Diabetes onset in ligand (–) recipients of donor $Ly49A^{+/-}$ T cells was not always identical

An adoptive transfer model was used where T cells were donated from single and double-transgenic mice to six Ins-HA/B10.HTG recipients (Figure 1). The T cells were harvested from spleen and lymph nodes and cell solution prepared as described previously (Appendix A, Protocol 1). Although each donor was found to have different total quantities of cells as indicated by the unequal cell count, the solutions were adjusted so that during each intravenous injection of 0.3 ml, each mouse received equal cell quantities (3.9×10^6).

Because the recipient mice where all of the B10.HTG background, $H\text{-}2D^b$ not $H\text{-}2D^d$, was expressed on their MHC Class I complex. Regardless of whether the recipients received T cells exhibiting the Ly49A inhibitory receptor or not, the necessary ligand for the receptor, $H\text{-}2D^d$ was not expressed by the APCs of the recipient mouse. Therefore, the onset of hyperglycemia was not expected to be statistically different between the two recipient

groups of mice. In other words, in this model the presence of Ly49A should not offer any protective benefit to the mouse that receives it.

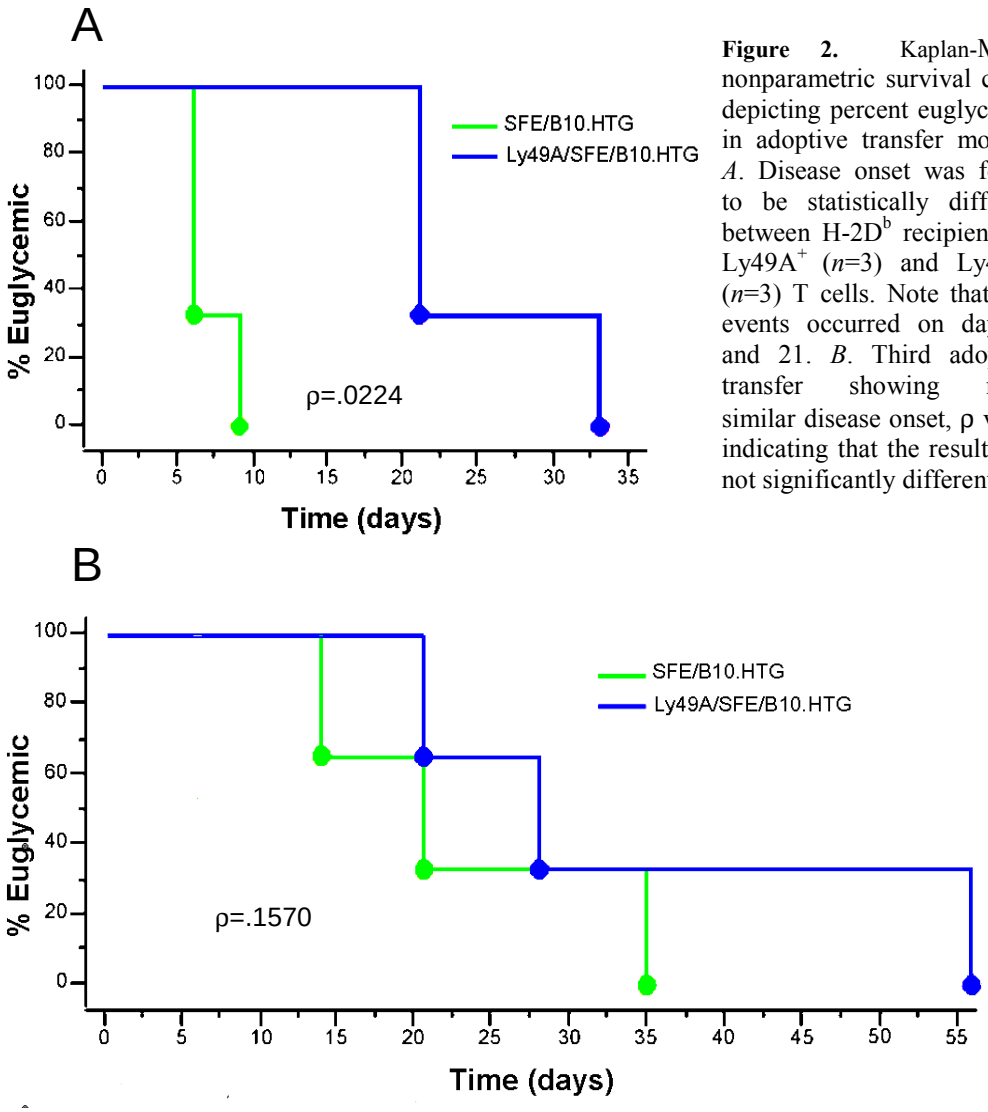

Figure 2. Kaplan-Meier nonparametric survival curve depicting percent euglycemia in adoptive transfer models. *A*. Disease onset was found to be statistically different between H-2D^{b} recipients of Ly49A^{+} (*n*=3) and Ly49A^{-} (*n*=3) T cells. Note that two events occurred on days 6 and 21. *B*. Third adoptive transfer showing more similar disease onset, ρ value indicating that the results are not significantly different.

However, as the results indicate (Figure 2A), a difference was observed (ρ=.0224, CI 95%) in the survival curves between the two recipient groups. The double-transgenic T cells (Ly49A^{+}) did confer a delayed onset of hyperglycemia (mean 7 days, SD 1.7) as compared to the recipient mice receiving the Ly49A^{-} T cells (mean 25 days, SD 7) (Table 2). As expected, all mice (n=6) did eventually become hyperglycemic (100% within 33 days, mean 16 days, SD 11).

Because of the unexpected results, the adoptive transfer was performed again using a modified version of the same model, this time being more careful to inject equal quantities of CD4^{+} T cells by determining the percentage of CD4^{+} T cells from each donor. As with

the first experiment the results showed that the disease onset was again dissimilar (Table 2) and the ρ value was also significant (ρ=.0499, data not shown) indicating that Ly49A$^{+/-}$ T cells induced different progression of disease.

This second adoptive transfer model was run again without modifications of the protocol and after again counting CD4^{+} T cells using flow cytometry, the cell suspensions were adjusted so that each recipient mouse received equal cell injections. Results are presented also in Table 2. While the experiment was not totally free of problems, a better outcome was achieved. The survival curves for this trial shows more similar diabetes onset and the ρ value also supports the results (Figure 2).

A

Ins-HA/B10.HTG recipient mice Trial 1

			Blood glucose measurements						
Mouse	Date of birth	T. cell injection on 5/19	6d	9d	14d	21d	23d	28d	33d
1587	02/08/2004	Ly49A/SFE/HTG (1605, DOB 1/13/04)	147		112	244	252	278	383
1588	"	"	86		200	503			
1589	"	"	100		173	522			
1590	"	SFE/HTG	162	355					
1592	"	"	529						
1594	"	"	452						

B

Ins-HA/B10.HTG recipient mice Trial 2

			Blood glucose measurements					
Mouse	Date of birth	T-cell injection on 6/14	7d	8d	9d	11d	14d	18d
1867	04/30/2004	Ly49A/SFE/HTG (1668, DOB 3/8/04)	73	28	died			
1868	"	"	126			160	192	164
1871	"	"	152			176	170	160
1872	"	SFE/HTG (1720, DOB 3/22/04)	165			376		
1873	"	"	172			193	310	
1875	"	"	336					

C

Ins-HA/B10.HTG recipient mice Trial 3

		Blood glucose measurements							
Mouse	T-cell injection	7d	14d	21d	28d	35d	42d	49d	56d
1997	Ly49A/SFE/HTG	89	128	283	267				
2003	"	126	141	380					
2004	"	143	254	247	229	236	226	293	223
2000	SFE/HTG	111	died						
2001	"	161	125	502					
2002	"	246	204	222	268	275			

Table 2. Blood glucose readings for adoptive transfer experiments. Values are in mg/dl. taken with OneTouch Ultra (LifeScan) monitor and OneTouch Ultra test strips. Blood glucose was taken approximately every 4-7 days post-injection and levels ≥300 mg/dl in one reading or ≥250 in two consecutive readings were considered diabetic. *A*. First adoptive transfer experiment. *B*. Second adoptive transfer experiment where T cells were injected after quantifying T cells using flow cytometry. Mice 1868 and 1871 were euthanized after never becoming diabetagenic. *C*. Repeat of transfer done in *B*. DOB, date of birth.

Intracellular staining of p44/42 MAP kinase (Erk1,2)

As previously described, several protocols were executed that involved various methods of T cell stimulation, all with the goal of quantifying the amount of Erk1,2 present intracellularly in the phosphorylated, or activated state. These were independent models from the adoptive transfer experiments with a goal of constructing a repeatable protocol in which T cells are successfully activated and phosphorylated Erk would be readily detected by flow cytometry. By doing so, the intracellular events that occur as Ly49A exerts its effect on suppressing autoreactive T cell effector function could be better defined. In the initial protocol the goal was to simply measure Erk1,2 phosphorylation. In an effort to closely mimic in vivo T cell activation, the in vitro assays used APC plus specific peptide to stimulate T cells. T cells were harvested from a TCR-SFE/HTG mouse and incubated with APCs from B10.HTG or B10.D2 mice in the presence of SFE peptide. Because T cells originate from a $Ly49A^{-}$ mouse, detectable difference in Erk phosphorylation when using different APCs was not anticipated.

The cells were counted and both T cells and APC donor cell solutions were adjusted to the same concentration of 5.0×10^6 cells/ml. This way, by adding equal volumes, there should have been approximately one APC for every $CD4^+$ T cell. SFE peptide was then added to the appropriate APC tubes as indicated in the protocol (Appendix A, Protocol 2) and the tubes were then incubated for 30 minutes at 37°C with 5% CO_2. This APC-SFE peptide solution was added to appropriate tubes containing T cells and immediately centrifuged at 3000 rpm for 30 seconds and incubated for 0, 5, 15, 30, and 60 minutes at 37˚C before adding the fix buffer. Staining of $CD4^+$ T cells and Erk was carried out as described in *materials and methods.*

The cytometric results gated for $CD4^+$ T cells showed that at best, 15.4% T cells with phosphorylated Erk (data not shown) was observed. At the same time, the positive control (T cells stimulated by PMA) exhibited Erk staining of 82%. Since control staining worked very well but little or no staining was observed with other T cell preparations, it is likely that T cell stimulation with APC plus specific peptide under the current protocol conditions does not generate sufficient ERK phosphorylation to be detectable in the flow cytometry assay.

Optimization of anti-p44/42 Alexa

Having identified a strong positive control for Erk activation and staining, staining with anti-Erk antibodies was optimized prior to proceeding with additional experiments. PMA, a known potent and direct activator of PKC, was used. Splenocytes were stimulated with PMA for 5 minutes at 37˚C. Staining of Erk was done with mAb dilutions of 1:50, 1:100, 1:200, 1:500, and 1:1000 for 60 minutes at room temperature, in the dark. Cells were also stained with anti-CD4 (Appendix A, Protocol 3).

