Egr-2 and PD-1 Are Required for Induction and Maintenance of T Cell Anergy: A Dissertation

Kenneth D. Bishop
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EGR-2 AND PD-1 ARE REQUIRED FOR INDUCTION
AND MAINTENANCE OF T CELL ANERGY

A Dissertation Presented

By

Kenneth D. Bishop

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

July 13, 2005

MD/PhD Program
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EGR-2 AND PD-1 ARE REQUIRED FOR INDUCTION
AND MAINTENANCE OF T CELL ANERGY

A Dissertation Presented

By

Kenneth D. Bishop

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July 13, 2005
I owe an undeniable debt of gratitude to my primary mentors, Michael Czech and Aldo Rossini, whose diverse backgrounds and experience have provided me with a vivid picture of how science and medicine are supposed to look. It has been my honor and privilege to study under them, and I will carry their lessons and example to my career's end. Also deserving of many thanks are Nancy Phillips, John Harris, and Mary Lively, without whom these endeavors would have been immeasurably more difficult and significantly less pleasant.

The other members of my committee, Dale Greiner, Leslie Berg, and Daniel Tracey, also deserve a note of recognition for their invaluable advice and guidance during this process.

Many thanks go to the members of the Czech and Rossini labs, who have succeeded in creating a collaborative and supportive environment. I have gained as much education during the past four years from conversations and advice received while at the bench as I have in classrooms and seminar halls, and this is a testament to the generosity of the members of these labs.

I also want to thank my family for their support, especially my wife Angela whose day-to-day presence during these four years I could not have done without.

Finally, I want to dedicate this to the memory of my Dad, who provided a model for life that I hope to live up to someday.
ABSTRACT

The prevalence of diabetes is approaching epidemic proportions worldwide. There is currently no cure for type 1 diabetes, and successful treatment requires constant monitoring of blood sugars and use of exogenous insulin to prevent hyperglycemia. Diabetes will be curable when pancreatic β-islet cells can be transplanted into diabetes patients without requiring long-term immunosuppression. This will require learning more about the induction of functional tolerance, a state that maintains the competence of the immune system to most antigens but protects graft-specific antigens from immune rejection, permitting transplantation. One known mechanism of peripheral tolerance is T cell anergy, a phenotype of hypo-reponsiveness in CD4+ T cells. The focus of this thesis is a description of factors shown to be specific to the induction and maintenance of T cell anergy, whose loss reverses the anergic phenotype, restoring the ability of the cells to proliferate in response to antigen. The first of these is Egr-2, a zinc-finger transcription factor, whose presence is required for the induction of anergy induced in T cell clones by TCR stimulation in the absence of costimulation. Egr-2 is shown to be important to anergy induction but not anergy maintenance. In contrast, a negative costimulation receptor, PD-1, is shown to be necessary for the maintenance of anergy. It is possible that learning more about the genetic factors that orchestrate T cell anergy will prove useful in the development of tolerance-based protocols for organ and tissue transplantation without the use of long-term immunosuppression.
TABLE OF CONTENTS

Copyright Information ........................................................................................................................................ ii
Approval Page .................................................................................................................................................. iii
Acknowledgements ......................................................................................................................................... iv
Abstract .......................................................................................................................................................... v
Table of Contents .......................................................................................................................................... vi
List of Figures .................................................................................................................................................. ix
List of Tables ................................................................................................................................................... x
Abbreviations .................................................................................................................................................. xi

Chapter 1: Diabetes Mellitus, Transplantation, and Immunological Tolerance ................................. 1

Impact of Diabetes ........................................................................................................................................... 1
Description of Type 1 Diabetes ..................................................................................................................... 1
Etiology of Type 1 Diabetes ........................................................................................................................... 2
Possible Triggers of Diabetes ....................................................................................................................... 5
Complications of Diabetes ........................................................................................................................... 6
Current Therapies for Type 1 Diabetes .......................................................................................................... 7
The Goal: β-cell Transplantation ................................................................................................................ 11
Transplantation: An Overview .................................................................................................................... 11
Pancreas/Islet Transplantation ..................................................................................................................... 14
Central Tolerance ......................................................................................................................................... 15
Peripheral Tolerance ..................................................................................................................................... 17
T Cell Activation .......................................................................................................................................... 19
Costimulation .................................................................................................................................................. 26
Chapter 1 Discussion .................................................................................................................................. 28

Chapter 2: T Cell Anergy .............................................................................................................................. 30

History of Anergy .......................................................................................................................................... 30
Signal Transduction in Anergic Cells ........................................................................................................... 34
Regulatory T cells, Anergy, and Immune Regulation .................................................................................. 39
In vitro and in vivo models for T cell anergy ............................................................................................... 45
  Immobilized Anti-CD3 Antibody ........................................................................................................... 45
  Altered Peptide Ligands .......................................................................................................................... 46
  Ionomycin .................................................................................................................................................. 46
  Division-Arest Anergy ............................................................................................................................. 47
  Intravenous Peptide Tolerization .......................................................................................................... 48
  Non-Mitogenic Anti-CD3 ......................................................................................................................... 48
Proposed Anergy Factors ............................................................................................................................ 50
The role of E3 Ligases in T Cell Anergy .................................................................................................... 53
Anergy and Peripheral Tolerance ............................................................................................................... 59
Chapter 2 Discussion .................................................................................................................................. 63
Chapter 3: Egr-2, a Zinc-finger transcription factor, is highly expressed in anergic T cells, and is required for full induction of anergy ................................................................. 76

Chapter 3 Introduction ................................................................. 76
Background of Egr-2 ................................................................. 79
Egr-2 is up-regulated in anergic T cells relative to untreated and stimulated populations ........................................................................................................ 80
Egr-2 protein levels decrease in the presence of IL-2 concurrently with the reversal of the anergic phenotype ........................................................................ 83
Depletion of Egr-2 with siRNA partially restores antigenic responsiveness and IL-2 production in early stages of anergy ............................................. 86
Egr-2 depletion in anergic cells restores ERK activation upon stimulation .... 94
Egr-2 depletion prevents anergy induction, but does not reverse established anergy ........................................................................................................... 99
Discussion of Chapter 3 ................................................................ 102

Chapter 4: PD-1, a negative costimulatory receptor, has an essential role in maintaining CD4+ clonal anergy ................................................................................ 104

Chapter 4 Introduction .................................................................. 104
PD-1 knockdown at late time-points results in the reversal of T cell anergy .. 106
PD-1 mRNA and protein levels are increased in anergic cells ...................... 111
PD-1 siRNA increases antigenic responsiveness in anergic A.E7 cells, but not in untreated or fully stimulated populations ............................................. 111
PD-1 depletion restores anergic cell responsiveness in an IL-2 dependent Fashion .............................................................................................................. 119
Transgenic T cells tolerized with intravenous peptide up-regulate PD-1 ...... 122
PD-1 expression in the DST + αCD154 tolerance induction protocol .......... 125
Chaper 4 Discussion ..................................................................... 131
Chapter 5: Comprehensive Discussion - Induction and Maintenance of T cell anergy

Chapter 5 Introduction ................................................................. 138
Proposed Phases of Anergy Induction and Maintenance .................. 138
Egr-2 and PD-1 in different phases of anergy .................................. 141
Discussion of In Vivo Anergy PD-1 Expression .............................. 149
Implications of PD-1 Expression in Costimulatory Blockade Protocols ........................................ 150
Future Questions ........................................................................ 151
Possible Roles for Anergy in Transplantation Tolerance ................. 152
Final Conclusions ...................................................................... 153
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>3</td>
</tr>
<tr>
<td>Figure 2</td>
<td>8</td>
</tr>
<tr>
<td>Figure 3</td>
<td>20</td>
</tr>
<tr>
<td>Figure 4</td>
<td>24</td>
</tr>
<tr>
<td>Figure 5</td>
<td>37</td>
</tr>
<tr>
<td>Figure 6</td>
<td>42</td>
</tr>
<tr>
<td>Figure 7</td>
<td>51</td>
</tr>
<tr>
<td>Figure 8</td>
<td>57</td>
</tr>
<tr>
<td>Figure 9</td>
<td>60</td>
</tr>
<tr>
<td>Figure 10</td>
<td>78</td>
</tr>
<tr>
<td>Figure 11</td>
<td>81</td>
</tr>
<tr>
<td>Figure 12</td>
<td>84</td>
</tr>
<tr>
<td>Figure 13</td>
<td>87</td>
</tr>
<tr>
<td>Figure 14</td>
<td>90</td>
</tr>
<tr>
<td>Figure 15</td>
<td>92</td>
</tr>
<tr>
<td>Figure 16</td>
<td>95</td>
</tr>
<tr>
<td>Figure 17</td>
<td>97</td>
</tr>
<tr>
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</tr>
<tr>
<td>Figure 19</td>
<td>109</td>
</tr>
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<td>Figure 20</td>
<td>112</td>
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<td>Figure 21</td>
<td>115</td>
</tr>
<tr>
<td>Figure 22</td>
<td>117</td>
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<tr>
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<td>Figure 25</td>
<td>126</td>
</tr>
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<td>Figure 26</td>
<td>129</td>
</tr>
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<td>Figure 27</td>
<td>139</td>
</tr>
<tr>
<td>Figure 28</td>
<td>144</td>
</tr>
<tr>
<td>Figure 29</td>
<td>147</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1 .............................................................................................................. 107
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-UTR</td>
<td>3' untranslated region</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activated Protein-1</td>
</tr>
<tr>
<td>BB/W</td>
<td>BioBreeding/Worcester</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinechonic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD28RE</td>
<td>CD28 response element</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxy-fluorescein diacetate, succinimidyl ester</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CSA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DST</td>
<td>Donor specific transfusion</td>
</tr>
<tr>
<td>ECDI</td>
<td>1-ethyl-3-(3-dimethylamino propyl) carbodiimide</td>
</tr>
<tr>
<td>Egr-2</td>
<td>Early Growth Factor-2</td>
</tr>
<tr>
<td>EGTA</td>
<td>(ethylenebis(oxyethylenenitrilo)) tetraethyleneglycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanidine diphosphate</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-induced tumor necrosis factor receptor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte and macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GRAIL</td>
<td>Gene related to anergy in lymphocytes</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanidine triphosphate</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible co-stimulator</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of NFkB</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
</tr>
<tr>
<td>IL-3</td>
<td>Interleukin-3</td>
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<td>IL-4</td>
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<td>IL-7</td>
<td>Interleukin 7</td>
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<td>Interleukin 10</td>
</tr>
<tr>
<td>IL-15</td>
<td>Interleukin 15</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IPEX</td>
<td>Immunodysregulation, polyendocrinopathy, enteropathy, X linked syndrome</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activating motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>ITSM</td>
<td>Immunoreceptor tyrosine-based switch motif</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun kinase</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility factor</td>
</tr>
<tr>
<td>MR1</td>
<td>aCD154 monoclonal antibody</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor ,B</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic (mouse)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCC</td>
<td>Pigeon cytochrome C</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death-1</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidyl inositol-4,5 biphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RasGAP</td>
<td>Ras GTPase activating protein</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RPM</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SHP-1</td>
<td>SH2 domain containing phosphatase 1</td>
</tr>
<tr>
<td>SHP-2</td>
<td>SH2 domain containing phosphatase 2</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal-transducing activator of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Tumor growth factor β</td>
</tr>
<tr>
<td>Th1</td>
<td>Type 1 helper T cell</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>Zeta associated protein, 70 kD</td>
</tr>
</tbody>
</table>
Impact of Diabetes

The prevalence of diabetes mellitus is approaching epidemic proportions. As of 2002, an estimated 18.2 million people in the United States suffered from diabetes, with one-third of them yet undiagnosed (1). Over the next fifty years, the number of diabetes cases in the United States is expected to at least double (2), with recent trends seeing an increase of 800,000 to 1.3 million new diagnoses each year. Diabetes is the fifth leading cause of death by disease in the United States (3). The rise seen in diabetes cases is not limited to the United States: estimates from the International Diabetes Federation report that 194 million people worldwide currently suffer from diabetes, a significant increase from the 1995 estimate of 134 million. There is currently no cure for diabetes, only the means to actively treat it. The high cost to patients’ personal lives from this disease and the imperfect nature of available treatments will make a cure for diabetes a welcome emergence.