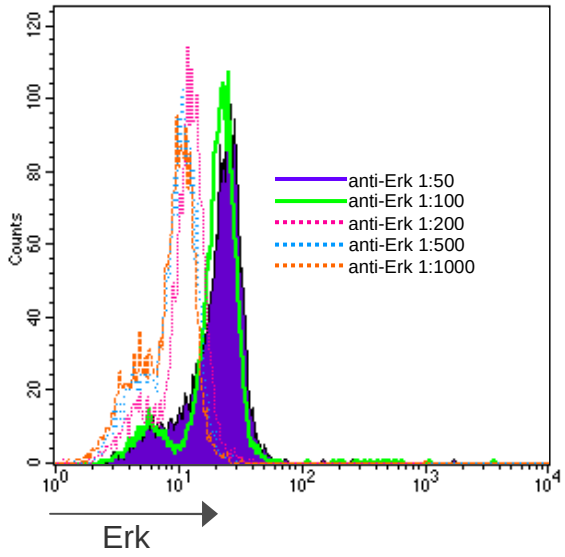

Figure 3. Overlay of histogram plots gated on $CD4^+$ T cells stained with anti-p44/42 Alexa Fluor 488. Staining is clearly most effective at 1:50 and 1:100 dilutions of the stock .222 mg/ml solution.

The cytometric results clearly show the effects of diluting the anti-p44/42 (Figure 3). Dilutions 1:50 and 1:100 show very similar results suggesting that using a concentration greater than 1:50 would not likely result in greater Erk staining and that using a lesser dilution than 1:100 is not necessary. There is an obvious, gradual decline in the percent Erk gated as the anti-p44/22 was diluted 1:50, 1:100, 1:200, 1:500, and 1:1000 (94.9, 94.3, 86.9, 79.4, and 73.0, respectively). Therefore, diluting anti-p44/42 more than the current 1:100 would not be useful.

Stimulation with plate-bound anti-CD3

Having established the ideal dilution of the anti-p44/42 mAb, focus was put on how to optimally stimulate the T cells. The first trial was utilized plate-bound anti-CD3 to stimulate the cells (Appendix A, Protocol 4). After preparing the 96-well plate as described

in *materials and methods*, the T cells were added to the wells and the plate was centrifuged at 1000 rpm for three minutes. The 96-well plate containing the bound anti-CD3 and cell solution was incubated at 37˚C. The contents of the wells where removed at the appropriate time points and cells were then fixed as described previously.

The best staining occurred with 30 minutes stimulation, but only 7.6% gated $CD4^+$ T cells exhibited Erk phosphorylation. Moreover, when compared to T cells that were added to wells not containing activating bound anti-CD3, there was little difference in detected Erk, suggesting low levels of Erk and very poor T cell activation.

Stimulation using anti-CD3 in solution

In an effort to maximize the number of T cells stimulated, anti-CD3 in soluble form was used. In addition to the same stimulation time points, two concentrations of anti-CD3: 1 µg/ml, and 10 µg/ml (Appendix A, Protocol 5) were used. Cell solution was incubated with anti-CD3 at 37˚C for the appropriate time points, and fixation was done as described in the previous protocols. As expected, staining was better when more anti-CD3 was used. Moreover, anti-CD3 stimulation for 60 minutes gave the best results, suggesting better activation at this time point. At 60 minutes and 10 µg/ml anti-CD3, gated $CD4^+$ T cells exhibited 33.2% Erk phosphorylation.

Stimulation using anti-CD3 (10 µg/ml) and anti-CD28 (20 µg/ml) with anti-Armenian hamster IgG crossbridging

Despite an improved ability to activate T cells as evidence by greater detection of phosphorylated Erk, the percentage of activated cells still remained relatively low. In an effort to further optimize T cell activation with anti-CD3 costimulation with anti-CD28 in addition to a crosslinking step was added to the protocol. The benefit of crosslinking anti-CD3 antibodies was suggested by Carey *et. al.*(45). The T cell solution was first incubated with anti-CD3 (10 µg/ml) and anti-CD28 (20 µg/ml) for 30 minutes at 4˚C (Appendix A, Protocol 6). The anti-hamster IgG was then added and cells were incubated for an additional 30 minutes at the same temperature allowing the anti-hamster antibody to

crosslink bound anti-CD3. Cells were then incubated at 37˚C for 0, 5, 15, 30, and 60 minutes before fixation.

The observed results suggested that the addition of anti-hamster IgG did not improve signal strength and Erk phosphorylation. When compared with parallel controls with equal stimulation but without the crosslinking, there were poorer results early on, but by 60 minutes there was actually more Erk phosphorylation detected in the group without the IgG (data not shown) crosslinking. Moreover, compared to the experiment before that used only anti-CD3 as the stimulation, (33.2% at 60 minutes), the addition of anti-CD28 costimulation in this protocol did exhibit more gated $CD4^+$-Erk staining (37.2% at 60 minutes) and was a more important factor contributing to TCR stimulation than was the anti-hamster IgG.

One explanation for the observed results may be because the cells spent 60 minutes in the presence of anti-CD3/anti-CD28 (30 minutes each before and after addition of the IgG). It is possible that the desired effect of the IgG may have already occurred shortly after its addition while incubating at 4˚C. Although incubation on ice should minimize intracellular signaling, this cannot be eliminated as a possibility.

Stimulation using anti-CD3/antiCD28 with anti-Armenian hamster IgG at 37˚C

Because it was suspected that holding the T cells in anti-CD3/anti-CD28 solution for 60 minutes with anti-hamster IgG at 4˚C was too long and that important intracellular events were likely occurring sooner than expected, the cell solution was removed from ice immediately after the initial 30 minutes with anti-CD3/anti-CD28 before adding the anti-hamster IgG. The cell solutions were then incubated at 37˚C in the presence of anti-hamster IgG for time points 0, 5, 15, 30, and 60 minutes and cells were fixed at appropriate times (Appendix A, Protocol 7).

Similar results were obtained in this experiment even when the cells in the presence of anti-CD3/anti-CD28 were removed from ice after 30 minutes before adding the anti-hamster IgG. The greatest Erk-phosphorylation occurred at 60 minutes of IgG crosslinking at 37˚C. In fact, the percent gated $CD4^+$ T cells showing Erk-phosphorylation was identical

to the previous protocol where anti-hamster IgG was added and cells remained on ice for the additional 30 minutes (Figure 4). The changes made in this trial had no beneficial effect on T cell activation and Erk staining. It remains possible that the real problem may be related to the 30-minute initial activation period with the anti-CD3/anti-CD28. Again, while the tubes are on ice, no crosslinking will occur making intracellular phosphorylation unlikely, however in the following protocol this time was reduced in half.

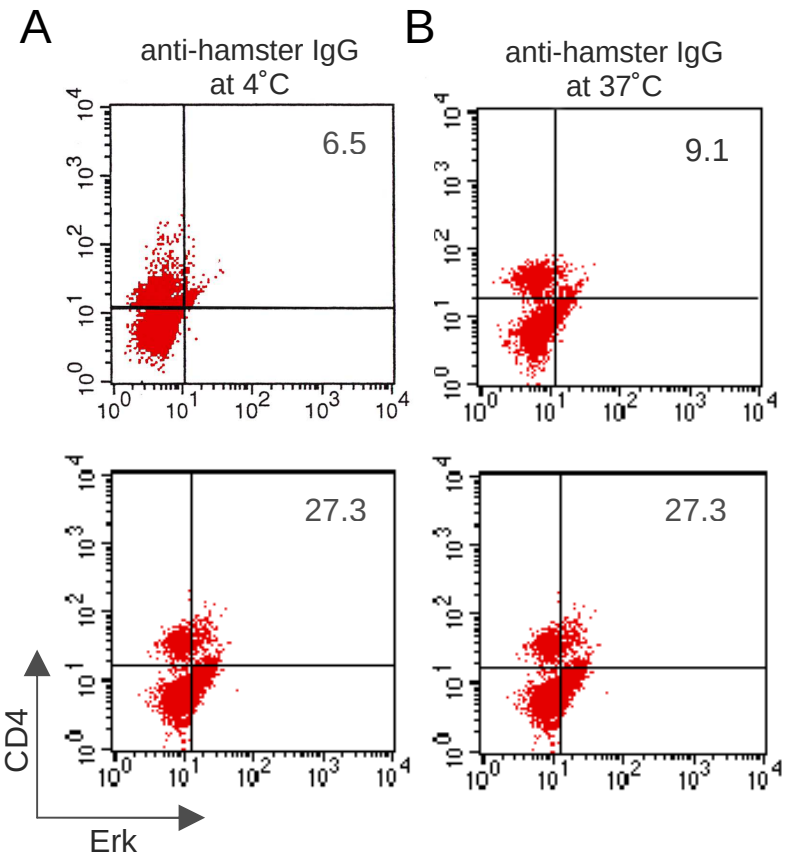

Figure 4. Dot plots showing $CD4^+$ T cells and phosphorylated Erk for two experiments of stimulation with anti-CD3/anti-CD28. When compared to plots that had no stimulation (*top*), a slight right shift of the $CD4^+$ population is noted in the stimulated cell plots (*bottom*). The change in protocol did not enhance results. Percentages indicated correspond to the quadrants containing $CD4^+$ T cells expressing Erk phosphorylation. *A*, Stimulation for 30 minutes on ice and 30 minute crossbridging with anti-hamster IgG on ice (Protocol 6). *B*, Stimulation for 30 minutes on ice and IgG crossbridging for 60 minutes and at 37˚C (Protocol 7).

Stimulation using anti-CD3/anti-CD28 for fifteen minutes at 4˚C

Focus remained on the events that were taking place while the T cell solution incubated with the anti-CD3/anti-CD28 at 4˚C. While it is desirable to have as much ligation as possible of anti-CD3/anti-CD28 mAbs to their respective TCRs, and it is likely that the amount of mAb ligation is directly proportional to incubation time, waiting for longer times may risk capturing the desired intracellular events, as they may have already come and gone.

For this reason the initial T cell stimulation at 4˚C with anti-CD3/anti-CD28 was reduced from 30 minutes to only 15 minutes (Appendix A, Protocol 8). A wash step using cold PBS was also added. By washing the T cells after the 15-minute stimulation with anti-CD3/anti-CD28, any unbound, free anti-CD3/anti-CD28 mAbs that may be usurping the anti-hamster IgG were removed. In the previous protocols 1µl of the IgG was added straight to the T cell solution while still containing excess anti-CD3 and anti-CD28. It was likely that free mAbs in solution would bind to anti-hamster antibody antagonizing its ability to effectively cross link surface bound anti-CD3. Therefore, the tubes were washed with 500 µl cold RPMI/FCS before the anti-hamster IgG was added. The cell solution was kept at approximately 4˚C during centrifugation.

A very small increase in gated $CD4^+$ T cells exhibiting Erk phosphorylation was observed, but the results remained inferior to those obtained previously where T cell stimulation with anti-CD3/anti-CD28 was for 30 minutes. The best Erk staining was at 60 minutes, however only 7.35% gated $CD4^+$ cells exhibited Erk phosphorylation. This was much lower than was observed while using either of the protocols that called for 30 minute stimulation on ice (27.3%, Figure 4) where T cells were stimulated for 30 minutes instead of 15 minutes as was done in this trial. The problem likely remains to be insufficient T cell stimulation and activation. Exceptional staining with the anti-p44/42 Alexa with PMA stimulation (92.2%, Figure 5) continued to be observed, indicating that the fixation and permeabilization procedures were likely adequate.