Description of Type 1 Diabetes

Diabetes mellitus is a group of disorders having in common the pathologic elevation of blood sugar levels (4). Although the lines distinguishing what have come to be known as type 1 and type 2 diabetes are blurring of late, a distinction can still be made with regard to the cause of hyperglycemia. This classification was first proposed in the
mid-1930s by Himsworth (5). Type 1 diabetes (until recently referred to as juvenile-onset diabetes) is marked by the destruction of the β cells of the pancreatic islet, most frequently due to autoimmunity. Approximately 5-10% of total diabetes cases fit into this category (6). In contrast, type 2 diabetes manifests as a relative insufficiency of insulin rather than an absolute insufficiency, due to the inability of peripheral tissues such as adipose tissue and skeletal muscle to make use of circulating sugars.

The real danger of type 1 diabetes is the damage it causes over the long term. Chronic high glucose levels can be toxic, causing loss of vision, kidney failure, and heart disease, and may lead to sufficient peripheral tissue damage to require amputation (4). A new diagnosis of diabetes also mandates some significant lifestyle changes including frequent insulin injections, constant monitoring of blood sugars, and careful attention to diet and activity. These things together more than justify the large body of research currently under way aiming to cure diabetes.

**Etiology of Type 1 Diabetes**

Most often, the root cause of type 1 diabetes is an autoimmune destruction of the insulin-producing cells in the pancreas, the β-islet cells (Figure 1). This has come to be known as type 1A diabetes, distinguishing it from those cases in which the cause of islet destruction is undetermined, type 1B. Why the immune system chooses to recognize the pancreas as foreign and attack it is a complicated question, and many studies are
Legend for Figure 1

The progression of diabetes is visible histologically. A. A normal pancreatic islet from a BioBreeder Diabetes-Prone rat B. Low levels of cellular infiltration mark the beginning of autoimmune islet destruction C. Complete functional destruction of the islet: a high degree of cellular infiltration and an almost complete loss of islet architecture are visible. Images courtesy of Aldo Rossini.
under way seeking an answer to why autoimmune responses take place. It is well accepted that many branches of the immune system are pathologically activated during an autoimmune attack; for example, a major pre-diabetic finding is the presence of anti-islet autoantibodies (7), suggesting the inappropriate activation of B cells. This is not the sole immune lesion in type 1 diabetes, and in a percentage of diabetic patients anti-islet antibodies are not even detectable (8). Studies have also implicated other compartments of the immune system including numerous subsets of T cells, natural killer cells, macrophages, and dendritic cells as being involved in the pathogenesis of diabetes (9, 10). In humans, autoimmune diabetes is often associated with other autoimmune disorders such as thyroiditis, celiac disease, Addison’s disease, and pernicious anemia (11), illustrating the scope and degree to which the immune system has missed its mark.

One arm of diabetes research aims to identify the factors responsible for triggering this autoimmunity, and discover ways to prevent its onset.

**Possible Triggers of Diabetes**

The factors that trigger diabetic autoimmunity are not yet known, though there are many hypotheses. A number of dietary factors have been implicated in diabetes, including cow’s milk (12), meat (13), and wheat and barley proteins (14), but none of these are widely accepted as stand-alone causes. Likewise, a number of viral infections have been implicated as triggers for autoimmune diabetes (15-17). In addition to the environmental factors thought to play a role in causing diabetes, there is almost surely a genetic predisposition to diabetes as well, complicating the story even further (18).
Discovering the environmental and genetic factors that trigger diabetes will provide areas of potential therapeutic intervention, eventually resulting in the means to identify and protect predisposed individuals from developing diabetes. It is unquestionable that research aimed toward preventing diabetes will be invaluable to current and future generations, but at the moment it is equally important that we discover how to cure existing cases of diabetes given the large number of people whose lives diabetes currently impacts. The costs of this disease are by no means limited to the financial burden on both diabetic individuals and the general public. More importantly, a diabetic’s quality of life is significantly decreased in both the short term and long term because of the disease’s numerous sequelae.

Complications of Diabetes

When islet β-cell destruction advances to such a degree that less than twenty percent of a patient’s β-cell mass remains, the remaining mass can no longer successfully manage blood sugar levels and post-prandial hyperglycemia becomes a risk. The early symptomatic manifestations of this can be frequent urination, excessive thirst, waking up to urinate, incontinence, and weight loss (6). For the most part, these can be brought under control by the administration of exogenous insulin. From this point on, managing diabetes is a constant tightrope walk of monitoring sugars, estimating the carbohydrate contents of meals, and administering enough insulin to prevent hyperglycemia without using too much insulin, which can result in dangerously low blood sugar levels and loss of consciousness.
Chronic hyperglycemia has debilitating long term effects, ultimately leading to the early mortality seen in type 1 diabetes (4). Hyperglycemia can affect most organ systems and lead to blindness due to retinopathy, kidney failure secondary to nephropathy, and a multitude of peripheral nervous system problems. These include loss of sensation of many types, loss of muscle strength, muscle wasting, decreased gut motility, decreased bladder function, and postural hypotension. In addition to these problems, known collectively as microangiopathies, diabetes is associated with hyperlipoproteinemia, which is thought to cause coronary, cerebral, and peripheral macrovascular disease. These can lead to heart attacks, strokes, and soft tissue ulcers requiring amputation.

Current Therapies for Type 1 Diabetes

Insulin therapy remains the indispensable tool for controlling blood sugar in patients with diabetes. First shown to decrease blood glucose levels in 1921 by Banting and Best (Figure 2) it is remarkable to see how little diabetes care has actually changed in concept since then (19). The basic strategy is mostly the same now as it was almost ninety years ago: provide sufficient exogenous insulin to restore normoglycemia. Specific insulin technologies have certainly improved, with the advent of many different formulations of insulin whose pharmacokinetics differ (20), and dramatic improvements have been made in home blood glucose monitoring, enabling the patient to achieve better precision during self-administration of insulin (21). A recent attempt at achieving even greater accuracy in insulin administration is the development of the insulin pump (22),
Figure 2

5:00 A.M. dog in good condition
Aug. 7 - 12 midnight (Aug. 6 - 7 AM)

Blood sugar - 143
Vol. urine from 2 P.M. till
12 midnight - 175 cc.

The last 20 cc hours catheterization
separate sugar determination

10 hour total sugar - 3.36 g

Hemoglobin - 1.20 g

G : N ratio 2.8

1. S.C. Delitin given
11 P.M. Blood sugar - .37
No urine obtained by catheter;
Drop about same, stands up and
walks about, has not vomited
since yesterday aft.

2. S.C. Delitin given

2 A.M. Blood sugar .33

3. No urine obtained

8 cc Delitin

3 A.M. Blood sugar .29

4. No urine obtained

8 cc Delitin

4 P.M. Blood sugar .21

The index of Aug. 1 stands the
Legend for Figure 2

Experiments in 1921 by Banting and Best discovered the presence of a substance (for which they coined the name “isletin”) in the pancreas that, when extracted, could regulate the blood sugar of a diabetic dog.
which is worn at all times with a direct subcutaneous infusion set supplying insulin at a consistent rate. The pump can be preset to deliver insulin at a specific basal rate with bolus administrations at set times to compensate for carbohydrate intake at meals. A significant advance will be the development of a real-time blood glucose monitoring system, which would give the patient the opportunity for great precision in insulin administration and significantly reduce the chance of exceeding normal levels of blood glucose. Just as importantly, it would reduce the risk of administering too much insulin and suffering the effects of low blood sugar (23). The dangers of hypoglycemia are mostly acute, as opposed to the more chronic damage caused by hyperglycemia. Symptoms can include hunger, shakiness, and weakness accompanied by peripheral vasoconstriction. In severe and prolonged instances, the brain can be deprived of glucose so that a patient’s condition can progress from difficulty in thinking and fatigue to loss of integrative function, obtundation, and without proper treatment, coma. Most hypoglycemic episodes can be remedied by ingesting some form of sugar.

Despite the importance of the recent advances in insulin technology, they only make diabetes care more convenient. It is important to keep in mind that they do not relieve a diabetic patient of the constant burden of blood glucose management.
The Goal: β-cell Transplantation

Even if future technologies enable diabetes patients to manage their blood sugar to increasingly precise degrees, they will never come close to the precision and balance achieved by the normal functioning of a non-diabetic pancreas. Thus, even better than developing new technologies and training patients to better manage their sugar levels would be to relieve them of the burden altogether. This will be achievable when medicine is able to introduce fully functional β-islet cells into a patient’s system where they can monitor glucose levels and deliver insulin as if they were the patient’s own cells.

The means for this are becoming a medical reality, thanks to the intensive studies currently under way aiming to describe novel transplantation protocols. Replacing a diabetic patient’s β-islet cells with a pancreas or islet graft will cure diabetes, but the transplantation protocols that currently exist have enough of a negative impact on quality of life that they are mostly reserved for life-threatening situations. The discovery of more benign methods of transplantation than those currently used will make curing diabetes a reality.

Transplantation: An Overview

European mythology has passed down a legend from the third century in which two physicians, the brothers Cosmas and Damien, remove a cancerous leg from a European man and replace it with that of a Moor (24). Whether this tale was born of fact or folklore, history offers Sts. Cosmas and Damien as the fathers of allo-transplantation. The potential therapeutic uses for transplantation are extensive, but the barriers that
prevent their realization are largely the same mechanisms we depend on to protect us from pathogens: the ability of the immune system to recognize and destroy anything it perceives to be other than ourselves. This conundrum has become a primary hurdle in successful allograft transplantation, especially since many of the anatomic technical hurdles have been cleared. The first major breakthrough in the field took place in 1954, when Merrill, Murray, and Harrison performed the first kidney transplant, using identical twins as donor and recipient (25). This was truly a landmark event in medicine, opening the gates for clinical opportunities whose potential is still being extended. Patients survive surgery and trauma because of the ability to type and match blood for transfusions, their vision is restored by cornea transplants, and the organs most fundamental to our lives, the heart and lungs, can now be grafted as well (26).

The initial success of Merrill, Murray, and Harrison can be partially attributed to the identical genetic makeup of the donor and recipient. Transplanting tissues and organs between genotypically mismatched individuals raises a myriad of problems, most of them involving the activation of the normal immune response. The goal of modern transplantation science is to learn to overcome these barriers to transplantation by means that are of diminishing harm to the recipient of a graft.

To date, transplantation medicine has protected grafted tissue from the host immune system (and the host from the immune cells in the graft) by long-term administration of immunosuppressive agents. For the most part, these regimens are necessary to stave off immune rejection for the remainder of the patient’s life. The evolution of immunosuppression has progressed from the use of cortisone and other
corticosteroids (27) to the most up-to-date agents such as mycophenolate mofetil and sirolimus (28). Although new agents are selected in part because of their increasingly benign side-effects profiles, the consequences of long term systemic immunosuppression remain: the burden of complying with a lifetime of numerous medications, increased vulnerability to infectious organisms (29), and an increased risk of neoplasia (30). A far preferable alternative to globally suppressing the immune system in order to allow the introduction of a limited set of antigens would be to tailor or train the immune system to allow only graft-specific antigens, while maintaining the competence to reject anything else deemed foreign. Ideally, this treatment would preserve a graft recipient’s ability to fend off the majority of commonly encountered pathogens while hosting a fully functional graft. The ultimate goal of transplantation research is to make it possible for a fully functional organ to be transplanted into an immune-competent host to survive in the absence of ongoing immunosuppression. Many studies toward this goal are underway, and it is conceivable that medicine is less than a generation away from its realization. Before this is a reality, however, there is much to be learned about how a normal immune system works, and how it interacts with its surrounding environment.

This goal of transplantation without immunosuppression has led researchers to envision a physiological state called transplantation tolerance. The induction of a state of functional tolerance would specify to a graft recipient’s immune system which antigens to permit but would leave the immune system competent to defend the recipient against the multitude of pathogens it was designed to protect against. The processes by which a host immune system rejects a graft are not yet fully elucidated, and they are surely
immensely complex. This complexity provides many possible points of modulation at which a rejection response could be dampened.

Pancreas/Islet Transplantation

The potential of replenishing a patient’s islet cell mass to cure diabetes was conceived as far back as 1966, when the earliest pancreas transplantations were performed (31). Until the advent of less harmful immunosuppressive agents such as cyclosporine and anti-T cell agents, however, pancreatic transplantation was not common. Since the late seventies, the number of pancreatic transplants has increased every year, with higher success rates and fewer complications overall (32). Many different surgical techniques and immunosuppressive regimens have been used and replaced by newer technologies, but transplantation medicine has yet to graduate from the use of immunosuppression altogether (33). Transplantation of pancreatic tissue specifically has additional drawbacks since some immunosuppressive agents have a negative impact on β cell function (34). Due to the side effects profiles of immunosuppressive agents and the grim reality that patient compliance is a far cry from one hundred percent (35), attempts to mitigate the impact of diabetes by transplantation are currently reserved for cases with the most difficult glycemic control.

In order to make less stringent the criteria justifying a pancreas or islet cell transplant, it will be necessary to understand the nature of functional tolerance, and determine the means by which it may be possible to circumvent the immune system’s natural defense mechanisms in order to persuade the body not to reject an allograft.
Developing a greater understanding of how the immune system permits some antigens and disallows others may provide pathways to this end. Some progress has been made in recent years. For example, several diabetes patients have become insulin-independent since receiving islet grafts facilitated by the Edmonton protocol. In these cases, steroids were not used, and newer immunosuppressive drugs such as tacrolimus, rapamycin, and anti-interleukin-2 receptor antibody were administered (36). With this breakthrough of the development of a repeatable protocol in which patients achieved insulin-independence, interest in islet transplantation is stronger than ever, bringing closer the reality of a true cure for diabetes (37).

Before it will be possible to harness the potential of functional tolerance to achieve immunosuppression-free transplantation, it will be necessary to learn more about what tolerance actually is, and by what means we might be able to control it. Systemic tolerance can be conceptually divided into central and peripheral tolerance. The induction and control of either has great therapeutic potential and it may be possible to modulate the nature of both in order to facilitate graft tolerance.

**Central Tolerance**

During normal T lymphocyte development, lymphocyte precursors emerge from the bone marrow as pluripotent stem cells, and undergo numerous stages of maturation after entering the thymus. The architecture of the thymus is intended to represent the library of antigens potentially found in the body, and is an effective training ground for T cells destined for the periphery. As T cell receptors are generated randomly in order to
achieve optimal antigenic diversity, it is possible for T cells to express receptors corresponding to self-antigens. The release of these T cells into the circulation could prompt an immune response against healthy tissue. Thus one role of the thymus is to educate the developing T cell repertoire as to the normal composition of the periphery and thereby define the outstanding set of antigens as non-self. When a T cell whose receptor corresponds to a self-antigen enters the thymus, it is deleted from the T cell repertoire to prevent its contribution to an autoimmune attack. This process is known as negative selection. Likewise, T cells whose receptors do not recognize antigens expressed in the thymus proceed to express normal CD4 or CD8 receptors and are permitted to exit the thymus and circulate in the periphery. This is termed positive selection.

It is primarily this process of T cell selection in the thymus that is exploited during the induction of central tolerance. By transplanting a graft donor’s bone marrow into a graft recipient, a state of hematopoietic chimerism can be achieved, specifically altering the cellular environment of the recipient’s thymus (38). At the point when a donor-specific T cell enters the thymus it is interpreted as self-specific due to the presence of graft-donor progenitor cells in the thymus, and is negatively selected. This depletes the emerging population of T cells of graft-specific as well as self-specific clones, creating an immunologically benign environment for the introduction of an organ graft (39).

The process of negative selection is by no means perfect. A high percentage of lymphocytes that undergo positive selection fall prey to a subsequent round of negative
selection (40). It is also well accepted that self-reactive T cells escape the thymus and are able to circulate in plain view of their cognate antigens (41). In pathological instances, this results in a situation of autoimmunity in which the self is inappropriately attacked. Luckily, this is not normally the case, as a safety net of mechanisms exists to maintain a non-inflammatory environment despite the presence of circulating self-reactive cells (42). These processes comprise peripheral tolerance.

**Peripheral Tolerance**

Despite decades of study, the processes that keep the immune system in check outside the thymus have not been fully described, nor have their respective relevance been assigned. There are numerous peripheral tolerance mechanisms that, most likely in combination, keep self-reactive cells in the periphery from developing into a pathological autoimmunity. One proposed mechanism of peripheral tolerance is termed immunological ignorance, a state in which immune cells circulate in the proximity of their cognate antigens, but for an unknown reason are not compelled to respond to their presence. This has been experimentally modeled by Ohashi, who showed an absence of cytolytic activity in LCMV-specific lymphocytes circulating in proximity to LCMV-epitope-expressing β-islet cells (43). When the mice in this study were infected with active LCMV, the lymphocytes became activated to confront and clear the infection, and incidentally attacked the epitope-expressing beta cells. It was only after the LCMV infection that these mice developed diabetes due to the cytolytic activity of the LCMV-specific memory population of T cells. Prior to infection, the virus-specific lymphocytes
circulated in a state of ignorance, possibly kept inactive because of an absence of
costimulation in the vicinity of the beta cells. This ignorance was broken when live virus
was introduced into the system, and the beta cells became casualties of the normal virus
clearing response.

Another proposed mechanism of tolerance in the periphery is extrathymic clonal
deletion, in which a population of lymphocytes specific to a given range of antigens is
directed into apoptosis in order to preserve the cells on which their cognate antigens
reside (44). This mechanism is usually most prevalent when high doses of antigen are
present (45). At lower doses, the emergence of cells that produce IL-4, IL-10, and TGF-
β is a more prevalent phenomenon, skewing the immune system toward a Th2 response.
This skewing results in decreased IL-2 production by T cells and decreased T cell
proliferation (46). These effects come under the umbrella term of immune deviation,
which encapsulates these processes and others.

Suppression of T cell responses has received a great deal of attention recently,
with most of the focus on the CD4⁺CD25⁺ regulatory T cells first described by Sakaguchi
(47). These have been shown to prevent T cell responses in a contact-dependent manner,
possibly orchestrated in some way by the transcription factor FoxP3 (48, 49) and the cell
surface protein GITR (50). The presence of these and other types of regulatory cells is
crucial to peripheral tolerance, as their absence results in the emergence of autoimmunity.

Another peripheral tolerance mechanism is T cell anergy, a state in which T cells
are actively rendered unresponsive to their cognate antigens (51). This form of peripheral
tolerance is the topic of this thesis and will be discussed at length in later sections.
Briefly, clonal anergy is a phenotype resulting from the sub-optimal activation of T cells, which renders them unresponsive to antigenic stimulation. It is possible that peripheral tolerance could be maintained over the long term by the rendering of specific clones of T cells anergic rather than facilitating their activation. In order to discuss anergy, it is useful to first describe the biochemical events that take place when a T cell is productively stimulated.

**T Cell Activation**

The anergic phenotype is best understood against the backdrop of T cell stimulation. During a normal immune response, CD4⁺ T cells are activated and have numerous responsibilities, such as activating B cells, providing IL-2 help for CD8⁺ T cells and producing interferon gamma (IFN-γ) to activate macrophages and dendritic cells. To prevent unsolicited T cell activity, T cells require two distinct signals through receptors on the cell surface (52). One of these receptors, called the T cell receptor, is unique to each T cell, and as a whole, the T cell repertoire is capable of recognizing and reacting to an incredible diversity of antigenic patterns. The other signal can be delivered through any of a number of receptors common to all T cells, generically known as costimulatory receptors. The most common of these is CD28. The sum effect of a T cell receiving these two signals is the production of IL-2, the primary growth cytokine for T cells (Figure 3). The IL-2 produced is secreted by the cell concurrently with the up-regulation of the IL-2 receptor, at which point IL-2 acts as an autocrine factor to drive clonal expansion. The biochemical mechanisms of IL-2 production are complex and the
Legend for Figure 3

T cells require two signals for activation. The sum effect of a signal through the T cell receptor and a signal through a costimulatory receptor is a transduction cascade resulting in the production of IL-2, a primary T cell growth cytokine. IL-2 is secreted from the cell and acts as an autocrine factor, signaling via the T cell’s own IL-2 receptor. The result of this is cell-cycle progression and clonal expansion.
subject of intense scrutiny. Upon receipt of a signal through the TCR from an MHC II molecule presenting its cognate peptide, along with a costimulatory signal from a B7 family member, there is a wave of protein tyrosine kinase (PTK) activity that commences T cell receptor signaling (53). The result of this activity is the initiation of downstream signaling pathways including the mitogen activated protein (MAP) kinase pathway, an influx of calcium, and the nuclear factor (NF) κB pathway. The sum of the contributions from these three major T cell signaling pathways determines whether the outcome is a stimulating or an ineffective signal.

Following TCR engagement, Src kinases Lck and Fyn are dephosphorylated by CD45 family members (54). Now activated, these kinases are available to activate ZAP-70 and Tec kinase family members (55), which can then activate immunoreceptor tyrosine-based activation motifs (ITAMs) found in the cytoplasmic subunits of the TCR complex (56). These phosphorylation events are subject to constant regulation by protein phosphatases, and only when this regulation is held at bay by formation of the immune synapse can successful TCR signaling occur.

One of the downstream consequences of PTK activation, as mentioned previously, is an influx of calcium into the cell cytoplasm. The signaling cascade leading to this biochemical event is initiated by the activation of phospholipase-Cγ1 (PLC-γ1), which cleaves membrane phosphatidyl inositol-4,5 biphosphate (PIP$_2$) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP$_3$) (57). An increase in cytoplasmic IP$_3$ concentration releases Ca$^{2+}$ from endoplasmic reticulum stores, followed by the opening of surface membrane Ca$^{2+}$-channels permitting additional Ca$^{2+}$ influx from the
extracellular space (58). A sustained increase in intracellular calcium activates calcineurin, a phosphatase capable of targeting the Nuclear Factor of Activated T cells (NFAT) (59). NFAT dephosphorylation exposes a nuclear localization signal, which permits its passage across the nuclear membrane, at which point it can drive gene transcription including that of the IL-2 gene.

The other by-product of PLC-γ1 activation, diacylglycerol, activates a number of signaling molecules including various isoforms of protein kinase C (PKC) (58). One result of PKC activation is the activation of p21<sup>ras</sup>, which requires the exchange of GTP for GDP to be activated. Ras activation triggers a cascade of kinase phosphorylation events resulting in activation of the MAP kinases ERK, JNK, and p38 (58). In various combinations, these activated factors migrate to the nucleus and up-regulate jun and fos transcription factors, which return to the nucleus and form a heterodimer named Activating Protein-1 (AP-1). AP-1 binds to numerous sites in the IL-2 promoter, either alone or complexed with NFAT, and is required for the up-regulation of IL-2 (60).

A third transduction pathway crucial to T cell activation is the NFκB pathway. It has been proposed that CD28 plays a crucial role in NFκB signaling, via the activation of PI-3-kinase acting in synergy with PKC (58). NFκB is permitted to cross the nuclear membrane upon ubiquitination of its inhibitory binding partner, IκB. NFκB, like NFAT and AP-1, contributes to T cell activation in part by binding the IL-2 promoter region and driving transcription of IL-2. Some of the major elements of the T cell activation signal transduction pathways are diagrammed in Figure 4.
Legend for Figure 4

T cell signaling is a complicated network of signal transduction cascades. IL-2 production requires input from at least three major signaling pathways: NFAT, the MAP kinase pathway, and NFκB. Each of these involves many intermediate steps that are subject to numerous regulatory mechanisms.
Costimulation

For a time, it was thought that the presence or absence of a signal through the prototypic costimulatory receptor CD28 was the primary determinant of the outcome of a tolerizing or immunizing stimulus. In the early nineties, the protein product of a gene on the same chromosomal band as CD28 was found to bind B7 family members with a greater affinity than CD28 (61). This protein, CTLA-4, was found to inhibit T cell responses, and its discovery ushered in the possibility that the decision between activation and inhibition when a T cell is stimulated depends on some balance of signals, modulated by both the lymphocyte and the antigen presenting cell. Since these studies were conducted, a number of other costimulatory and coinhibitory receptors have been found. The mechanisms by which these groups of signaling proteins act are quite diverse (62). Although the mechanisms are still under investigation, a number of interesting characteristics of these pathways are known. CD28 and CTLA-4 bind the same receptors, B7.1 and B7.2 (CD80 and CD86, respectively), although it has been shown that CTLA-4 can exert inhibitory effects independently of these (63). Other costimulatory and coinhibitory receptors have unique ligands although most are members of the B7 family, which has grown significantly in the past decade (62). Another T cell costimulatory receptor is ICOS, which binds to ICOS Ligand in order to activate T cells. Unlike CD28, ICOS does not signal to NFκB or up-regulate CD40 (64), but it does stimulate PI3K activity to a greater extent than CD28 (65). This apparent lack of redundancy illustrates the complex interplay of signals involved in costimulation, the full extent of which is continuing to emerge. Other than CTLA-4, one of the most well studied coinhibitory
receptors is PD-1. Originally discovered in apoptosis models (66), PD-1 has garnered much more attention for its inhibitory effect on T cell signaling. The mechanisms of PD-1 inhibition have been the subject of many studies since its discovery, which will be discussed at length in later sections of this dissertation.