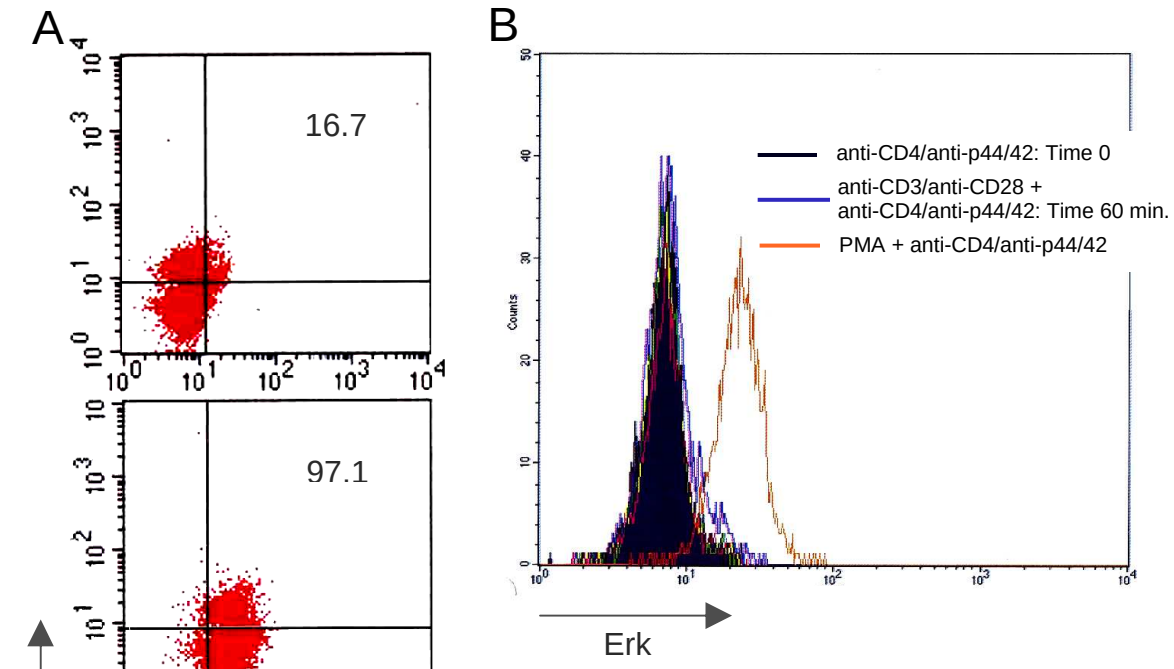

Figure 5. Cytometric analysis depicting exceptional T cell activation by PMA and staining by anti-p44/42 Alexa Fluor. *A*, Dot plots of staining with anti-CD4/anti-p44/42 with no TCR stimulation (*Top*), and with PMA stimulation (*Bottom*). A very clear right shift is noted in the $CD4^+$ population upon stimulation with PMA. Percentages are $CD4^+$ T cells exhibiting Erk phosphorylation. *B*, Overlay of histograms showing much better Erk phosphorylation when T cells are stimulated with PMA, rather than anti-CD3/anti-CD28 in which even at 60 minutes there was very little right shift.

This trial did add one important caveat, when the T cells were washed with cold PBS all unbound anti-CD3/anti-CD28 was removed, which was advantageous, however at the same time the exposure time of the T cells to the mAbs while was reduced assuming that 15 minutes was ample time for all mAbs to bind to all TCRs. It would therefore be sensible to revisit the previous protocol where anti-CD3/anti-CD28 stimulation occurred for 30 minutes on ice, and then adding the PBS wash step at that time.

Discussion

We constructed an adoptive transfer model of autoimmune diabetes to provide an in vivo model to observe the onset of diabetes in mice that receive T cells expressing the transgenic Ly49A NK inhibitory receptor. The model produces rapid disease onset; all mice are typically hyperglycemic within 4 weeks of transfer.

Upon binding of the Ly49A TCR with its specific ligand present on the MHC class I molecule of B10.D2 (H-2D^d, ligand +), onset of diabetes has been delayed more than 75 days in previous adoptive transfer models, and decreased IL-2 secretion and alteration of normal T cell surface expression upon antigen stimulation suggests the inhibitory function of the Ly49A TCR (Pauza and Smith, unpublished data). Here, an in vivo model was used of B10.HTG mice that express H-2D^b (ligand –) with donated T cells from both Ly49A positive and Ly49A negative mice. In both cases, normal T cell function is expected to result in pancreatic β cell destruction and hyperglycemia. Previously, in vitro studies using the same mice showed normal upregulation of CD25, CD40L, and CD69, downregulation of CD62L, and secretion of IL-2, indicating typical T cell activation in the ligand negative group.

Diabetes onset was expected to be equal in both T cell recipient groups. The presence of Ly49A TCR on the T cells donated to one group of Ins.HA/B10.HTG mice should not confer delayed hyperglycemia because all recipient mice are B10.HTG (Ly49A ligand –) mice. However, as revealed by statistical analysis, the Kaplan-Meier survival curves indicated significantly different onset of diabetes in the first two trials, ρ=.0224 and ρ=.0499, respectively (95% CI).

The most obvious explanation was that the SFE/HTG recipients received more CD4$^+$ T cells. A solution was prepared using total cells (without RBCs) from spleens and lymph nodes from two different mice, making it entirely possible that the percentage of CD4$^+$ T cells found in the harvested tissue from one mouse is not equal to that of the other. That was the reason for running the same adoptive transfer protocol again, but instead, equalizing the number of CD4$^+$ T cells for each sample.

This was accomplished by having the donor cells stained with anti-CD4PE to determine the percentage of CD4 cells in each cell suspension using flow cytometry. Indeed, the Ly49A$^–$ donor mouse had 28% CD4$^+$ T cells compared to 22% in the Ly49A$^+$ donor (Figure 6). Each cell solution was adjusted so that each mouse received 3.9 X 10^6 CD4$^+$ T cells per 0.3 ml injection.

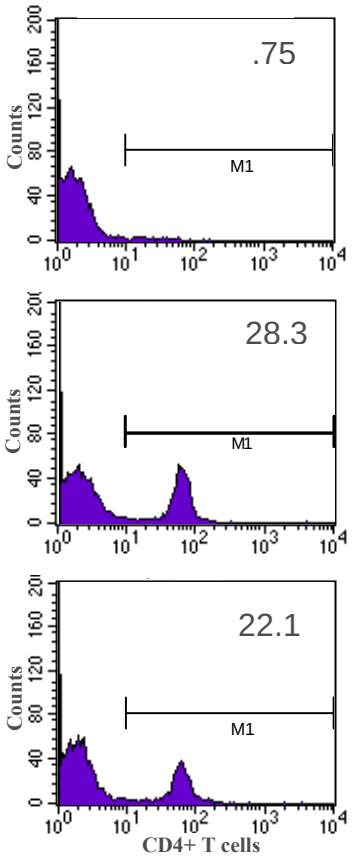

Figure 6. Histograms showing gated $CD4^+$ T cells in negative control (top), SFE/B10.HTG (middle), Ly49A/SFE/HTG (bottom). Numbers in upper right corners are percentages of cells in the M1 gate. This analysis was used during the second adoptive transfer to assure transfer of equal numbers of $CD4^+$ T cells.

As with the first experiment the results showed that the disease onset was again dissimilar (Table 2) and the ρ value was also significant (ρ=.0499, data not shown) indicating that $Ly49A^{+/-}$ T cells induced different progression of disease. During the follow-up of this group, one mouse of the $Ly49A^-$ group died very soon after the first week accompanied by very low glycemia levels while one mouse of the $Ly49A^+$ group was hyperglycemic at 7 days. In addition, the two remaining mice of the $Ly49A^+$ group never became diabetic. As the results of this second adoptive transfer model were likely due to technical difficulties, it was decided to rerun the same model without modifications.

Alternatively, it may be a possibility that the homing properties of SFE/B10.HTG T cells are more efficient than for cells that express Ly49A. Histological sections from the pancreata of the mice may be helpful in this instance, where differences in the degree of insulitis would suggest better infiltration properties of SFE/B10.HTG T cells. The mere presence of the Ly49A TCR should not have any effect over the aggressiveness of the T cell effector function because in this model it is never activated so signals never are initiated. In other words, we have no compelling reason to consider both T cell populations as unequal in their reactivity and response to stimulation, once present in the pancreas. As mentioned previously, many factors are involved in the homing and transmigration of T cells to antigenic tissue. For this reason it would be helpful in viewing stained pancreatic tissue as a means of trying to evaluate the efficiency of T cell homing and infiltration between the two T cell population. If T cell reactivity is indeed equal, the limiting factor in disease onset and severity may very well be the homing properties. To be consistent with this hypothesis, staining of the pancreata of the Ins.HA/B10.HTG mice that received the

SFE/B10.HTG T cells would reveal vast T cell infiltration while the pancreata of the Ly49A$^+$ recipients to a much lesser extent for any given time post-injection.

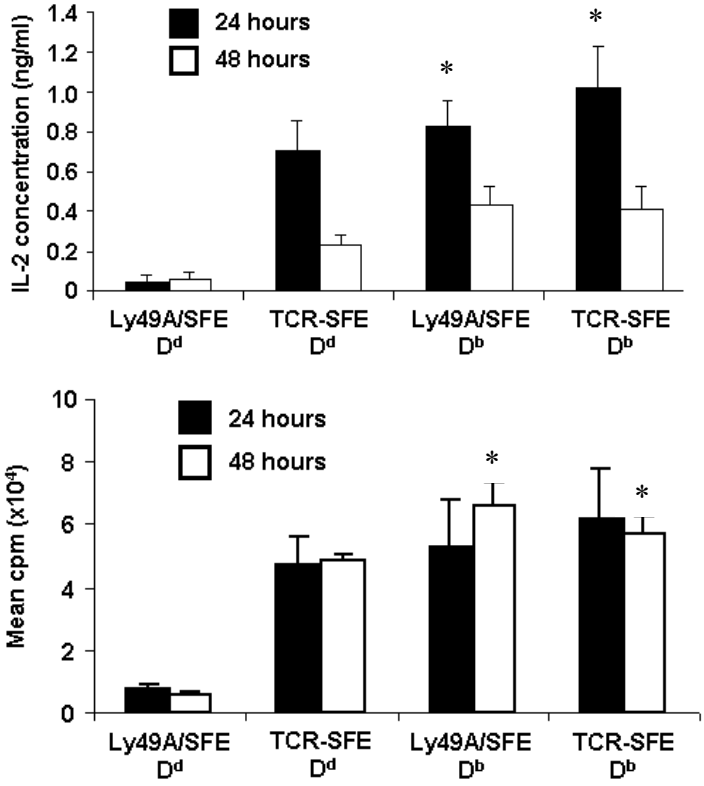

Figure 7. Graphs of IL-2 secretion (*top)* and T cell proliferation (*bottom)* 24 and 48 hours after in vitro stimulation of Ly49A$^+$ and Ly49A$^-$ T cells by SFE stock peptide presented by H-2D^d (Ligand +) and H-2D^b (Ligand –) APCs.

Figures courtesy of Mary Pauza and Sherry Smith

In contrast, in a previous *in vitro* model of the adoptive transfer used in this paper, where harvested B10.HTG APCs (H-2D^b, ligand –) presented stock SFE peptide to Ly49A$^{+/-}$ T cells (similar to the first intracellular staining protocol), it was found that an initial surge of IL-2 at 24 hours post transfer was greater from SFE/B10.HTG T cells when compared to Ly49A/SFE/B10.HTG (Figure 7).

This might reflect a slightly different behavior during early activating events by the Ly49A$^-$ T cells. While cell counts simultaneously parallel the difference in IL-2 secretion between Ly49A$^{+/-}$ T cells during the first 24 hours (greater IL-2 secretion by Ly49A$^-$ T cells results in greater Ly49A cell counts), by 48 hours Ly49A$^+$ T cells proliferated and surpassed Ly49A$^-$ T cells in number, which had actually declined. This pattern in the TCR-SFE T cells may be a consequence of the dual action of IL-2 secretion, which initially promotes cell proliferation in an autocrine fashion, but later potentiates Fas-mediated apoptosis of antigen-activated T cells. Indeed, in the same experiment, Annexin V (a

marker of cellular apoptosis) levels were slightly higher at 24 and 48 hours in the higher IL-2 secreting TCR-SFE T cells (data not shown).

While the difference is small, this initial IL-2 surge in SFE/B10.HTG may promote earlier and more rapid β cell destruction (via IL-2's function on NK cells and B cells) just before it's regulatory function commences. This is another possible explanation of what we were observing in the first adoptive transfer experiment where the Ly49A receptor appears to be offering a protective innate function (regardless of it's activation via the H-2D^{d} ligand) when in actuality it is only a reflection of a more destructive SFE/B10.HTG (Ly49A^{-}) T cell population.

It was reassuring that the third trial of the adoptive transfer was able to achieve an in vivo model more like what we expected, that is, showing that the presence of the inhibitory receptor Ly49A on T cells is not all that is required (it requires ligation) for it's protective effect to delay insulitis, insulin deficiency and hyperglycemia. At the same time, problems are expected to arise in these types of experiments because operator experience plays a significant role. Perhaps the most sensitive step in these protocols is the actual injection of the prepared cell suspension intravenously into the recipients. The method used here is widely accepted, but hardly an easy process. Any deviance from the established injection volume consequently will expose the recipient to different quantities of CD 4^{+} T cells. Injecting retroorbitally is also a stressful event for the animal, which in itself may have hormonal effects that can differ between animals. Moreover, it is difficult to predict the consequences of two needle sticks in one recipient versus only one.

A small experimental population is also a difficult, but sometimes unavoidable, obstacle in achieving statistical significance. In these models, only six mice were used, three in each population. Consequently, and as was not uncommon in the trials, the death of a subject may have reduced statistical power. While the cause of death was probably unrelated to autoimmunity, it eliminates the subject from the study's endpoint, in this case hyperglycemia.

In the first two adoptive transfer experiments (those with suboptimal results) greater T cell quantities than what are generally used were injected. This was because of time

constraints. It was hoped that the higher cell counts would accelerate the disease onset. This may have been excusable had the experiment been free of any other technical complications, however in actuality it may have just added another unknown variable. For this reason, in the third adoptive transfer protocol, a smaller amount of $CD4^+$ T cells were transferred to recipients (2.0×10^6 vs. 3.9×10^6). This is a quantity that has been used previously in similar adoptive transfers.

The ρ value greater than .05 in the third trial signifies that the progression to hyperglycemia in the two groups ($Ly49A^+$ and $Ly49A^-$) was indeed similar, or in other words, it was not significantly different. In the most perfect model where the individual subjects become hyperglycemic on the same day, the ρ value will approximate 0.3. While the results here certainly are not perfect, they are acceptable for a model that used only 3 mice in each experimental group and techniques inherent with technical difficulties.

Much remains to be learned concerning the intracellular events that are taking place during Ly49A mediated T cell inhibition of autoreactivity. During Ly49A ligation and concomitant TCR activation, phenotypic consequences include greatly reduced secretion of IL-2, expression of cell-surface receptors CD25, CD40L, CD69, as well as persistence of CD62L all suggesting reduced T cell activation and proliferation with preservation of a naïve cell phenotype. Anergic T cells, via altered signaling patterns, are found to express similar deviations in surface membrane receptor expression. Anergy is one means of maintaining peripheral T cell tolerance and describes T cells that remain unresponsive to antigenic restimulation by failing to proliferate and secrete IL-2. If it is found that the intracellular events during Ly49A T cell inhibition are very similar to those of anergic cells, the Ly49A receptor may prove to be a means of restoring unresponsiveness, or peripheral tolerance, to self antigens.

For several years the theory of proofreading at the TCR-antigen complex has been used to describe how antigens with varying affinities for the TCR result in unique intracellular cascade events that are kinetically different from each other. It proposes that rapid dissociation of the antigen from the TCR stimulates only early phosphorylation events while longer, sustained interaction results in further downstream signaling and ultimately, gene transcription. In other words, the higher the affinity and persistent ligation, the better

the ligand is at inducing T cell responses. Recently, modifications have been suggested for this model where, even weak antagonists and partial agonists have been shown to induce downstream events at the same intensity as strong agonists, only less efficiently. It is proposed that these weak, low affinity interactions and subsequent transient early phosphorylation events cumulatively result in T cell activation, albeit at a slower rate (47). Furthermore, as many of the early phosphorylation events are rapidly reversible, they may be difficult to biochemically detect. If Ly49A influences the TCR signal in this manner, observation of phosphorylated Fyn and Zap70 could be difficult to observe, and for this reason we began studying the effects of Ly49A ligation on phosphorylation of p44/42 (Erk1/2), as it is more downstream.

We suspect that the presence of Ly49A on T cells results in consequences that are similar to anergic cells or T cells with responses that are similar TCR stimulation by partial antagonists or weak agonists. We set out to examine the difference in Erk phosphorylation between Ly49A negative T cells in B10.HTG (ligand –) and B10.D2 (ligand +) APCs presenting SFE peptide antigen expecting to see comparable Erk phosphorylation.

Unfortunately, the failure to detect significant staining of p44/42 prompted us to focus more on optimizing our protocol. Experiments of intracellular staining in vitro are difficult, it involves proper stimulation of the TCR cascade events, fixating the cell at the correct point in time to capture the desired intracellular phosphorylated events, permeabilizing the cell membrane allowing the entry of the mAb, and finally adequate staining of the desired kinase.

It seemed fairly obvious that the problems we were having were related to not having sufficient T cell activation and that are permeabilization and staining procedures were indeed adequate. This is suggested by consistent, excellent Erk-phosphorylation and detection on flow cytometry when stimulated using PMA. As a result we tried various methods of T cell activation with anti-CD3 mAbs, costimulation with anti-CD28, and finally crossbridging with anti-hamster IgG. The best result obtained was by using anti-CD3/anti-CD28 for 60 minutes on ice, followed by 60-minute incubation at 37˚C (37.2% Erk-phosphorylation). These T cells never were in the presence of anti-hamster IgG. Interestingly, in this same protocol, the T cells that received treatment with anti-hamster

IgG did not fair as well in Erk staining. This may reflect a phenomena by which the addition of the anti-hamster IgG was binding to free anti-CD3/anti-CD28 that only needed additional time to eventually find it's CD3 and CD28 receptors.

The use of anti-hamster IgG crosslinking has been shown to be affective in other protocols, and our negative results in this case should not discourage their continued used. In contrast, optimal T cell stimulation by anti-CD3/anti-CD28 may require longer incubations times. After all CD3 and CD28 are occupied by the mAbs, washing with PBS seems appropriate, this will remove from the solution any anti-CD3 and anti-CD28 mAbs that are no longer needed. Addition of the anti-hamster IgG at this time should allow satisfactory linking of the bound anti-CD3 and anti-CD28 and provide increased signal strength.

Conclusion

Among other strategies that are being applied to support the curbing of T cell autoreactivity and autoimmune disease is the transgenic use of inhibitory receptors expressed on T cells. Other strategies have included: tissue-specific induction of tolerance (in this case, pancreatic β cells), alteration of cytokine environments (IL-4, IL10, IL13, and TGF-β) T cell differentiation, and disruption of costimulation (CD28, CD40L). While impressive, these approaches are complicated by inconsistent findings, variations in genetic backgrounds, and significant alteration of normal immune regulatory function.

Recent work done at Southern Illinois University on the use of Ly49A in keeping autoreactivity in check, or more specifically, pancreatic β cell destruction, has had favorable results. In their murine models, Ly49A expressed on T cells has had various effects. Two of the most important findings are that Ly49A will indeed delay diabetes onset, and that the presence of the receptor on the T cell creates more of a tolerant, or anergic, cell.

In this way, by creating T cells that are unresponsive to antigenic stimulation, it is thought that due to their exceptional homing capabilities and extensive interaction with APCs, they will be beneficial in curbing autoimmunity by competing for space and nutrients at the site of autoimmune destruction and by influencing local APCs to down-regulate important

coreceptors. All of this would be done while not interfering with normal immunologic activity.

The experiments that were performed in this paper also involved the use of Ly49A. We tried to show that the function of Ly49A is dependent on its ligation with the specific MHC class I ligand, H-2D^{d}. We also set out to elucidate some of the important intracellular events that take place as Ly49A is ligated. We began by assessing the level of p44/42 MAP kinase phosphorylation by using intracellular phospho-specific antibodies and flow cytometry. It was unfortunate though, that we weren't able to proceed past the studies of Erk phosphorylation because of the difficulty in achieving satisfactory T cell activation.

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Glossary:

Addressin: *Molecules expressed on endothelial cells in different anatomic sites that bind to counterreceptors on lymphocytes called homing receptors and that direct organ-specific lymphocyte homing. Mucosal addressin cell adhesion molecle-1 (MadCAM) is an example of an addressin expressed in Peyer's patch in the intestinal wall that binds to the integrin $\alpha_4\beta_7$ on gut-homing T cells.*

Antigens: *A molecule that binds to an antibody or a T cell receptor (TCR). Antigens that bind to antibodies include all classes of molecules. TCRs only bind peptide fragments of proteins complexed with MHC molecules; both the peptide ligand and the native protein from which it is derived are called T cell antigens.*

Antigen presenting cells (APCs): *A Cell that displays peptide fragments of protein antigens, in association with MHC molecules, on its surface, and activates antigen-specific T cells. In addition to displaying peptide-MHC complexes, APCs must also express costimulatory molecules to optimally activate T lymphocytes.*

Apoptosis: *A process of cell death characterized by DNA cleavage, nuclear condensation and fragmentation, and plasma membrane blebbing that leads to phagocytosis of the cell without inducing an inflammatory response. This type of cell death is important in lymphocyte development, regulation of lymphocyte responses to foreign antigens, and maintenance of tolerance to self antigens.*

Autoantibodies: *An antibody produced in an individual that is specific for a self antigen. Autoantibodies can cause damage to cells and tissues and are produced in excess in systemic autoimmune diseases such as systemic lupus erythematosus (SLE).*

Autocrine: *Denoting self-stimulation through cellular production of a factor and a specific receptor for it.*

B lymphocyte: *The only cell type capable of producing antibody molecules and therefore the central cellular component of humoral immune responses. B lymphocytes develop in the bone marrow, and mature B cells are found mainly in lymphoid follicles in secondary lymphoid tissues, in bone marrow, and in low numbers in the circulation.*