Since it was discovered that two signals are required for full T cell activation, studies have attempted to describe the individual contributions of the TCR and the costimulatory receptor to IL-2 production. These studies have revealed that both transcriptional and post-transcriptional regulatory mechanisms are most likely in place to control the amount of IL-2 being produced. Studies in human T cell blasts have demonstrated that CD28 ligation by B7 family members in the absence of TCR triggering results in AP-1 and NFκB activation, but not NFAT translocation (67). These activation events were not sufficient to produce IL-2 suggesting that these signals serve mostly to complement signals delivered by the TCR. CD28 ligation on resting T cells effected no change in these studies. These findings were confirmed by a group examining the effect of individual TCR and CD28 receptor engagement, who showed that very little JNK is activated when these are stimulated individually, whereas it is strongly activated in the presence of both signals, suggesting a synergism rather than an additive relationship between the two (68). The importance of JNK as a possible point of cross-talk between the TCR and CD28 signals has been confirmed by studies showing that its activity is necessary for activation of disparate elements on the IL-2 promoter region, primarily the AP-1 binding sites and the composite CD28RE/AP-1 site. This implicates JNK in the NFκB pathway as well as the AP-1 pathway via the MAP kinases (69).
In addition to the regulatory mechanisms in control of IL-2 transcription, there is evidence that there are post-transcriptional factors that determine levels of IL-2 protein production. Studies examining the effect of costimulation on IL-2 production have shown that the half-life of IL-2 mRNA is prolonged in the presence of costimulation, compared with cells stimulated through the TCR only (70). It is likely that this prolongation is due to the blockade of destabilizing elements located in the 3' UTR region of the mRNA by CD28 receptor engagement, although the mechanism of this has not been shown. It has been proposed that the ability to produce IL-2 conferred by CD28 signaling is in large part due to the ability of CD28-derived signals to stabilize IL-2 mRNA rather than providing a signal required for IL-2 transactivation (71).

Chapter 1 Discussion

Curing type 1 diabetes will require protocols that enable the successful transplantation of β-islet cells into diabetic patients. Because existing transplantation procedures have a significant negative impact on patient health, it will be necessary to develop protocols more benign than those currently used, preferably avoiding the use of immunosuppressive agents altogether.

T cell activation is necessary to an effective immune response, including the rejection response against a transplanted graft. It is the global suppression of this response that enables current transplantation procedures, but preferable would be to tailor the immune system not to respond to the set of antigens found on a graft while maintaining its competence toward any other foreign invader. It is conceivable that this
tailoring process will involve the incapacitation of graft-specific T cell clones, blocking their ability to attack a graft and thus protecting the graft from immune rejection. Inducing anergy in graft-specific T cells may aid in dampening their ability to reject a graft.

There is an indissoluble relationship between IL-2 production, which is required for T cell activation, and T cell anergy. It is useful to discuss the details of IL-2 production in order to provide context for the known biochemical characteristics of anergic T cells. The absence of an IL-2 signal upon T cell stimulation ushers a T cell into an anergic phenotype, and once anergy is established, it is rendered incapable of IL-2 production and therefore unable to proliferate. The characteristics of T cell anergy are the focus of Chapter 2.
Chapter 2 -

T Cell Anergy

History of Anergy

Clonal anergy is a T cell phenotype characterized by the inability to produce IL-2 (72). Nossal and Pike borrowed the term ‘anergy’ from Von Pirquet in 1980, to describe a phenotype of hypo-responsiveness in B lymphocytes (73). Jenkins and Schwartz described an analogous phenomenon in T cells resulting from stimulation with ECDI-fixed splenocytes (74). Although numerous biochemical defects have been found in anergic cells as the result of two decades of research, it has not yet been conclusively determined what genetic programs are in place to institute and maintain the prevention of IL-2 production.

Early anergy studies observed that ECDI-treatment of antigen presenting cells (APC) resulted in the establishment of an unresponsive phenotype in T cell clones, rather than the expected stimulation (75). These studies were performed prior to the discovery that CD28 signaling is required for full T cell stimulation, but it was hypothesized at the time that the result of the ECDI treatment was the fixation of some ligand whose receptor was required for IL-2 production. The studies also showed that chelation of calcium from the culture medium with EGTA prevented the hyporesponsive phenotype completely. Conversely, addition of the calcium ionophore ionomycin induced this unresponsive phenotype. These studies implicated intracellular calcium signals as crucial to the induction of anergy. Follow-up studies showed that MHC-containing planar lipids were not capable of stimulating T cell clones, and also rendered the cells unresponsive to
subsequent stimulation with normal APC and antigen (76). This impaired stimulation could not be overcome by the addition of exogenous IL-3, IL-1, IL-4, IFN-γ, or GM-CSF, and could not be induced in the presence of cyclosporine A, confirming the importance of calcium in the induction of the hyporesponsive phenotype.

In the ECDI-fixation studies, the addition of allogeneic splenocytes to the anergizing culture restored the ability of the ECDI-treated syngeneic splenocytes to fully stimulate the T cells. This finding provided evidence for the hypothesis that the induction of the hyporesponsive state is due to the absence of some non-MHC restricted signal that is obliterated by APC fixation with ECDI. Further studies found that the addition of T cell clones to αCD3 antibody immobilized on plastic could induce an identical anergic phenotype, confirming the finding that stimulation of T cells through the TCR alone results in a long lasting anergic phenotype rather than an activation response (77). At the time these early studies were performed, it was not known which receptor or receptors on the T cell were capable of providing this necessary additional signal. Shortly following these studies, it was demonstrated that CD28 signaling was capable of augmenting IL-2 production in conjunction with TCR signaling, and Jenkins was able to block the induction of anergy in T cell clones by the addition of αCD28 antibody (78). These studies demonstrated that it is possible to both induce anergy and stimulate T cells by selectively engaging specific receptors; they also provided models with which clonal anergy and stimulation can be studied under controllable circumstances.

Once anergy is established, it persists for seven to ten days if the cells are unmanipulated after induction (76). Studies by Beverly, et al (79), demonstrated that in a
ConA-induced anergy model (shown to be phenotypically similar to previously described methods), the addition of exogenous IL-2 to the anergized cells restored their ability to proliferate at levels similar to those of previously untreated cells. These results were confirmed by at least one other group (80). The results indicated that anergic T cells are defective in their ability to produce IL-2, but are capable of responding to exogenous IL-2. This raises the important question as to what specifically leads to the induction of anergy: given that IL-2 is the primary growth cytokine for Th1 T cells, what is the relationship between proliferation and the induction of anergy? More specifically, does the addition of the costimulatory signal to the TCR stimulus actively counter the anergizing program that is established, or does it rather augment IL-2 production to levels sufficient for proliferation, thus overcoming whatever conditions are present to maintain anergy? This question was answered by blocking IL-2 signaling in T cells stimulated with APC and antigen. αIL-2 and αIL-2R antibodies were used to neutralize IL-2 activity, resulting in the prevention of T cell proliferation, as expected (81). Importantly, after the cells were rested for five days, they were unresponsive to normal antigen-pulsed APC. This result shows that T cells can be anergized even in the presence of a costimulatory signal, though it was not known at the time of these experiments whether this was due to the absence of IL-2R signaling or cell cycle paralysis.

Later studies sought to investigate this phenomenon further, dissecting apart the IL-2 receptor pathway with pharmacological agents whose activities block the cell cycle in different phases. The important findings from these studies demonstrated a contrast between the effects on cell cycle progression of hydroxyurea, a drug that arrests cell-
cycle progression after the cells reach S phase, and those of rapamycin (sirolimus), which arrests the cells prior to the G1→S phase transition (82). In light of the body of prior literature, Powell et al. proposed the existence of an anergy pathway parallel to the stimulation pathway that negatively regulates T cell activation in the absence of IL-2 signaling. In these studies, CD4+ T cell clones fully stimulated in the presence of hydroxyurea were arrested after transition to the S phase, but after removal of the drug they were not anergic upon restimulation. This was not the case when activated T cells were treated with rapamycin. Rapamycin treatment permits T cells to make normal amounts of IL-2 (83), but the IL-2 receptor signal is blocked at the mammalian Target of Rapamycin (mTOR). When the rapamycin was removed from the stimulated T cells, they were unresponsive to antigenic stimulation; blocking the IL-2R signal anergized the cells. The importance of these data is that they show that anergy is not a passive phenotype, and that its abrogation is dependent on signals downstream of the IL-2 receptor.

The importance of mTOR to anergy abrogation was later demonstrated by stimulating T cell cultures in the presence of drugs that block the G1→S phase transition by different mechanisms. These experiments concluded that the ability of rapamycin to induce anergy was specific to its targeting of mTOR, rather than just its ability to block cell cycle progression (84). This conclusion was confirmed by experiments showing that IL-2-independent T cell proliferation is unable to reverse anergy (85). These findings further implicate the IL-2 receptor signal as being primarily responsible for the reversal of anergy in T cell clones, and suggest the existence of a pathway parallel to the TCR-
dependent stimulation pathway, that, in the absence of IL-2 signaling, results in the anergic phenotype.

Signal Transduction in Anergic Cells

The inability of anergic T cells to produce IL-2 has prompted investigators to examine signaling differences between these and proliferation-capable lymphocytes. Examination of other cytokines such as IL-3 (81) and IFN-γ (75) revealed negligible differences between the two. Importantly, it was found that frequently changing the culture medium following anergy induction or productive stimulation resulted in anergy in both cell populations (79). These data suggest that it any factors responsible for establishing anergy are not soluble. It is possible that IL-2 production is prevented by the presence of a biochemical block in signal transduction upstream of IL-2 gene transcription, or that IL-2 transcription is actively repressed at its gene promoter. These have been questions about the mechanism of anergy since its discovery. Neither question has been definitively answered, although a number of biochemical changes have been found in anergic cells.

There are many points in the IL-2 transcription pathway at which IL-2 production could be disrupted. As mentioned above, not only is the presence of calcium essential to productive stimulation, but its chelation prevents anergy induction: therefore in early studies it was deemed unlikely that NFAT was defective in anergic cells. Indeed, more recent studies have shown that NFAT1−/− T cells cannot be anergized (59). Crucial for IL-2 production is a complex of NFAT bound to AP-1, consisting of jun and fos
heterodimers. Thus, the inactivation of jun and fos would result in impaired IL-2 production, and possibly prevent NFAT from binding to crucial elements in the IL-2 promoter region. Analysis of nuclear extracts from anergic cells showed a decrease in c-fos and JunB protein expression levels (86), and promoter region studies showed that transcriptional activity at both the AP-1 and the NFAT/AP-1 binding sites was impaired. Follow up studies examining upstream members of the MAP kinase pathway showed that ERK and JNK total protein levels were equal in anergic cells and stimulated cells, but the enzymatic ability of both kinases was tested and found to be impaired in anergic cells (87, 88). Since ERK and JNK are intermediate steps in the MAP kinase pathway, the question remained as to the upstream cause of these defects.

p21ras is a GTP-ase shown to be a crucial regulatory point for the MAP kinase pathway (58). This makes it a clear candidate for studies hoping to identify a biochemical switch toggling between productive stimulation and anergy. Studies contemporary to the ERK and JNK experiments mentioned above discovered that the inactive state of these kinases was due to a lack of phosphorylation on key activating tyrosines (89). The investigators pursued the cause of this by examining the ability of Ras to bind GTP, the primary indicator of its activation status. They found that Ras is unable to bind GTP in anergic cells whereas previously stimulated cells could activate Ras normally. Interestingly, stimulating anergic cells with PMA and ionomycin could activate Ras normally. This is perhaps the same mechanism by which these reagents can reverse anergy. As to the cause of this inability of anergic cells to activate Ras, a number of proteins known to regulate Ras including Shc, Grb-2, and mSOS were examined. Their
protein levels and activation statuses were found to be equal in anergic and stimulated cells (89), ruling out any specific role for them in anergic cell populations. Crespi et al. further investigated the role of defective Ras in anergy by expressing a constitutively-active Ras in T cells, showing that they were still able to be anergized despite the reversal of the ERK phosphorylation defect (90). Interestingly the constitutive activation of Ras enables anergic cells to produce IL-2 in response to ionomycin alone, bypassing the need for PMA stimulation. This suggests that a major role of PMA in productive stimulation is the activation of Ras. It remains to be determined by what mechanisms anergic cells hold Ras in an inactive state. It has been proposed that a yet-unidentified RasGAP is active in anergic cells, thwarting upstream attempts to activate Ras by driving the autocatalytic ability of Ras to cleave GTP to GDP (72). The identification of such a factor would possibly provide a point for therapeutic manipulation of anergic T cells, and is currently the goal of many studies. A depiction of the known signaling defects in anergic T cells is shown in Figure 5.
Legend for Figure 5

Anergic T cells are unable to make IL-2. It is possible that this is a result of defects found in signaling pathways known to be required for productive T cell stimulation. It is believed that the primary barrier to IL-2 production in anergic cells is a defect in the MAP kinase pathway, specifically the inability to activate Ras, ERK, and JNK.
Regulatory T cells, Anergy, and Immune Regulation

The concept of T cells with suppressor or regulatory capabilities has reemerged in recent years, and has gained a great deal of momentum since the discovery that CD4^+CD25^+ cells have the ability to regulate other T cell populations. For a time, the existence and relevance of T cell-mediated active suppression was controversial, but studies have demonstrated well the importance of regulatory T cells in peripheral tolerance, transplantation, and autoimmunity (91). Interestingly, studies have shown that not only do regulatory T cells have the ability to suppress distinct populations of T cells, but they also manifest signs of anergy. It is possible that the anergic phenotype is important to the function of regulatory T cells, and learning more about anergy and the role that regulatory T cells play in peripheral tolerance may aid in developing safer methods of inducing transplantation tolerance.