CD molecules: *Cell surface molecules expressed on various cell types in the immune system that are designated by the "cluster of differentiation" or CD number.*

CD28: *Expressed on T cells (most $CD4^+$, some $CD8^+$ cells) this is a TCR for costimulatory molecules CD80 (B7-1) and CD86 (B7-2).*

CD3: *Molecule that is noncovalently associated with the TCR $\alpha\beta$ heterodimer and is involved in TCR signal transduction; consists of three nonpolymorphic proteins that are designated CD3γ, δ, and ε.*

CD45: *Tyrosine phosphatase whose main cellular expression is on hematopoietic cells and plays a critical role in T and B cell antigen receptor-mediated signaling.*

CD5: *CD72 binding signaling molecule expressed mainly on T cells, thymocytes, and B cells.*

CD62L: *Found on B cells, T cells, monocytes, granulocytes, and some NK cells whose function is in leukocyte-endothelial adhesion and homing of naïve T cells to peripheral lymph nodes.*

Chemokines: *A large family of structurally homologous, low molecular weight cytokines that stimulate leukocyte movement and regulate the migration of leukocytes from the blood to tissues.*

Congenic: *A strain is congenic if the mutation or gene of interest was transferred from another strain or stock by repeated backcrossing.*

Cytotoxic Lymphocyte-Associated Antigen-4 (CTLA-4): *Also CD152, expressed in activated T lymphocytes its proposed function is in inhibitory signaling in T cells and binds CD80 (B7-1) and CD86 (B7-2) on antigen-presenting cells.*

Cytokines: *Proteins produced by many different cell types that mediate inflammatory and immune reactions. Cytokines are principal mediators of communication between cells of the immune system.*

Dendritic cells: *Bone marrow-derived immune accessory cells found in epithelial and lymphoid tissues that are morphologically characterized by thin membranous projections. Dendritic cells function as APCs for naïve T lymphocytes and are important for initiation of adaptive immune responses to protein antigen.*

Dephosphorylation: *Removal of phosphate moieties form tyrosine residues, mainly by protein tyrosine phosphatases (PTPs) such as Src homology phosphatase-1 and 2 (SHP-1 and SHP-2), countering the actions of PTKs.*

Extracellular Receptor-Activated Kinase (Erk): *Final activated component of the mitogen-activated protein (MAP) kinases responsible for phosphorylation of Elk*

and subsequent transcription of Fos, a component of the activation protein-1 (AP-1) transcription factor.

Fyn ($p59^{fyn}$): *Cytoplasmic tyrosine kinase that is found in physical association with the TCR and CD3, similar to Lck it is involved in phosphorylation of CD3 and TCRζ ITAMs, perhaps to a lesser extent.*

G proteins: *Proteins that bind guanyl nucleotides and act as exchange molecules by catalyzing the replacement of bound guanosine diphosphate (GDP) by guanosine triphosphate (GTP). G proteins with bound GTP can activate a variety of cellular enzymes in different signaling cascades. Trimeric GTP-binding proteins are associated with the cytoplasmic portions of many cell surface receptors, such as chemokine receptors. Other small soluble G proteins, such as Ras and Rac, are recruited into signaling pathways by adapter proteins.*

Human leukocyte antigens (HLA): *Major histocompatibility complex (MHC) molecules expressed on the surface of human cells. Human MHV molecules were first identified as alloantigens on the surface of white blood cells that bound serum antibodies from individuals previously exposed to other individuals' cells.*

Interferon-γ (IFN-γ): *Immune, or type II interferon, it is a cytokine produced by T lymphocytes and natural killer (NK) cells whose principal function is to activate macrophages in both innate immune responses and adaptive cell-mediated immune responses.*

Interleukin-1 (IL-1): *A cytokine produced mainly by activated mononuclear phagocytes whose principal function is to mediate host inflammatory responses in innate immunity. The two forms of IL-1 (α and β) bind to the same receptors and have identical biologic effects, including induction of endothelial cell adhesion molecules, stimulation of chemokine production by endothelial cells and macrophages, stimulation of the synthesis of acute phase reactants by the liver, and fever.*

Interleukin-2 (IL-2): *A cytokine produced by antigen-activated T cells that acts in an autocrine manner to stimulate T cell proliferation and also potentiates the apoptotic cell death of antigen-activated T cells. Thus IL-2 is required for both the induction and self-regulation of T cell-mediated immune responses. Il-2 also stimulates the proliferation and effector functions of natural killer (NK) cells and B cells.*

Interleukin-4 (IL-4): *A cytokine produced manly by the T_H2 subset of $CD4^+$ helper T cells whose functions include induction of differentiation of T_H2 cells from naïve $CD4^+$ precursors, stimulation of IgE production by B cells, and suppression of IFN-γ-dependent macrophage functions.*

Interleukin-5 (IL-5): *A cytokine produced by $CD4^+$ T_H2 cells and activated mast cells that stimulates the growth and differentiation of eosinophils and activates mature eosinophils.*

Interleukin-6 (IL-6): *A cytokine produced by many cell types, including activated mononuclear phagocytes, endothelial cells, and fibroblasts, that functions in both innate and adaptive immunity. IL-6 stimulates the synthesis of acute phase proteins by hepatocytes, as well as the growth of antibody-producing B lymphocytes.*

Interleukin-10 (IL-10): *A cytokine produced by activated macrophages and some helper T cells whose major function is to inhibit activated macrophages and therefore maintain homeostatic control of innate and cell-mediated immune reactions.*

Interleukin-12 (IL-12): *A cytokine produced by mononuclear phagocytes and dendritic cells that serves as a mediator of the innate immune response to intracellular microbes and is a key inducer of cell-mediated immune responses to these microbes. IL-12 activates natural killer (NK) cells, promotes IFN-γ production by NK cells and T cells, enhances the cytolytic activity of NK cells and CTLs, and promotes the development of T_H1 cells.*

Insulin: *A protein hormone, produced in the pancreas by the β cells of the islets of Langerhans, that is important for regulating the amount of glucose in the blood.*

Islets of Langerhans: *Small groups of endocrine cells, scattered throughout the material of the pancreas. There are three main histological types of cells: α, β, and δ cells: the cells produce glucagons, insulin, and somatostatin, respectively.*

Integrins: *Heterodimeric cell surface proteins whose major functions are to mediate the adhesion of leukocyte to other leukocytes, endothelial cells, and extracellular matrix proteins. Integrins are important for T cell interactions with APCs and for migration of leukocytes from blood into tissues. The ligand-binding affinity of the integrins can be regulated by various stimuli, and the cytoplasmic domains of integrins bind to the cytoskeleton.*

Ketoacidosis: *Acidosis, as in diabetes or starvation, caused by the enhanced production of ketone bodies.*

Lck ($p56^{lck}$): *Src family tyrosine kinase that is associated with the cytoplasmic tails of CD4 and CD8 that upon activation phosphorylates the ITAMs of CD3 and ζ chains.*

Locus: *The position that a gene occupies on a chromosome.*

Lymph nodes: *One of numerous round, oval, or bean-shaped bodies located along the course of lymphatic vessels, varying greatly in size (1-25 mm in diameter) and usually presenting a depressed area, the hilum, on one side through which blood vessels enter and efferent lymphatic vessels emerge. Lymph nodes are the sites where adaptive immune responses to lymph-borne protein antigens are initiated.*

Lymphopenia: *A decrease in the cumber of lymphocytes in the blood, which may occur in a wide variety of diseases.*

Lymphotoxin (LT, TNF-β): *A cytokine produced by T cells that is homologous to and binds to the same receptors as TNF-α. Like TNF-α, LT has proinflammatory effects, including endothelial and neutrophil activation. LT is also critical for the normal development of lymphoid organs.*

Macrophage: *A tissue-based phagocytic cell derived from blood monocytes that plays important role in innate and adaptive immune responses. Macrophages are activated by microbial products such as endotoxin and by T cell cytokines such as IFN-γ. Activated macrophages phagocytose and kill microorganisms, secrete proinflammatory cytokines, and present antigens to helper T cells.*

Major histocompatibility complex (MHC): *A heterdimeric membrane protein encoded in the MHC locus that serves as a peptide display molecule for recognition by T lymphocytes. Two structurally distinct types of MHC molecules exist. Class I MHC molecules are present on most nucleated cells, bind peptides derived from cytosolic proteins, and are recognized by $CD8^+$ T cells. Class II MHC molecules are restricted largely to professional APCs, bind peptides derived from endocytosed proteins, and re recognized by $CD4^+$ T cells.*

Mitogen-activated protein (MAP) kinase cascade: *A signal transduction cascade initiated by the active form of the Ras protein and involving the sequential activation of three serine/threonine kinases, the last one being MAP kinase. MAP kinase in turn phosphorylates and activates other enzymes or transcription factors.*

Naïve lymphocyte: *A mature B or T lymphocyte that has not previously encountered antigen, nor is the progeny of an antigen-stimulated mature lymphocyte. When*

naïve lymphocytes are stimulated by antigen, they differentiate into effector lymphocytes, such as antibody-secreting B cells or helper T cells and cytolytic T lymphocytes (CTLs).

Natural Killer (NK) cells: *A subset of bone marrow-derived lymphocytes, distinct from B or T cells, that function in innate immune responses to kill microbe0infected cells by direct lytic mechanisms and by secreting IGN-γ. NK cells do not express clonally distributed antigen receptors like Ig receptors or TCRs, and their activation is regulated by a combination of cell surface stimulatory and inhibitory receptors, the latter recognizing self-MHC molecules.*

Peptide: *A molecule consisting of two or more amino acids linked by bonds between the amino group and the carboxyl group.*

Phosphorylation: *Referring to tyrosine residues, the protein-protein interactions and the activities of cellular enzymes are often regulated by phosphorylation principally by protein tyrosine kinases (PTKs) such as Src, Yes, Fgr, Fyn, Lck, Lyn, Hck, and Blk.*

Spleen: *A secondary lymphoid organ located in the left upper quadrant of the abdomen. The spleen is the major site of adaptive immune responses to blood-borne antigens.*

T lymphocyte: *The cell type that mediates cell-mediated immune responses in the adaptive immune system. T lymphocytes mature in the thymus, circulate in the blood, populate secondary lymphoid tissues, and are recruited to peripheral sites of antigen exposure.*

T cell receptor (TCR): *The clonally distributed antigen receptor on $CD4^+$ and $CD8^+$ T lymphocytes that recognized complexes of foreign peptides bound to self-MHC molecules on the surface of APCs.*

T helper 1 (Th1) cells: *A functional subset of helper T cells that secretes a particular set of cytokines, including IFN-γ, and whose principal function is to stimulate phagocyte-mediated defense against infections, especially with intracellular microbes.*

T helper 2 (Th2) cells: *A functional subset of helper T cells that secretes a particular set of cytokines, including IL-4 and IL-5, and whose principal functions are to stimulate IgE and eosinophil/mast cell-mediated immune reactions and to downregulate T_H1 responses.*

Thymocytes: *A precursor of a mature T lymphocyte present in the thymus.*

Thymus: *A bilobed organ situated in the anterior mediastinum, which is the site of maturation of T lymphocytes from bone marrow-derived precursors. Thymic tissue is divided into an outer cortex and an inner medulla and contains stromal thymic epithelial cells, macrophages, dendritic cells, and numerous T cell precursors (thymocytes) at various stages of maturation.*

Tolerance: *Unresponsiveness of the adaptive immune system to antigens, as a result of inactivation or death of antigen-specific lymphocytes, induced by exposure to the antigens. Tolerance to self antigens is a normal feature of eh adaptive immune system, but tolerance to foreign antigens may be induced under certain conditions of antigen exposure.*

Transcription: *Transfer of genetic code information from one kind of nucleic acid to another, especially with reference to the process by which a base sequence of messenger RNA is synthesized (by an RNA polymerase) on a template of complementary DNA.*

Transgenic mouse: *A mouse that expresses an exogenous gene that has been introduced into the genome by injection of a specific DNA sequence into the pronuclei of fertilized mouse eggs. Transgenes insert randomly at chromosomal break points and are subsequently inherited as simple mendelian traits. By designing transgenes with tissue-specific regulatory sequences, mice can be produced that express a particular gene only in certain tissues.*

Tumor necrosis factor (TNF-α): *A cytokine produced mainly by activated mononuclear phagocytes that functions to stimulate the recruitment of Neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes. TNF stimulates vascular endothelial cells to express new adhesion molecules, induces macrophages and endothelial cells to secrete chemokines, and promotes apoptosis f target cells. (TNF-β, or LT, is a closely related cytokine wit identical biologic effects to TNF-α but is produced by T cells)*

Zeta-associated protein of 70kD (ZAP70): *An Src family cytoplasmic protein tyrosine kinase that is critical for early signaling steps in antigen-induced T cell activation. ZAP-70 binds to phosphorylated tyrosines in the cytoplasmic tails of the ζ chain off the TCR complex and in turn phosphorylates adapter proteins that recruit other components of the signaling cascade.*

ζ chain: *Protein dimer found within TCR, each dimer containing three immunoreceptor-based tyrosine activation motifs (ITAMs) is necessary for TCR-mediated activation via protein tyrosine kinase (PTK) phosphorylation.*

Appendix A

Protocol 1

1. Euthanize and harvest lymph nodes and spleen from 2 adult (≥6 wks) mice, 1 SFE//HTG, the other Ly49A/SFE/HTG
    a. Label 2 sterile Petri dishes Ly49A(+) and add 5 ml of RPMI+10%FCS to each
    b. Extract lymph nodes from Ly49A(+) mouse, place in one sterile dish
    c. Extract spleen from mouse 1, place in other sterile dish
    d. Label 2, 15 ml plastic tubes Ly49A(+)SP and Ly49A(+)LN
    e. Grind LN in Petri dish with rough side of slides and transfer initial contents to LN tube, then wash the slides and dish with additional 5 ml of RPMI/FCS solution and add to same tube
    f. Grind SP in Petri dish and transfer initial contents to SP tube, then wash slides and dish with additional 5 ml of RPMI/FCS and add to same tube
    g. Repeat steps a-f for Ly49(-) mouse for a total of four tubes
2. Vortex the four tubes thoroughly and let sit for 3 minutes, transfer only supernatant to clean 15 ml, labelled tubes as above.
3. Centrifuge tubes with cellular solution for 3 minutes at 1600 rpm. Discard this supernatant
4. Red cell lysis
    a. Break up pellets and to each of the LN tubes add 1ml of 1.44% NH_4Cl and to the SP tubes, add 1.5 ml
    b. Vortex and let sit for 5 minutes
    c. Add 10 ml RPMI/FCS and mix
    d. Centrifuge for 3 minutes at 1600 rpm
    e. Discard supernatant
5. Break up pellets in SP tubes, add 5 ml of normal RPMI (no FCS) and transfer to LN tubes. Wash SP tubes with additional 5 ml of media.
6. Count cells of each cell solution
    a. Break up pellets, vortex, take out 2µl of cell solution of each tube and put in own well on 96-well plate.
    b. Add 18µl of TB to each well and count cells on Neubauer slide using dilution factor of 10
    c. # cells x 2 x 10^4 / # squares counted = # cells/ml
    d. (# cells/ml) x total volume of cell mixture in ml = total cell yield
7. Label 3, 12x75 FACS tubes (1. Neg. Control, 2. Ly49A(+), 3. Ly49A(-) and add 1 x 10^6 cells
8. To each tube add 500µl FACS buffer, vortex, centrifuge at 1600 rpm for 3 minutes.
9. Dump and blot the tubes
10. If not available, prepare 1:2000 dilution of anti-CD4 antibodies by adding 0.5µl of anti-CD4PE stock to 1 ml of FACS buffer in Eppendorf tube, vortex, store remaining dilution.
    a. Add 100 µl antibody dilution to tubes 2 and 3
    b. Add 100µl FACS buffer to tube 1
    c. Vortex tubes and put on ice, cover, and let sit for 20 to 60 minutes.

11. Wash once by adding 500µl FACS buffer to tubes, vortex, centrifuge at 1600 rpm for 3 minutes
12. Dump and blot the tubes
13. Break up pellets and resuspend in 300µl FACS/FIX, vortex, and run cytometry
14. Calculate % CD4+ cells and multiply by total cell count to find total CD4+ donor cells
15. Based on cell count, prepare solution of 3.9 X 10^6 or 2.0 X 10^6 CD4+ T cells, enough for three injections of 0.3cc plus a little extra for transfer loss.
16. IV inject previously irradiated insHA/HTG mice with cell solution

Protocol 2

1. Harvest lymph nodes from one mouse: TCR-SFE/HTG strain
2. Isolate and purify CD4+ T cells from extracted nodes according to following method:
    a. Grind LN and wash plate and grinding slides with RPMI 10% (5ml). Allow debris to settle, retain cells, and centrifuge to pellet for 3 min at 1600 rpm. Discard supernatant.
    b. For magnetic bead depletion of non-CD4+ cells, first incubate cells in a solution of antibodies. Calculate the volume of antibody solution needed. Cells should be @10^7 cells/ml (generally 7 ml/lymph node)

Antibody	Dilution	Cells removed	Volume
B220	1:1000 (1µg/ml)	B cells	7 µl
M5/114	1:50	APC	140 µl
F4/80	1:50	MQ	140 µl
a-CD8	1:200	CD8+ T cells	35 µl

    c. Pellet the cells, remove wash solution and add antibodies, mix.
    d. Incubate 30 minutes at 4 degrees Celsius (on ice)
    e. Wash magnetic beads 3X with cold PBS/2%HS, wash once and allow separation, than wash twice more.
        i. Use 1ml bead suspension for each 10^7 cells, this is typically the same volume as was used for the antibody solution above in step 3.
    f. Centrifuge cells and discard supernatant and retain the pellet.
    g. Mix cells and washed beads (i.e. maintain 10^7 concentration)
    h. Place mix on magnet for 10 min.
    i. Centrifuge cells and discard supernatant and resuspend in 10 ml of RPMA/2%FCS
    j. Count cells (20 µl dye + 20 µl cells)
3. Once purified, T cells will be allowed to rest at 37C, 5% CO_2 in RPMI1640 without FCS for 1-2 hours.
4. APC preparation:
    a. Inject B10-HTG and B10-D2 mice i.p. with sterile 3% thioglycolate on day -4
    b. On day 0 (today), harvest APC by peritoneal lavage
        i. Euthanize mouse with isoflourane
        ii. Pin down mouse
        iii. Carefully expose peritoneal cavity without puncturing the peritoneal lining
        iv. Inject 5-10ml cell dissociation solution (22-25 gauge needles work best), avoid blood vessels and organs
        v. Message cavity for ~1 minute.
        vi. Remove lavage fluid
    c. Cfg. Cells and count
    d. Transfer APC cells into polypropylene tubes and adjust cell concentration to 5X10^6 cells/ml with RPMI+10%FCS (PSGH, 2ME). This will yield 2.5X10^5 cells per 50µl. Preincubate one half of each APC population in

RPMI(PSGH, 2ME) at plus 10μg/ml stock SFE peptide for 30 minutes at 37C, 5% CO_2.

e. Cfg. TCR-SFE CD4 cells at 1600 rpm for 3 minutes. Resuspend in prewarmed RPMI+10%FCS (PSGH, 2ME) at a concentration of $5X10^6$ cells/ml. This will yield $2.5X10^5$ B10.D2 or B10.HTG APC +/- peptide, IMMEDIATELY cfg at 3000 rpm for 30 seconds, and incubate at 37C for 0, 5, 15, and 30 minutes.

0 min	5 min	15 min	30 min
Tube 1: Just T cells	Tube 6: T + PMA		
Tube 2: Just D2 APC			
Tube 3: Just HTG APC			
Tube 4: T + D2 APC	Tube 7:T + D2 APC	Tube 11: T + D2 APC	Tube 13: T + D2 APC
T + D2 APC + PEP	Tube 8 : T + D2 APC + PEP	Tube 12 : T + D2 APC	Tube 14 : T+D2 APC + PEP
	Tube 9: T+HTG APC		
	Tube 10 : T + HTG APC + PEP		

*This assay will require a minimum of $1.8X10^7$ CD4 cells and $4.5X10^7$ HTG APC and $4.5X10^7$ D2 APC.

*Additional controls are needed for flow cytometry compensation: T cells without antibody, T+anti-CD4PE, T+p44/42 Alexa488.

f. At the indicated time points, fix cells immediately by adding 100μl of 1X cytofix/cytoperm (BD PharMingen) and placing cells at 37C for 10 min.
g. Pellet by centrifugation 5000 rpm for 1 minute and remove the supernatant.
h. Wash the cells twice with PhosFlow Perm/Wash Buffer I then centrifuge at 5000 rpm for 1 minute. And remove supernatant.
i. Resuspend cells in PhosFlow Perm/Wash Buffer I at $10X10^6$ cell/ml.
j. Aliquot 100μl of cell suspension ($1X10^6$) to each tube and add the recommended volume of PhosFlow antibody. Incubate at room temperature for 1 hour in the dark.
k. Wash cells once with PhosFlow Perm/Wash Buffer I; resuspend in PhosFlow Perm/Wash Buffer I prior to flow cytometric analysis.