Early studies demonstrated the importance of regulatory T cells in autoimmune models by periodically transfusing whole blood from non-diabetic rats into diabetes-prone strains and preventing diabetes in these rats (92). Importantly, depleting the T cells from these transfusions reversed the protective effect (93). Further studies explained this reversal by identifying a population of W3/25^+ T cells whose presence in blood transfusions protects the diabetes-prone BB/W rat from autoimmunity. This led to the speculation that a contributing factor to diabetes in the diabetes-prone rat is a defect in this cell population (9).

Recently, the number of studies focused on regulatory T cell populations has increased dramatically, and this cell population has been found to be important to many
facets of autoimmunity, natural peripheral tolerance, tumor immunity, and transplantation (94). A result of this increased attention has been the discovery of a number of subpopulations of regulatory T cells. These subpopulations include those emerging from the thymus expressing CD4 and CD25, which are referred to as naturally occurring CD4⁺CD25⁺ T_{reg} cells; T_{reg} that do not express CD25 as a surface marker; and IL-10 producing T_{reg} cells (95).

The relationship between these populations and their individual roles in immune regulation has not yet been fully determined. It has been shown that the transcription factor FoxP3 functions in some capacity as a master regulatory switch for the development of T_{reg} cells, and that its forced expression in naïve T cells confers on them the properties of a regulatory T cell (96). Defects in FoxP3 have been found in IPEX/XLAAD mice, which develop an X-linked lymphoproliferative disease caused by defective T cell tolerance, supporting its importance in immune regulation.

Although the details of how regulatory T cells exert their suppressive effects are not fully known, TGF-β has recently been shown to play an important role. Freshly isolated naïve CD4⁺ cells treated with TCR stimulation and exogenous TGF-β develop the ability to suppress CD4⁺CD25⁻ responder cells in a contact-dependent fashion. The development of this ability may be due to the increase in their cell surface expression of TGF-β. (97). It has also been shown that TGF-β treatment of naïve CD4⁺CD25⁻ cells increases their expression of Foxp3, suggesting their conversion to regulatory T cells (98). The importance of TGF-β is not limited to its ability to induce regulatory cell properties; it has been shown to play an important role in the actual suppression process.
This has been demonstrated in part by studies showing that cells without the ability to respond to TGF-β are not subject to suppression by naturally arising regulatory T cells (99).

It is possible that the anergic phenotype is important for the proper functioning of regulatory T cells. Sakaguchi has shown that naturally arising CD4⁺CD25⁺ regulatory T cells are phenotypically anergic, in that they do not proliferate in response to TCR stimulation (100). Rather than eliciting a proliferative response as it would for a naïve T cell, triggering the TCR on a CD4⁺CD25⁺ suppressor cell activates its capacity to suppress the proliferative response of CD4⁺CD25⁻ T cells in its proximity. Other studies have found the anergic and suppressive phenotypes in the same cell population – these cells don’t proliferate or produce IL-2, and have the ability to prevent proliferation in nearby CD4⁺CD25⁻ lymph node cells (101).

With regard to biochemical signaling, similarities have been found between the form of anergy seen in regulatory cells, and the form seen in cells anergized by conventional methods, but the extent of their equivalence remains to be seen (102). Apart from the fact that there may be phenotypic similarities between these populations of cells (Figure 6), it has also been shown that regulatory T cells may exert their suppressive effects not only by transiently preventing proliferation in other populations of T cells, but by inducing an anergic phenotype that persists even in the presence of subsequent costimulation. This form of suppression has been found to correlate with one of the factors currently thought to be essential to anergy induced by traditional anergizing methods (103).
Expression of PO-Egr-E3 Ligases
Characterized by a unique transcriptional profile

Anergic T Cell
Hyporesponsive to TCR Stimulation
Unable to Produce IL-2
Expression of CD4 and CD25

Regulatory T Cell
Expression of FoxP3, LAG3, TGFβ, RT6
Characterized by a unique transcriptional profile

Figure 6
Legend for Figure 6

Anergic cells and regulatory T cells share characteristics. Both cell populations are refractory to TCR stimulation, and neither population is able to make IL-2 in a costimulatory environment.
The importance of suppressor T cells in providing protection against autoimmunity has been known for some time. Recently, their immunoregulatory capabilities have been implicated in transplantation models, functioning both systemically (104) and at the site of a tolerated allograft (105). Increasing our knowledge of these types of cells may not only provide insight into preventing diabetes; there is also the chance that learning to direct the ability of these cells to suppress specific sets of antigen-responsive immune cell populations may provide the opportunity to cure diabetes by facilitating peripheral tolerance without general immunosuppression.
In vitro and in vivo models for T cell anergy

There have been a number of models developed to study anergy. Though they are mostly dissimilar in the means by which they induce anergy, the end result is the same: the inability to produce IL-2 and proliferate upon antigenic stimulation. The following section describes a number of methods of anergy induction, in vitro and in vivo.

Immobilized Anti-CD3 Antibody

When Jenkins and Schwartz first began to examine T cell anergy, they stimulated T cell clones with splenocytes fixed with ECDI (74). Subsequent experiments led to the discovery that T cell clones could be anergized with immobilized αCD3 antibody, and were productively stimulated when soluble αCD28 antibody was added to the culture medium in addition to αCD3 (77). The in vitro model we have chosen to study is the T cell clone A.E7, provided by the Schwartz laboratory. A.E7 T cells were derived from B10.A mice immunized with pigeon cytochrome C and carried by stimulation and expansion every two weeks, by a method modified from that described previously (106). The ability to maintain a pure population of cells throughout an experiment provides the opportunity to lyse cells for Western blotting or RNA purification for RT-PCR, which would not be possible if a model involving the use of APCs was chosen. Although the use of antibodies for stimulation is less physiological than some other models, the opportunity to use pure clone cultures is invaluable for answering biochemical questions.
Altered Peptide Ligands

Shortly following the studies demonstrating the effect of fixed splenocytes on T cell activation, studies using modified TCR ligands (alterations in the cognate peptide sequence) showed that T cells could be anergized even in the presence of costimulation (107). By changing amino acid residues in the peptide sequences, the authors could weaken TCR signaling sufficiently to induce a state of hypo-responsiveness similar to that seen with fixed antigen presenting cells. It was hypothesized that in this model of anergy induction, it was not the lack of a signal through costimulatory receptors that determined the activation status of the cell, but rather the inability of the TCR to provide sufficient signal to make IL-2, even though full costimulation was present. This form of anergy induction has recently been shown to have potential therapeutic value in the treatment of autoimmunity; the use of altered peptide ligands in a myasthenia gravis-susceptible mouse strain resulted in the amelioration of autoimmune symptoms and a phenotypic change in lymphocytes, which became anergic (108). It was subsequently shown that this hypo-responsiveness was due to altered CD3-ζ chain phosphorylation and an inability to recruit phosphorylated ZAP70 to the TCR signalosome (109).

Ionomycin

Ionomycin is a calcium ionophore that triggers the release of calcium from the endoplasmic reticulum and an influx of calcium from the extracellular space (110). The importance of the calcium pathway to anergy induction has been shown (77) and is substantiated by the fact that a strong calcium signal in an otherwise unstimulated cell
can confer an anergic phenotype to a T cell (59, 111). This method of anergy induction has proven effective in T cell clones and ex vivo purified CD4+ T cells (112). There is some evidence that this method anergizes cells by a mechanism different from that of conventional TCR-signal-only anergizing methods (59).

Division-Arrest Anergy

An IL-2-refractory model of anergy has been described by Wells and Turka, who stimulated CFSE-stained CD4+ T cells ex vivo and found a correlation between a lack of initial proliferation and the entry of the cells into a hyporesponsive state (113). This fits with currently understood models of anergy: stimulated cells that don’t proliferate become anergic. Interestingly, unlike most other forms of anergy, the anergic subpopulation of cells does not respond to exogenous IL-2, but can be restored to full reponsiveness by treatment with PMA and ionomycin. It has not yet been shown what specific disconnect exists biochemically in these cells to prevent the IL-2 receptor from delivering a proliferative signal, but the authors do correlate the presence of the phenotype with increased expression of p27kip1, a known cell-cycle inhibitor implicated in other forms of anergy (114). Whether the presence of p27kip1 is simply a correlation or the cause of cell-cycle inhibition in this case is not discussed by the authors and is not yet known.
Intravenous Peptide Tolerization

The model of \textit{in vivo} anergy induction most analogous to anergy observed in cell clones was developed by Jenkins while in the Schwartz laboratory (72). This model consists of the adoptive transfer of small numbers of transgenic cells, identifiable by a clonotypic antibody, into a non-irradiated, immune competent, syngeneic recipient. After transfer, cells are tolerized with an intravenous injection of their cognate peptide, or immunized with an injection of peptide with lipopolysaccharide. Cells are permitted to circulate for twelve days and are then purified out of the spleen and lymph nodes for FACS staining and rechallenge with peptide (115). There is at least one example in the literature of this model being used to confirm the function of a proposed ‘anergy factor’ (112), and the cells exhibit behavior very similar to that in the above described model of anergy induction \textit{in vitro}.

Non-Mitogenic Anti-CD3

The ability of mitogenic anti-CD3 antibodies to prolong graft survival has been shown (116-118), but in many cases the acute activation of immune cells prior to the tolerizing effect of the antibody proved problematic (119). This led to the search for ways to make this reagent less mitogenic and more tolerogenic, a goal achieved by decreasing the affinity of the antibody $F_c$ region for $F_c\gamma$-receptors (120, 121). Further investigation into the consequences of this alteration showed that this decreased ability to bind $F_c$ receptors resulted in impaired cross-linking of the TCR complexes, an event necessary for full TCR activation. This prevented the T cells from making sufficient IL-2 to drive
cell proliferation, and subsequently led them into an anergic state from which they could be rescued with exogenous IL-2 (122). In contrast to other anergy models, this form was found to manifest deficient TCR signaling, insufficient PLC-γ activation, and an impaired calcium flux in response to TCR triggering. Biochemically, it appears that the type of anergy induced by non-mitogenic αCD3 is somewhat distinct from the clonal anergy first described by Schwartz, et. al.

The use of non-mitogenic αCD3 antibody in the regulation of peripheral tolerance has proved to be promising (123-126). The precise mechanism by which αCD3 antibody is able to modulate autoimmunity remains controversial, but it has been proposed that it is stimulating a population of regulatory T cells (127) or inducing a functional tolerance similar to anergy (128, 129).

These findings suggest that in some capacity, anergy may be an important contributory mechanism to successful transplantation, and thereby merits study to further elucidate the biochemical nature of the phenotype.
Proposed Anergy Factors

Early studies of anergy hypothesized that a unique signaling pathway might be involved in its orchestration. This idea was first proposed first by Quill and Schwartz, who demonstrated that cyclohexamine treatment could prevent the induction of anergy (72) (Figure 7). The findings were extended by Powell, et al (82), who showed that cell cycle progression from G1 to S phase incapacitates some proposed molecular factor or pathway whose presence is necessary for the anergic phenotype to persist. Many studies have attempted to characterize this putative pathway and identify these factors. The following is a summary of these attempts.

Boussiotis et al. found that p27kip1 was specifically expressed in hypo-responsive T cell populations both in vitro and in vivo, and further showed that p27kip1 overexpression prevented jun activation in anergic cells, resulting in defective AP-1 transcription and IL-2 production (114). Subsequent to these studies, it was shown that the amounts of IL-2 required to reduce p27kip1 levels were far lower than those required to reverse anergy, and that cells from p27kip1/- mice were fully capable of being anergized, calling into question its importance to the anergic phenotype. It is interesting to note that Boussiotis employed overexpression of p27kip1 to demonstrate IL-2 protein suppression, and later studies that reduced the amount of p27kip1 expression to more physiological levels did not suppress IL-2 production upon T cell rechallenge (130).

Boussiotis continued her studies and subsequently discovered that Tob-1, a known cell cycle inhibitory gene, is expressed in both anergic and quiescent populations of T cells, and that its depletion with antisense oligonucleotides reduced the need for
Legend for Figure 7

The T cell receptor signals via two distinct pathways. If costimulatory signals are present, they are able to synergize with the TCR activation pathway, resulting in IL-2 production and proliferation. In the absence of costimulation, a second pathway institutes the anergic phenotype, which is then capable of preventing IL-2 production, even in the presence of subsequent costimulation.
costimulation in making sufficient IL-2 to drive proliferation. These studies showed that Tob-1 acts not by inhibiting the MAP kinase pathway, whose defects in anergic cells have been well demonstrated, but rather by collaborating with Smad to bind the -105 negative regulatory element of the IL-2 promoter (131). Despite their seeming relevance, these studies have not been followed up, and no further details regarding the role of Tob-1 in anergy have appeared in publication since these studies.

Another proposed anergy factor is lymphotactin, a cytokine whose high expression has been reported in unresponsive Th1 T cell clones. It was initially proposed to be a member of the putative anergy pathway, but it was subsequently found that this protein is down-regulated by CD28 signaling prior to IL-2 production. It is likely that lymphotactin was not pursued in this context because the putative anergy factor proposed by Powell would be down-regulated by IL-2R signaling after the production of IL-2, not by CD28 signaling prior to IL-2 transcription (132).

The role of E3 Ligases in T Cell Anergy

Despite the numerous studies claiming to have identified the sought-after 'anergy factor', the precise biochemical picture of anergy continues to elude us. The majority of studies mentioned above claim to have discovered a factor crucial to the anergic phenotype, yet none has definitely demonstrated that their factor is essential to anergy, and each factor is implicated indirectly at best. An exception to this trend is a class of signaling molecules termed E3 ubiquitin ligases, whose functions are necessary for
shuttling proteins to the proteasome for degradation. Recent studies have been quite definitive in demonstrating the importance of E3 ligases in anergy.

The importance of the proteasome degradation pathway to anergy has been well illustrated in the past few years, beginning with the discovery of GRAIL (Gene Related to Anergy in Lymphocytes), a novel E3 ligase (133). Briefly, the proteasome degradation pathway is a post-translational regulatory mechanism that disposes of proteins by mechanically breaking them down. This pathway is initiated by an E1 ubiquitin-activating enzyme that charges a ubiquitin molecule so it can bind an E2 ubiquitin carrier protein/E3 ubiquitin ligase complex (134). The E3 ubiquitin ligase can then transfer the ubiquitin to the target protein, labeling it to be shuttled to the proteasome where it is degraded.

GRAIL was found in an in vitro artificial APC anergy model to be highly expressed in anergic T cells after four hours of stimulation, and almost undetectable in unstimulated T cells (133). As an alternative method of anergy induction, cells were anergized with ionomycin and it was determined that GRAIL expression peaked after six hours of ionomycin treatment and had decreased significantly by eighteen hours, suggesting a very early window of activity for GRAIL in the anergy induction process (112). Constitutively-expressed GRAIL was shown to inhibit IL-2 production in T cell hybridomas, an effect successfully abrogated by mutating GRAIL’s RING domain, thus abolishing its ability to transfer ubiquitin molecules to other proteins. Follow up studies showed GRAIL to be capable of inhibiting T cell proliferation in stably-transduced GRAIL-expressing cells ex vivo (112). These studies further showed that GRAIL-
expressing CD4+ T cells manifested signs of being anergic, which were reversed upon mutation of GRAIL’s RING domain.

This first example of the potential importance of E3 ligases ushered in a new era in anergy research (135). Until the discovery of GRAIL, it was not known what prevented proliferation early in the anergy induction phase, permitting the full establishment of the phenotype. After the GRAIL studies were published, another group published studies using ionomycin to induce anergy and showed that GRAIL, along with two other E3 ubiquitin ligases, Itch and Cbl-b, was transcriptionally up-regulated as a result of strong calcineurin activation (136). These studies further demonstrated the importance of E3 ligases in anergy induction by showing a correlation between the transactivation of these factors, primarily Itch and Cbl-b, and the degradation of a number of signaling factors known to be crucial for successful TCR signaling. Among these is PLC-γ1, which is responsible for the cleavage of PIP2 into IP3 and DAG, an early event in the TCR signaling pathway. The authors also showed the degradation of PKC-θ, a regulatory point in Ras and MAP kinase pathway activation. It has also been speculated that this E3-ligase-driven degradation program also prevents T cell activation by weakening the interaction between the T cell and the APC, thus cutting short the time required for full signaling (137). These correlations suggest that the presence of E3 ligases in the early induction of anergy preempts successful T cell receptor signaling due to their ability to direct crucial signaling proteins such as PLC-γ1 and PKC-θ to the proteasome for degradation rather than permitting their participation in T cell signaling cascades. The importance of Cbl-b to anergy induction was confirmed \textit{in vivo} by studies
showing a lack of proliferation in peptide tolerized adoptive transfers of Cbl-b+/+ CD4+ T cells, an effect reversed when the same experiments were done with Cbl-b/- cells (138). A simplified depiction of the role of E3 ligases in anergy induction is shown in Figure 8.

The discovery of the importance of E3 ligases in anergy has provided some insight into the early events in T cell signaling that dictate the decision between productive stimulation and anergy. It is likely that the predominant role of these factors is very early in the signaling process, and their activity is one of the criteria by which a cell chooses to become anergic rather than proliferate and expand. Further studies of these and other E3 ligases will aid in understanding the importance of E3 ligases to the immune response as a whole.
Legend for Figure 8

E3 ligases prevent IL-2 production early in anergy induction. A. PLC-γ1 and PKC-θ, known to be important in the IL-2 production pathway, are activated upon TCR + CD28 stimulation. B. The presence of E3 ubiquitin ligases early in the anergy induction process leads to ubiquitination of PLC-γ1 and PKC-θ, resulting in their degradation. This disrupts IL-2 production and proliferation.
Anergy and Peripheral Tolerance

Studying the biochemical characteristics of anergic cells provides interesting insight into T cell function, but against a physiological backdrop studying anergy is only useful to the extent that it fits into the dynamics of the immune system as a whole. There have been studies offering evidence that anergy may be an active process in numerous instances of peripheral tolerance, including the dampening of allo-immune responses. Studies using primary cells to investigate the CD4⁺ T cell response to allo-splenocytes have shown a significant decrease in allo-responsiveness when αCD154 (CD40L) antibody was included in the MLR culture, an effect abrogated by the addition of mitogenic doses of IL-2 to the culture (139).

It is well established that αCD154 antibody treatment concurrent with a donor specific transfusion (DST) prolongs allograft survival (140-142); the mechanism by which this occurs is still being investigated. One function of CD40 on APCs is to signal the up-regulation of costimulatory molecules, and it is tempting to speculate that graft survival prolongation is due to the induction of an anergic state in T cells that recognize graft antigens. The proposed mechanism of αCD154 treatment is shown in Figure 9. It remains to be seen whether anergy is an active process in the induction of tolerance seen with αCD154 treatment.

Clinical transplantation studies have shown evidence suggesting a role for anergy in graft tolerance. Peripheral CD4⁺ T cells purified from the blood of patients with tolerated kidney grafts (successful engraftment for at least one year) were hyporesponsive to antigenic rechallenge, and the proliferative response of these cells was dramatically
Figure 9

A

Antigen Presenting Cell

MHCI TCR CD40 CD154

T cell activation

T cell

B

Antigen Presenting Cell

MHCI TCR CD154

No activation

T cell
Legend for Figure 9

A. Tolerance induction with αCD154 mAb in the context of the three-signal hypothesis of T cell activation. When the TCR is engaged by an MHC molecule, the T cell upregulates CD154, which engages CD40 on the APC and induces the up-regulation of B7 family members. These are now able to deliver a signal through CD28, the result of which is T cell activation. B. The use of αCD154 monoclonal antibody prevents the up-regulation of B7, rendering the APC incapable of fully stimulating the T cell.
increased following treatment with IL-2 (143). This is reminiscent of a conventionally anergized cell’s response to IL-2, and suggests that there may be a functional role for anergy in transplantation tolerance. A very recent study has shown what appears to be a definitive link between anergy and graft tolerance (144). The authors stimulated recipient splenocytes with donor splenocytes and added αB7.1 and αB7.2 antibodies to block costimulation of the recipient T cells. After culture, the cells were transfused back into the recipient followed by a kidney transplant, which engrafted successfully following a short course of immunosuppression. The authors suggest that some property of the anergic cells generated by blocking costimulation in the mixed lymphocyte reaction is what enabled the successful engraftment. It will certainly be necessary to further characterize the nature of the transfused cells prior to any emphatic claims.
Chapter 2 Discussion

T cell anergy may play an important role in peripheral tolerance, and it may be possible to anergize T cells in order to quell an undesired immune response. It is clear that T cell anergy is a complex phenotype involving a biochemical disconnect in the IL-2 production pathway. This break in signaling is likely due to the presence of anergy-specific proteins whose functions in some way impede proper T cell signal transduction. I propose that it is possible to identify these proteins and demonstrate their importance to the anergic phenotype, gaining additional insight into the biochemistry of anergic cells, and potentially providing markers for the identification of anergic cell populations. Perhaps more importantly, knowledge of these anergy factors could provide insight into how to direct specific populations of cells into a state of anergic hypo-responsiveness, possibly enabling us to tailor the immune response to react to some antigens and not to others.
Thesis Goals and Specific Aims

Given the evidence that there is a distinct biochemical pathway that establishes the anergic phenotype in T cells, it stands to reason that the members of this pathway might be identified and their expression levels modulated in order to control T cell responsiveness. With this in mind, the following specific aims were addressed:

Specific Aim 1: Test the hypothesis that there is a TCR-dependent anergy pathway. Utilize Affymetrix RNA transcriptional profiles to identify factors specifically expressed in anergic T cell populations.

Specific Aim 2: Confirm the importance of selected anergy factors by depleting their protein levels in anergic cells with siRNA to test for the reversal of the phenotype.

Specific Aim 3: Test various models of tolerance for the involvement of factors identified in Specific Aims 1 and 2.
Materials and Methods

Animals

B10.BR (H2^k), Balb/c (H2^d), DO11.10/Balb/c (H2^d), and C57BL/6 (H2^b) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). DO11.10/Balb/c is a transgenic strain of BALB/c mice whose T cell repertoire is restricted to a single T cell receptor cognate to OVA peptide 323-339. They are hereafter referred to as DO11.10.

All animals were certified to be free of Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, ectromelia, LDH elevating virus, mouse poliovirus, Reo-3 virus, mouse adenovirus, lymphocytic choriomeningitis virus, polyoma, Mycoplasma pulmonis, and Encephalitozoon cuniculi. They were housed in a specific pathogen free facility in microisolator cages, and given autoclaved food and acidified water ad libitum. All animal use was in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School and recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

A.E7 T Lymphocytes

The A.E7 cell line (a gift from Ronald Schwartz’s lab, NIH) is a clonal population of CD4+ T lymphocytes whose T cell receptor recognizes residues 81-104 of the pigeon cytochrome C (PCC) protein presented in the context of an IE^k MHC class II molecule. Cells were carried in a medium consisting of 50% RPMI 1640 (Invitrogen #11879-020)
and 50% EHAA (Biosource #P120-500), with 10% fetal bovine serum (HyClone #SH30070.03) and supplemented with 0.1% 1000X β-Mercaptoethanol (Gibco #21985-023) and 2mM L-glutamine/20 U/ml penicillin/20ug/ml streptomycin (Sigma-Aldrich #G1146). To prevent activation of the complement cascade, fetal bovine serum was heat inactivated at 56°C for 30 minutes. The medium was 0.2 μM sterile-filtered before use.