Protocol 3

1. Euthanize and harvest lymph nodes from one mouse
2. Grind lymph nodes with 5 ml of RPMI + 10%FCS using an empty sterile Petri dish and two frosted microscope slides.
    a. Transfer initial contents of Petri dish to 15 ml plastic tube. Wash with an addition 5 ml of RPMI/FCS solution.
    b. Vortex, let sit for approx. 3 minutes, transfer supernatant to clean 15 ml tube
3. Centrifuge tube with cells for 3 minutes at 1600 rpm. Discard this supernatant.
4. Break-up the pellet, suspend in 10 ml normal RPMI (no FCS)
    a. Vortex, take out 20µl and mix with 20µl of TB and count cells on Neubauer slide with dilution factor of 2.
    b. # cells x 2 x 10^4/ # squares counted = # cells/ml
    c. (# cells/ ml) x total volume of cell mixture in mililiters = total cell yield
5. Make a solution of 1.5 ml normal RPMI with a concentration of 10^7 cells/ml.
6. Place 100 µl of cell solution into each of 12 Eppendorf tubes. Set aside.
7. Prepare in one 50 ml conical tube 10X Perm/Wash buffer and dH_2O (mix 2.5ml Perm/Wash (white bottle) in 25ml dH_2O). Using this dilutions prepare aliquot of 1:2000 anti-CD4 by adding 0.75µl stock anti-CD4 in 1.5 ml 1X PermWash dilution in Eppendorf tube. The remaining 1X Perm/Wash will be used for washes later.
8. Label 5 Eppendorf tubes 1:50, 1:100, 1:200, 1:500, 1:1000 and to each add 100µl, 100µl, 100µl, 300µl, and 300µl respectively of anti-CD4PE in Perm/Wash (as prepared in 7 above). Note: do not discard the remaining anti-CD4PE in Perm/Wash, you will need it in step 16 for tube #10. Then add 2µl, 1µl, .5µl, .6µl, and .3µl of anti-Erk (anti-p44/42 Alexa 488) stock solution to the tubes in the same order, set aside. You will also need 1 Eppendorf tube with 100 µl 1X Perm/Wash plus 1 µl anti-Erk only (for tube #3).
9. Label eight tubes 1-12 and to each add 100µl of cell suspension (done already in 6). Then add to tube 1-8 and 11-12, 1µl of 100 µl/ml stock PMA solution, vortex, and incubate for 5 minutes at 37°C. Do not add PMA to tubes 9 and 10, but instead just incubate them at 37C for 5 minutes.
10. Immediately add 100µl of cytofix/cytoperm solution (brown bottle, don't dilute), vortex and incubate for 10 minutes at 37°C.
11. Transfer all tubes to FACS tubes.
12. Add 200µl 1X Perm/Wash solution prepared earlier, vortex, centrifuge for 5 minutes at 1600 rpm.
13. Dump and blot
14. Add 200µl Perm/Wash solution, vortex, centrifuge for 5 minutes at 1600 rpm (washing again)
15. Dump and blot
16. Add antibodies according to the following scheme:

Tube 1	Tube 2	Tube 3
Nothing Just 100 ul 1XPerm/Wash	-only 100ul 1:2000 aCD40PE in Perm/Wash	-100ul a-Erk 1:100 in Perm/Wash

Tube 9	Tube 10
Nothing Just 100 ul 1XPerm/Wash No PMA	-only 100ul aCD40PE in Perm/Wash No PMA

Tube 4 100 ml aby dilution 1:50
Tube 5 100 ml aby dilution 1:100
Tube 6 100 ml aby dilution 1:200
Tube 7 300 ml aby dilution 1:500
Tube 8 300 ml aby dilution 1:1000

17. Immediately incubate the eight tubes for 1 hour in dark at room temperature.
18. Wash each tube with 200µl of same Perm/Wash dilution as before
19. Centrifuge for 5 minutes at 1600 rpm and resuspend in 250µl of Perm/Wash for Cytometry

Protocol 4

1. Before harvesting lymph nodes, prepare anti-CD3-bound 96-well plate
    a. Add 10 µg/ml of anti-CD3 in 300 µl PBS
    b. To each well requiring it, add 30µl of anti-CD3/PBS (see diagram)
    c. Incubate plate for 60 minutes at 37˚C
2. Euthanize and harvest lymph nodes from one mouse of SFE/HTG lineage
3. Grind lymph nodes with 5 ml of RPMI+10%FCS using an empty sterile Petri dish and two frosted microscope slides.
    a. Transfer initial contents of Petri dish to 15 ml plastic tube. Wash with an addition 5 ml of RPMI/FCS solution.
    b. Vortex, let sit for approx. 3 minutes, transfer supernatant to clean 15 ml tube
4. Centrifuge tube with cellular solution for 3 minutes at 1600 rpm. Discard this supernatant
5. Red cell lysis
    a. Break up pellet and add 1ml of 1.44% NH_4Cl
    b. Vortex and let sit for 5 minutes
    c. Add 10 ml RPMI/FCS and mix
    d. Centrifuge for 3 minutes at 1600 rpm
    e. Discard supernatant
6. Break-up the pellet, suspend in 10 ml normal RPMI (no FCS), count cells
    a. Vortex, take out 20µl and mix with 20µl of TB and count cells on Neubauer slide with dilution factor of 2.
    b. # cells x 2 x 10^4/ # squares counted = # cells/ml
    c. (# cells/ ml) x total volume of cell mixture in milliliters = total cell yield
7. Make a solution of RPMI/FCS with a concentration of 5 x 10^6 cells/ml.
8. Hold 3 aliquots at 37˚C
9. After plate has incubated for 1 hour, wash with PBS
10. To 4 wells add PMA + PBS at appropriate dilutions (see diagram)
    a. Add 9µl PBS to each of 3 wells in c, d, e
    b. well 1: 1µl PMA(stock)
    c. well 2: 1µl well 1
    d. well 3: 1µl well 2
    e. well 4: 1µl well 3
11. Prepare 16 FACS tubes with 200µl cytofix/cytoperm (brown bottle, no dilution) solution, label each tube 1-16 according to diagram
12. Using the three aliquots prepared in step 8, add cell solution +/- antibody to appropriate wells (see diagram). Do this as quickly as possible.
13. Centrifuge plate for 3 minutes at 1000rpm
14. Put <u>time 0</u> wells in cytofix/cytoperm tubes prepared in step 12 (watch numbering), vortex
15. Incubate plate at 37˚C and set timers
16. At five minutes remove contents from appropriate cells and put into FACS tubes prepared in step 12
    a. Also remove all PMA wells prepared in step 11 and put in FACS tubes
17. Run same procedure for 15 and 30 minutes wells

    a. After 30 minutes time, begin washing tubes by adding 200 µl 10X? Perm/Wash (white bottle)
    b. Vortex, centrifuge for 3 minutes at 1600rpm
    c. Dump and blot
    d. Do this twice
18. After 60 minute tubes have incubated for 10 minutes in cytofix/cytoperm and washed twice with Perm/wash, add antibodies to appropriate FACS tubes with correct Ab dilutions
    a. Anti-CD4PE 1:2000
    b. Anti-p44/42Alexa488 1:100
19. Incubate FACS tubes at 37˚C for 60 minutes
20. Wash tubes once with 200µl Perm/Wash, vortex, centrifuge for 3 minutes at 1600rpm
21. Dump and blot
22. Resuspend in 250 µl Perm/Wash for cytometry

Protocol 5

1. Euthanize and harvest lymph nodes and spleen from one mouse of SFE/HTG lineage
    c. add 5 ml of RPMI+10%FCS to two Petri dishes
    d. Extract lymph nodes from mouse, place in one sterile dish
    e. Extract spleen from mouse, place in other sterile dish
    f. Label 2, 15 ml plastic tubes SP and LN
    g. Grind LN in Petri dish with rough side of slides and transfer initial contents to LN tube, then wash the slides and dish with additional 5 ml of RPMI/FCS solution and add to same tube
    h. Grind SP in Petri dish and transfer initial contents to SP tube, then wash slides and dish with additional 5 ml of RPMI/FCS and add to same tube
2. Vortex the tubes thoroughly and let sit for 3 minutes, transfer only supernatant to clean 15 ml, labelled tubes as above.
3. Centrifuge tubes with cellular solution for 3 minutes at 1600 rpm. Discard this supernatant
4. Red cell lysis
    i. Break up pellets and add 1ml of 1.44% NH_4Cl to LN, and 1.5 ml to SP.
    j. Vortex and let sit for 5 minutes
    k. Add 10 ml RPMI/FCS to each tube and mix
    l. Centrifuge for 3 minutes at 1600 rpm
    m. Discard supernatant
5. Break-up the pellet, suspend in 10 ml normal RPMI (no FCS), count cells
    n. Vortex, take out 2µl and mix with 18µl of TB and count cells on Neubauer slide with dilution factor of 10.
    o. # cells x 2 x 10^4/ # squares counted = # cells/ml
    p. (# cells/ ml) x total volume of cell mixture in milliliters = total cell yield
6. Make a solution of RPMI/FCS with a concentration of 1 x 10^7 cells/ml.
7. Prepare aliquot of stock anti-CD3 in Perm/Wash at 1µg/ml and 10µg/ml. This aliquot will be added to appropriate tubes and incubated at 37C for different time points 0, 5, 15, 30, and 60 minutes.
8. After 60 minute tubes have incubated for 10 minutes in cytofix/cytoperm and washed twice with Perm/wash, add antibodies to appropriate FACS tubes with correct Ab dilutions
    c. Anti-CD4PE 1:2000
    d. Anti-p44/42Alexa488 1:100
9. Incubate FACS tubes at 37˚C for 60 minutes
10. Wash tubes once with 200µl Perm/Wash, vortex, centrifuge for 3 minutes at 1600rpm
11. Dump and blot
12. Resuspend in 250 µl Perm/Wash for cytometry

Protocol 6

1. Euthanize and harvest lymph nodes and spleen from one mouse of SFE/HTG lineage
    a. add 5 ml of RPMI+10%FCS to two Petri dishes
    b. Extract lymph nodes from mouse, place in one sterile dish
    c. Extract spleen from mouse, place in other sterile dish
    d. Label 2, 15 ml plastic tubes SP and LN
    e. Grind LN in Petri dish with rough side of slides and transfer initial contents to LN tube, then wash the slides and dish with additional 5 ml of RPMI/FCS solution and add to same tube
    f. Grind SP in Petri dish and transfer initial contents to SP tube, then wash slides and dish with additional 5 ml of RPMI/FCS and add to same tube
2. Vortex the tubes thoroughly and let sit for 3 minutes, transfer only supernatant to clean 15 ml, labelled tubes as above.
3. Centrifuge tubes with cellular solution for 3 minutes at 1600 rpm. Discard this supernatant
4. Red cell lysis
    a. Break up pellets and add 1ml of 1.44% NH_4Cl to LN, and 1.5 ml to SP.
    b. Vortex and let sit for 5 minutes
    c. Add 10 ml RPMI/FCS to each tube and mix
    d. Centrifuge for 3 minutes at 1600 rpm
    e. Discard supernatant
5. Break up pellets in SP tube, add 5 ml of normal RPMI (no FCS) and transfer to LN tubes. Wash SP tubes with additional 5 ml of media.
6. Count cells
    a. Vortex, take out 2µl and mix with 18µl of TB and count cells on Neubauer slide with dilution factor of 10.
    b. # cells x 10 x 10^4/ # squares counted = # cells/ml
    c. (# cells/ ml) x total volume of cell mixture in milliliters = total cell yield
7. Make a solution of RPMI/FCS with a concentration of 2 x 10^7 cells/ml.
8. Prepare aliquot of anti-CD4 and anti-p44/42Alexa488 in PermWash
    a. To 1400 µl of perm wash add 0.7 µl of stock anti-CD4PE and 14 µl stock anti-Erk, vortex and put on ice.
9. Prepare aliquot of anti-CD3 and anti-CD28 in RPMI with 10%FCS
    a. To 500 µl of RPMI/FCS add 5.5 µl stock 1µg/ml anti-CD3 and 11 µl stock 1µg/ml (**verify**) anti-CD28, vortex and put on ice. Final anti-CD3 10µg/ml, final anti-cd28 20µg/ml
10. Label 16 FACS tubes 1-16 and add 50 µl cell solution to each tube
11. Add 50 µl of anti-CD3/anti-CD28 aliquot to tubes 6-10, and 12-16, vortex
12. Add 50 µl of RPMI/FCS from separate aliquot (don't contaminate stock) to tubes 5 and 11, vortex
13. Put tubes 5-16 on ice for 30 minutes (start timer), have anti-Armenian Hamster IgG on hand in ice and also fix/perm and perm/wash dilution
14. Working with tubes 1-4
    a. Add 100 µl RPMI/FCS to cell solution in tube 1
    b. Add 100 µl anti-CD4PE in PermWash (my dilution) to tube 2
    c. Add 1 µl stock PMA (wait 5 min) and 1 µl anti-Erk to tube 3