The A.E7 is not a transformed T cell line, and therefore needs to be stimulated every two to three weeks to remain viable. To carry the line, RBC-depleted B10.BR (H2K) splenocytes were Cesium-135 irradiated with 3000 rad and added in culture to a subpopulation of A.E7 at a 10:1 ratio (B10.BR:A.E7). The stimulation culture was pulsed with 8 μM whole cytochrome C (from pigeon breast muscle, Sigma #C4011) and incubated for 40-48 hours at 37°C 5% CO2. Following stimulation, cells were expanded 1:40 (cells:medium) in 10% ΔFBS/RPMI 1640/EHAA (referred to hereafter as growth media) supplemented with 15 U/ml recombinant mouse IL-2. Cultures were incubated for 10 days following addition of IL-2 to allow proliferation and return to quiescence. Every six to eight weeks, a stock vial of A.E7s was thawed from liquid N2 storage and stimulated by the above methods.

**Anergy Induction and Stimulation of A.E7s**

To induce anergy in A.E7s, anti-CD3 antibody (clone 145-2C11, BD Pharmingen #553057) was immobilized on plastic tissue culture flasks or dishes at a concentration of 1 μg/ml in Dulbecco’s PBS (Gibco #14040075). Flasks were incubated at 37°C for at least one hour after addition of antibody, or at 4°C overnight to permit coating of the
plastic. After coating, plates were washed three times with PBS to avoid carryover of any soluble anti-CD3. Viable A.E7 cells were purified over a Lympholyte M gradient (CedarLane #CL5030), washed twice in 1% ΔFBS/RPMI1640/EHAA/PSQ (referred to hereafter as wash medium) and brought into suspension in growth medium. Cells were plated at a density of $1 \times 10^6$/ml and incubated at 37°C for 12-16 hours. As a control population, used as a proliferation-capable comparison to the anergic A.E7s, a sub-population of cells was treated as above and supplemented with 1 μg/ml anti-CD28 antibody (clone 37.51, BD Pharmingen #553295) to provide costimulation. After 12-16 hours of incubation, culture flasks were placed on ice to suspend receptor signaling. The supernatant was removed to a centrifuge tube and replaced with wash medium, and the cells were scraped to remove them from the plate-bound antibody. The cells in medium were then removed to a fresh centrifuge tube, and the flasks were rinsed with wash medium, which was added to the same centrifuge tube as the scraped cells. Cells were washed twice in wash medium and added to new tissue culture flasks at 1-2x10⁶/ml. Cells were left un-manipulated in culture for at least five days to permit the full induction of anergy before experimental use.

**Restimulation of Anergic Cells**

To assess the degree of hypo-responsiveness induced by the energizing stimulus, anergic A.E7 cells were rechallenged in a 96-well plate with B10.BR splenocytes. To prepare the APCs, B10.BR spleens were homogenized with frosted slides, filtered through a 100-μm sieve, and treated with Gey’s solution to lyse the red blood cells. The
remaining leukocytes were then washed twice with wash medium and irradiated with 3000 rads in a cesium gamma-irradiator. The cells were washed again with wash medium, resuspended in growth medium, and counted. Untreated, anergic, and costimulated A.E7 cells were added to the splenocytes at a ratio of 5:1 (B10.BR:A.E7) and triplicate wells were treated with increasing doses of PCC protein (Sigma) ranging from a starting concentration of 0.6 μM and doubling each triplicate to a final concentration of 20 μM. A.E7s were stimulated for 64 hours. Forty-eight hours after the start of stimulation, 1 μCi of [3H]-thymidine was added to each well in order to measure DNA synthesis, an approximation of cellular proliferation. At 64 hours, the contents of each well were transferred to a glass fiber filtermat (Wallac #1450-421) in a Tomtek Mach III Harvester 96 and dried for one hour under a heat lamp. After drying, the filter was heat-sealed in a Perkin Elmer sample bag (#1450-432), coated with β-scintillant (Perkin Elmer #1205-441), and counted on a Microbeta 1450 Trilux microplate counter.

Transfection of siRNA

Short inhibitory RNA duplexes (siRNA) were purchased from Dharmacon RNA Technologies (Layfayette, Colorado) in the form of individual duplexes (Egr2-1 and Egr2-2, and SCR control siRNA) or SMARTPool formulations (Egr2, Neuritin, PAC1, AK011178, DGKz, and PD-1). SiRNAs were resuspended in RNase-free H2O (provided by Dharmacon) at a concentration of 1 nmol/μl. A.E7 cells were centrifuged to pellet and resuspended at a concentration of 30-60x10^6 in growth medium. 500 μl of cell culture was added to a 0.4-cm cuvette (BioRad #165-2091) and incubated on ice for 10 minutes.
Following incubation, 5 nmol of siRNA (single duplexes) or 20 nmol of siRNA (SMARTPools) was added to the cuvette, which was immediately electroporated with 310 V at a capacitance of 950 μF in a BioRad Gene Pulser II electroporator. Cells were placed back on ice to recover for 5-10 minutes, added to 3-6 ml of growth medium per cuvette, and incubated at 37°C 5% CO₂ for at least 48 hours before use in experiments.

**siRNA Sequences**

siRNAs with the sequences gug acc acc uua cua cuc adt dt (Egr2-1) and gtt tgc cag gag tga cga adt dt (Egr-2) were used to direct the degradation of Egr-2 mRNA transcripts. An siRNA with the sequence cag ucg cgu uug cga cug gdt dt was used as a non-specific control. The siRNA duplexes making up the PD-l (NM_008798) SMARTPool were (sense strand) duplex 1: uau cau gag ugc ccu agu guu; duplex 2: gau gcc cgc uuc cag auc auu; duplex 3: gaa cug gaa ccg ccu gag uuu; duplex 4: gca agg acg aca cuc uga auu.

**Western Blotting**

To assess protein levels, cells were washed in PBS, lysed in SDS lysis buffer (130 μM Tris-Cl pH 6.8, 4% SDS, 10% glycerol), and immediately boiled to prevent protease degradation. 5-10 μl of boiled lysate was saved for quantitation by BCA assay (Pierce #23225), and calculated by comparison to a BSA standard curve. Samples were reduced with 14M β-mercaptoethanol, stained with 0.1% bromophenol blue, and boiled for 5-8 minutes to denature any residual tertiary protein structure. Samples were loaded in equal total protein quantities (10 - 20 μg) onto a 4% acrylamide Tris-Cl stacking gel poured on
top of a 10% acrylamide Tris-Cl resolving gel in a BioRad Mini-Protean II Western blot apparatus. Samples were run for 1 hour at a voltage of 150 V at a constant current of 500 mA. After resolution, protein samples were transferred from the gel to an Immobilon-P PVDF membrane (Millipore #IPVH07850) previously activated for 15 seconds with 100% methanol, and washed for one minute in deionized water. Samples were transferred for 1 hour at a voltage of 100 V with a constant current of 500 mA.

For Egr-2 blotting, membranes were blocked for 30 minutes with 5% nonfat milk (Biorad #170-6404) dissolved in TBS with 0.5% Tween-20 (TBS-T). Primary antibody against Egr-2 (Covance #PRB-236P) was diluted 1:200 in 5% milk/TBS-T and membranes were incubated with shaking at room temperature for 1-2 hours or at 4°C overnight. After primary antibody incubation, membranes were washed twice in 200 ml TBS-T and incubated in anti-rabbit secondary antibody (Chemicon #AP132P) conjugated to horseradish peroxidase diluted 1:10,000 in 5% milk TBS-T for 30 minutes with shaking at room temperature. Following secondary antibody incubation, membranes were washed twice in 200 ml TBS-T and developed in Western Lightning Plus Chemiluminescence reagent (Perkin Elmer #NEL103) or SuperSignal Pico Enhanced Chemiluminescence reagent (Pierce #34077) for one minute. BioMax Light or BioMax XAR (Kodak) film was exposed to the developed blot for times ranging from 1 to 30 minutes and developed in a Kodak X-OMAT developer.

For PD-1 blotting, samples were prepared, resolved, and transferred as above, and blocked in 8% BSA TBS-T for 30 minutes. Membranes were then washed twice in 200 ml TBS-T and incubated with shaking in 0.1 µg/ml anti-mouse-PD-1 antibody (R & D
Systems #AF1021) overnight at 4°C, then washed twice again with TBS-T. Membranes were incubated in chicken anti-goat-HRP antibody (Chemicon #AP163P) diluted 1:50,000 in TBS-T for 30 minutes with shaking at room temperature, then washed and developed as above.

**Flow Cytometry**

FITC-conjugated anti-PD-1 antibodies (#11-9981, clone RMP1-30) were obtained from eBiosciences. PE-conjugated anti-DO11.10 TCR (#MM7504 clone KJ1-26) antibodies were obtained from Caltag Laboratories, and APC-conjugated anti-CD4 (#553051, clone L3T4) was obtained from BD Pharmingen. For staining, RBC-lysed primary splenocytes and lymphocytes were washed in PBS with 1% BSA, 0.1% sodium azide. Fc receptors were blocked for 10 minutes with FcγIII/II antibody (clone 2.4G2, Invitrogen #553142), washed, and treated with diluted conjugated antibodies for 1 hour on ice in the dark. FITC-conjugated anti-PD-1 antibody was diluted 1:10 in PBS with 1% FBS, and PE-conjugated anti-DO11.10 TCR antibody was diluted 1:5 in PBS with 1% FBS. APC conjugated anti-CD4 antibody was diluted 1:25 in PBS with 1% FBS. Cells were washed and fixed in a solution of PBS-1% paraformaldehyde, and read on a BD FACScan flow cytometer.

**qRT-PCR**

Total RNA was purified from A.E7 cells over an RNeasy Mini column (Qiagen #74104) according to the manufacturer’s instructions. A QiaShredder (Qiagen #79654)
column was used to homogenize the sample prior to purification. cDNA was transcribed with oligo-dT_15 primer and Transcriptor brand reverse transcriptase (Roche #03531287001) Real time PCR was performed in a LightCycler (Roche) using a SYBR Green I PCR Kit (Roche 11988131001) and primers amplifying a 100-1000 base pair region of the transcript of interest. PD-1 transcript levels were quantitated by amplifying the region flanked by primers 5' CCG CTT CCA GAT CAT ACA G 3' and 5' CTC TGG CCT CTG ACA TAC TTG 3'. Egr-2 transcript levels were quantitated by amplifying the region flanked by primers 5' TGA CAG CCT CTA CCC GGT GGA 3' and 5' GTG GAT GGC GGC GAT AAG AA T 3'. Unstimulated or ConA-stimulated mouse splenocytes were used to generate a standard curve and calculate units of expression. Fluorescence was normalized to HPRT or GAPDH.

**ELISA**

To measure IL-2 cytokine levels in culture supernatants, 96 well plates were coated (100 µl/well) with purified rat anti-mouse IL-2 capture antibody (BD Pharmingen, #554424) diluted 1:500 in 0.1M Na_2HPO_4 pH 9. Plates were incubated overnight at 4°C, capture antibody was removed, and wells were washed twice with PBS-T. Wells were then blocked for 1 hour with 1% BSA in PBS-T. After blocking, plates were washed four times with PBS-T and 150 µl of culture supernatant was added to each well, typically with three serial dilutions of each sample. For a standard curve, recombinant mouse IL-2 was serially diluted from 500 to 7.8 pg/ml. Samples were allowed to bind the antibody for two hours at room temperature, and supernatants were washed out of the wells. Plates
were washed six times with PBS-T and biotinylated rat anti-mouse IL-2 detection antibody was added to each well (100 μl/well) diluted 1:500 in 1% BSA PBS-T. After a one hour incubation, plates were washed four times with PBS-T and 100 μl HRP-conjugated avidin (Vector Labs, #A-2004) diluted 1:2500 in 1% BSA PBS-T was added to each well. The avidin was allowed to bind biotin for 30 minutes, and then the plates were washed six times with PBS-T and twice with PBS. Following the washes, 100 μl of OPD buffer (0.05M Na₂HPO₄, 0.025M NaCitrate dihydrate, pH 5, 1 mg/ml o-phenylenediamine (Sigma #P1526), 2.5 μl/ml H₂O₂) was added to each well. Color was permitted to develop over 5-30 minutes and 25 μl of 3M HCl was added to each well to stop the colorimetric reaction. Plates were read at 490 nm on a Molecular Devices eMax plate reader.