    d. Add 1 µl stock PMA (wait 5 min) and 100 µl anti-CD4/anti-Erk (step 8)
    e. Vortex tubes, they are done until fix
15. After tubes 5-16 have set for 30 minutes in ice add 1 µl anti-hamster to each tube except for tube 12 and place tubes back in ice for additional 30 minutes.
16. After tubes have incubated on ice for thirty minutes in the presence of the IgG, put all tubes in 37C oven.
    a. Tubes 5,6, and 12 can be grouped with 1-4
    b. Add 100 µl Cytofix/cytoperm to tubes 1-6, and 12, vortex
17. Fix tubes 7-9, 12-15 at correct times
18. After 30 minute tubes (9 and 15) have fixed for 10 minutes begin washing tubes 1-9, 12-15 (all but 3 tubes in oven) with 200 µl Perm/Wash
    a. Centrifuge for 3 minutes at 1600 rpm, dump and blot
    b. Wash again
19. Fix 60 minute tubes (10,11,16) for ten minutes
    a. Wash with 200 µl Perm/Wash, centrifuge, dump and blot
    b. Wash again
20. Add 100 µl anti-CD4/anti-Erk to tubes 4-16, vortex, put all tubes in drawer for 60 minutes
21. Wash all tubes with 200 µl Perm/Wash
    a. Centrifuge for 3 minutes at 1600 rpm
    b. Dump and blot
22. Resuspend in 250 µl Perm/Wash for cytometry

Protocol 7

1. Euthanize and harvest lymph nodes and spleen from one mouse of SFE/HTG lineage
    a. add 5 ml of RPMI+10%FCS to two Petri dishes
    b. Extract lymph nodes from mouse, place in one sterile dish
    c. Extract spleen from mouse, place in other sterile dish
    d. Label 2, 15 ml plastic tubes SP and LN
    e. Grind LN in Petri dish with rough side of slides and transfer initial contents to LN tube, then wash the slides and dish with additional 5 ml of RPMI/FCS solution and add to same tube
    f. Grind SP in Petri dish and transfer initial contents to SP tube, then wash slides and dish with additional 5 ml of RPMI/FCS and add to same tube
2. Vortex the tubes thoroughly and let sit for 3 minutes, transfer only supernatant to clean 15 ml, labelled tubes as above.
3. Centrifuge tubes with cellular solution for 3 minutes at 1600 rpm. Discard this supernatant
4. Red cell lysis
    a. Break up pellets and add 1ml of 1.44% NH_4Cl to LN, and 1.5 ml to SP.
    b. Vortex and let sit for 5 minutes
    c. Add 10 ml RPMI/FCS to each tube and mix
    d. Centrifuge for 3 minutes at 1600 rpm
    e. Discard supernatant
5. Break up pellets in SP tube, add 5 ml of normal RPMI (no FCS) and transfer to LN tubes. Wash SP tubes with additional 5 ml of media.
6. Count cells
    a. Vortex, take out 2µl and mix with 18µl of TB and count cells on Neubauer slide with dilution factor of 10.
    b. # cells x 10 x 10^4/ # squares counted = # cells/ml
    c. (# cells/ ml) x total volume of cell mixture in milliliters = total cell yield
7. Make a solution of RPMI/FCS with a concentration of 2 x 10^7 cells/ml.
8. Prepare aliquot of anti-CD4 and anti-p44/42Alexa488 in PermWash
    a. To 1000 µl of perm wash add 0.5 µl of stock anti-CD4PE and 10 µl stock anti-Erk, vortex and put on ice.
9. Prepare aliquot of anti-CD3 and anti-CD28 in RPMI with 10%FCS
    b. To 350 µl of RPMI/FCS add 3.5 µl stock 1µg/ml purified anti-CD3 and 7 µl stock 1µg/ml purified anti-CD28, vortex and put on ice. Final anti-CD3 10µg/ml, final anti-cd28 20µg/ml
10. Label 12 FACS tubes 1-12 and add 50 µl cell solution to each tube
11. Add 50 µl of anti-CD3/anti-CD28 aliquot to tubes 6-10, and 12, vortex
12. Add 50 µl of RPMI/FCS from separate aliquot on ice (don't contaminate stock) to tubes 5 and 11, vortex
13. Put tubes 5-12 on ice for 30 minutes (start timer), have anti-Armenian Hamster IgG on hand in ice and also fix/perm and perm/wash dilution (make more this time)
14. Working with tubes 1-4
    a. Add 50 µl RPMI/FCS to cell solution in tube 1, put in oven with tubes 3, 4
    b. Nothing done to tube 2, but put in oven with tubes 3, 4

    c. Add 1 µl stock PMA (wait until you start timer for tubes 5-12 and are ready for 37˚C) to tube 3 and put in 37˚C for 5 min.
    d. Add 1 µl stock PMA (wait until you start timer for tubes 5-12 and are ready for 37˚C) to tube 4 and put in 37˚C for 5 min.
15. After tubes 5-12 have set for 30 minutes in ice add 1 µl anti-hamster to each tube except for tube 12 and place tubes in 37˚C oven and start timer
    a. Tubes 5,6, and 12 can be grouped with 1-4
    b. Add 100 µl Cytofix/cytoperm to tubes 1-6, and 12, vortex
16. Fix tubes 7-9 at correct times
17. After 30 minute tubes (9 and 15) have fixed for 10 minutes begin washing tubes 1-9, and 12 (all but 2 tubes in oven) with 200 µl Perm/Wash
    a. Centrifuge for 3 minutes at 1600 rpm, dump and blot
    b. Wash again
18. Fix 60 minute tubes (10,11) for ten minutes
    a. Wash with 200 µl Perm/Wash, centrifuge, dump and blot
    b. Wash again
19. Add 100 µl anti-CD4PE in Perm/Wash to tube 2
20. Add 100 µl P/W and 1 µl stock anti-Erk
21. Add 100 µl anti-CD4/anti-Erk to tubes 4-12, vortex, put all tubes in drawer for 60 minutes
22. Wash all tubes with 200 µl Perm/Wash
    a. Centrifuge for 3 minutes at 1600 rpm
    b. Dump and blot
23. Resuspend in 250 µl Perm/Wash for cytometry

Protocol 8

1. Euthanize and harvest lymph nodes and spleen from one mouse of SFE/HTG lineage
    a. add 5 ml of RPMI+10%FCS to two Petri dishes
    b. Extract lymph nodes from mouse, place in one sterile dish
    c. Extract spleen from mouse, place in other sterile dish
    d. Label 2, 15 ml plastic tubes SP and LN
    e. Grind LN in Petri dish with rough side of slides and transfer initial contents to LN tube, then wash the slides and dish with additional 5 ml of RPMI/FCS solution and add to same tube
    f. Grind SP in Petri dish and transfer initial contents to SP tube, then wash slides and dish with additional 5 ml of RPMI/FCS and add to same tube
2. Vortex the tubes thoroughly and let sit for 3 minutes, transfer only supernatant to clean 15 ml, labelled tubes as above.
3. Centrifuge tubes with cellular solution for 3 minutes at 1600 rpm. Discard this supernatant
4. Red cell lysis
    a. Break up pellets and add 1ml of 1.44% NH_4Cl to LN, and 1.5 ml to SP.
    b. Vortex and let sit for 5 minutes
    c. Add 10 ml RPMI/FCS to each tube and mix
    d. Centrifuge for 3 minutes at 1600 rpm
    e. Discard supernatant
5. Break up pellets in SP tube, add 5 ml of normal RPMI (no FCS) and transfer to LN tubes. Wash SP tubes with additional 5 ml of media.
6. Count cells
    a. Vortex, take out 2µl and mix with 18µl of TB and count cells on Neubauer slide with dilution factor of 10.
    b. # cells x 10 x 10^4/ # squares counted = # cells/ml
    c. (# cells/ ml) x total volume of cell mixture in milliliters = total cell yield
7. Make a solution of RPMI/FCS with a concentration of 2 x 10^7 cells/ml.
8. Prepare aliquot of anti-CD4 and anti-p44/42Alexa488 in PermWash
    a. 1000 µl of perm wash
    b. 0.5 µl of stock anti-CD4PE
    c. 10 µl stock anti-Erk, vortex and put on ice.
9. Prepare aliquot of anti-CD3 and anti-CD28 in RPMI with 2%FCS
    a. 300 µl of RPMI/2%FCS
    b. 3.0 µl stock purified anti-CD3
    c. 6.0 µl stock purified anti-CD28, vortex and put on ice.
10. Prepare aliquot of anti-hamster in RPMI/2%FCS
    a. 750 µl of RPMI/2%FCS
    b. 7.5 µl anti-armenian hamster antibody
11. Label 11 FACS tubes 1-11 and add 50 µl cell solution to tubes 5-11
12. Add 50 µl of anti-CD3/anti-CD28 aliquot to tubes 6-10, vortex.
13. Add 50 µl of RPMI/FCS from separate aliquot on ice (don't contaminate stock) to tubes 5 and 11, vortex
14. Put tubes 5-11 on ice for 15 minutes (start timer), have anti-Armenian Hamster in RPMI/10%FCS on hand in ice and also fix/perm and perm/wash dilution

15. After tubes 5-11 have set for 15 minutes in ice, wash the tubes with cold RPMI/2%FCS 500µl, vortex, centrifuge for 3 minutes at 1600 rpm with temperature set to 4˚C, dump and blot
16. Add 100µl anti-ham/RPMI/FCS2% to tubes 5-11, put in 37°C, tubes 5 and 6 get fixed and washed, use timer
17. Fix and wash tubes 7-9 at correct times
18. After 30 minute tube is done fixing for 10 minutes and has been washed once, to tubes 1-4:
    a. Nothing done to tube 1, put in oven with tubes 3, 4
    b. Nothing done to tube 2, but put in oven with tubes 3, 4
    c. Add 1 µl stock PMA to tube 3 and put in 37˚C for 5 min.
    d. Add 1 µl stock PMA to tube 4 and put in 37˚C for 5 min.
    e. Add 100µl cytofix/cytoperm to tubes 1-4 for 10 minutes
    f. Wash the tubes with 200 µl of PermWash, vortex, dump and blot
    g. Wash again
19. Fix 60 minute tubes (10,11) for ten minutes
    a. Wash with 200 µl Perm/Wash, centrifuge, dump and blot
    b. Wash again
20. Add 100 µl of PermWash to tube 1
21. Add 100 µl anti-CD4PE in Perm/Wash to tube 2
22. Add 100 µl P/W and 1 µl stock anti-Erk to tube 3
23. Add 100 µl anti-CD4/anti-Erk to tubes 4-11, vortex, put all tubes in drawer for 60 minutes
24. Wash all tubes with 200 µl Perm/Wash
    a. Centrifuge for 3 minutes at 1600 rpm
    b. Dump and blot
25. Resuspend in 250 µl Perm/Wash for cytometry