**Transgenic T Cell Adoptive Transfers**

Spleens and inguinal, superficial and deep axillary, cervical, and mesenteric lymph nodes were removed from DO11.10 OVA TCR transgenic mice into wash medium, homogenized between frosted glass slides, and washed in wash medium. RBCs were lysed by a five-minute incubation on ice in Gey’s lysis solution, and cells were washed three times in wash medium then resuspended at 133.33×10⁶ cells/ml of sterile, endotoxin-free PBS. It was determined by flow cytometry that approximately 15% of splenocytes and lymphocytes were transgenic, as assessed by staining with the KJ126+ clonotypic antibody (Caltag, #MM7504). Thus, 500 μl of cell suspension at a concentration of 133.33×10⁶ cells/ml containing 10⁷ KJ126+ cells was injected
intravenously into the recipient mouse. Recipients were age- and sex-matched, non-irradiated BALB/c mice (syngeneic to the DO11.10). One day following cell injections, recipients were given another intravenous injection consisting of 300 μg OVA323-339 peptide solubilized in sterile, endotoxin-free PBS. Mice were left unmanipulated for 12 days following cell injections, at which point their spleens, inguinal lymph nodes, axillary lymph nodes, and cervical lymph nodes were removed, Gey's-treated, washed twice in wash medium, and resuspended in growth medium. A cohort of cells was stained with anti-CD4, anti-DO11.10 TCR (KJ126), and anti-PD1, and analyzed by flow cytometry. Another cohort was rechallenged with irradiated syngeneic splenocytes as described above, pulsed with increasing doses of OVA323-339. Cells were stimulated for 88 hours, the last 16 hours of which included 1 μCi of [3H]-thymidine. The proliferative response to antigen was normalized to the percentage of KJ126+ cells in the splenocytes and lymphocytes of each recipient.

**Tolerance Induction with DST and αCD154 monoclonal antibody**

To prepare DST, spleens were removed from Balb/C mice and mashed between glass slides in RPMI 1640 medium (without serum and antibiotics). Cells were filtered through a 100-μm sieve and washed twice in RPMI 1640. After the last wash, cells were counted and diluted to 20x10⁶/ml in RMPI 1640. 500 μl of cell suspension was administered intravenously into C57BL/6 recipients, either alone or concurrently with 500 μg of anti-CD154 mAb (MR1) delivered intraperitoneally. Spleens were removed 3 or 7 days after transfusion and stained with APC-conjugated anti-CD4 (BD Phamingen
#553051) and FITC-conjugated anti-PD-1 (eBiosciences #11-9981-82) for analysis by flow cytometry. The mice whose spleens were removed on day 7 were given an additional dose of αCD154 on day 3 following DST.
Egr-2, a Zinc-finger transcription factor, is highly expressed in anergic T cells, and is required for full induction of anergy

Introduction to Chapter 3

It is well established that there exists a phenotypic distinction between T cell clones receiving a signal through the TCR, and those receiving signals through both the TCR and CD28 (72) (Figure 10). The first aim of this project, work done primarily by John Harris, consisted of optimizing a system in which mRNA profiles from untreated, anergized, or stimulated A.E7 T cell clones were compared using Affymetrix GeneChips. Samples for analysis were prepared in biological triplicates from each of these populations at three time points: immediately following the 12-hour antibody treatment with αCD3 or αCD3+αCD28 (day 0), day 2, and day 5 after removal from the antibody. It was determined that day 5 was the earliest time at which anergized cells and stimulated cells responded differently to antigenic rechallenge (145). Prior to this time, the cells treated with αCD3 and αCD28 stimulation were hypo-responsive to antigen, similarly to the anergic cells. Analysis of the data from these mRNA profiling experiments produced a list of gene products more highly expressed in anergic cells than in either untreated cells or productively stimulated cells. From these we chose Egr-2 as a potentially important gene in the anergic phenotype.
Figure 10

A

[3H]-Thymidine (CPM)

±M Antigen (PCC)

- Untreated
- Anergic (TCR)
- Stimulated (TCR+CD28)

B

Exogenous IL-2 Treatment

[3H]-Thymidine (CPM)

<table>
<thead>
<tr>
<th></th>
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<th>Anergic</th>
<th>Stimulated</th>
</tr>
</thead>
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<tr>
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</table>
Legend for Figure 10

A. A.E7 CD4+ T cell clones stimulated through their TCR with immobilized αCD3 antibody develop a phenotype of hypo-responsiveness to antigenic stimulation. The addition of soluble αCD28 antibody to the stimulation culture prevents the cells from developing this anergic phenotype. Cells were initially stimulated (αCD3 or αCD3+αCD28) for 12-16 hours, removed from the antibody, and left in culture for at least five days before rechallenging them with irradiated (3000 rad) syngeneic splenocytes pulsed with increasing doses of pigeon cytochrome C. These two populations are shown in reference to a previously untreated population. B. Anergized A.E7 clones are equally as responsive as untreated or fully stimulated clones to exogenous IL-2, demonstrating that their capacity to proliferate in response to some stimuli is not impaired.
Background of Egr-2

Members of the Early Growth Response family of genes (Egr-1-4) are induced very rapidly following cell stimulation. A hallmark feature of the Egr family is a DNA binding domain consisting of three zinc-finger motifs (146). This domain has been demonstrated to bind the DNA sequence GCGGGGGCG, whose presence in gene promoter regions has been shown to facilitate the transactivation of Egr family target genes (147). Egr-2 has been studied most extensively in the nervous system, and its role in the immune compartment has not been the focus of much study to date; its targeting in knockout mice results in early lethality due to defects in hindbrain patterning, peripheral nerve myelination, and bone formation (148). In T cells, it has been shown that Egr-2 up-regulation is dependent on members of the NFAT family, and that Egr-2 may play a role in the NFAT-dependent regulation of Fas ligand (147). Given the demonstrated importance of NFAT in T cell anergy (59), we attempted to further characterize what role Egr-2 may play in this phenotype.
Egr-2 is up-regulated in anergic T cells relative to untreated and stimulated populations

Given the relatively high number of mRNAs differentially expressed by anergic cells, especially at the day 2 time-point, it was necessary to consider the known characteristics of these gene products in order to prioritize them. It was our initial goal to identify a primary point of regulation for anergy, and a transcription factor seemed likely to be a good candidate. Our hypothesis was that a transcription factor active early in the processes that distinguish anergy from stimulation could activate a network of genes whose cumulative actions result in resistance to antigenic stimulation. As shown in Figure 11A, Affymetrix GeneChips show that Egr-2 mRNA is expressed to a greater degree in anergic T cells than in either untreated or stimulated cells. These data were confirmed by purifying total RNA from untreated, anergic, and stimulated cells, at the same three time-points as were examined in the Affymetrix screen, synthesizing cDNA from these, and RT-PCR amplifying a region of the Egr-2 message to confirm the Affymetrix data. As shown in Figure 11B, elevated Egr-2 mRNA in anergic cells was detected by RT-PCR. It is important to note that mRNA levels are not an exact representation of the protein profiles of a given cell population, due to phenomena such as differences in mRNA stability, post-transcriptional regulation, and post-translational regulation. Thus, it was important to confirm these mRNA data by assessing the levels of Egr-2 protein in all three populations of interest.
Figure 11

A

Egr2
Affymetrix GeneChip

Relative Fluorescence

Untreated  Anergic  Stimulated  Untreated  Anergic  Stimulated  Untreated  Anergic  Stimulated

t=Day 0  t=Day 2  t=Day 5

B

Egr2
RT-PCR

25
20
15
10
5
0

Egr2/HPRT

Untreated  Anergic  Stimulated  Untreated  Anergic  Stimulated  Untreated  Anergic  Stimulated

t=Day 0  t=Day 2  t=Day 5
Legend for Figure 11

Egr-2 is up-regulated in anergic T cell populations. A. Affymetrix GeneChip analysis shows Egr-2 to be up-regulated in anergic cells. B. RT-PCR confirms that Egr-2 RNA is increased in anergic cells, compared with both untreated and fully stimulated populations.
Western blotting for Egr-2 protein revealed that these mechanisms were not significantly altering Egr-2 protein levels, since the relative band intensities of Egr-2 protein reflect mRNA levels reasonably closely. As shown in Figure 12A, Egr-2 protein is expressed at similar levels in both anergic and stimulated A.E7s at the earliest time-point assessed (12 hours, or day 0). Over time, after removal from antibody, Egr-2 can be seen to dissipate rapidly in the stimulated A.E7s, but persists in the anergic cells. Egr-2 can be detected in anergic cell lysates to day 9, far longer than in stimulated samples. Elevated levels of Egr-2 correlate with the anergic phenotype, as seen in Figure 12B.

**Egr-2 protein levels decrease in the presence of IL-2 concurrently with the reversal of the anergic phenotype**

An important characteristic of the anergic phenotype is that it is not permanent; over time anergic cells return to an antigen-responsive state. The addition of exogenous IL-2 to the culture medium reverses anergy quickly, supporting the hypothesis that IL-2 receptor signaling prevents anergy in a costimulatory environment. Given the correlation of Egr-2 expression and the anergic phenotype thus far, we sought to determine the effect of exogenous IL-2 on Egr-2 protein expression. We considered the outcome important because it was not clear from existing data whether the decrease in Egr-2 levels in stimulated cells was due to the same signaling events leading to IL-2 production, a process impaired in anergic cells, or the result of IL-2 receptor signaling, which anergic cells are capable of transducing
Figure 12

A

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Egr-2

B

Day 9

Proliferation (CPM x 10^-4)

0 0.3 0.6 1.3 2.5 5 10

[IL-2] uM

- Untreated
- Anergic
- Stimulated
Legend for Figure 12

A. Egr-2 protein expression is high in anergic and stimulated populations at early times, but is rapidly down-regulated in stimulated populations, while expression persists in anergic populations. B. The degree of hypo-responsiveness correlates with the level of Egr-2 expressed as can be seen when cells are rechallenged at day 9. The anergic cells, which are still expressing Egr-2 protein, are unresponsive to antigenic rechallenge, while the stimulated cells, which have down-regulated Egr-2, respond well. Western blot and $[^3]$H-thymidine plot courtesy of John Harris.
As seen in Figure 13, the addition of exogenous IL-2 to anergic cultures following their removal from the anergizing stimulus results in a rapid decrease in Egr-2 protein levels. This suggests a direct relationship between the presence of Egr-2 protein and the persistence of the anergic phenotype. Importantly, following 10 days of rest, the anergic cells treated with exogenous IL-2 were markedly more responsive to antigenic stimulation, demonstrating a reversal of anergy.

Depletion of Egr-2 with siRNA partially restores antigenic responsiveness and IL-2 production in early stages of anergy

Given the nature of Egr-2 as a transcription factor, we hypothesized that Egr-2 might play a role in inducing some of the early biochemical changes required to prevent IL-2 production. To test this, we employed RNA interference, a means of degrading mRNA first characterized in Caenorhabditis elegans (149) and optimized extensively in mammalian systems (150). The value of being able to modulate protein levels without changing the nature of the signals received by the lymphocytes lies in its specificity. To establish a causative relationship between Egr-2 protein expression and the anergy phenotype, we sought to reduce Egr-2 protein levels during anergy induction. 22-mer siRNA duplexes corresponding to the Egr-2 mRNA sequence were transfected into A.E7 T cells by electroporation, after which the cells were allowed to recover in medium at 37°C for 4-6 hours, and anergized as described in Materials and Methods. Using a fluorescent-tagged non-specific siRNA duplex, it was determined by flow
Figure 13

A

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</tbody>
</table>

IL-2 → Egr-2

B

[Graph showing proliferation vs. IL-2 concentration (µM)]

- ● Control
- ○ Control+IL-2
- ▼ Anergized
- ▲ Anergized+IL-2
Legend for Figure 13

A. Egr-2 protein levels decrease in the presence of exogenous IL-2, concurrently with the reversal of the anergic phenotype. Levels of Egr-2 protein were assessed in untreated and anergic samples by Western blotting. Cells were processed immediately after removal from stimulus, and 1, 3, 5, 7 and 10 days thereafter, either in medium or in medium supplemented with 10 U/ml exogenous IL-2 to induce proliferation. B. Ten days after removal from the anergizing stimulus, cells were rechallenged with syngeneic splenocytes pulsed with increasing doses of pigeon cytochrome C. Western Blot and [³H]-thymidine plot courtesy of John Harris.
cytometry that electroporation was 90-99% effective in delivering duplexes to the cell cytoplasm. Fluorescence microscopy was used to confirm that siRNA was internal to the cell and not sticking to the cell membrane (Figure 14). Five days following the induction of anergy, Egr-2 levels in the siRNA transfected cells were detectably lower than those in non-transfected anergic cells, or anergic cells transfected with a non-specific siRNA (Figure 15). The use of the non-specific siRNA demonstrated that the presence of double stranded RNA in the cell did not change Egr-2 levels. Upon antigenic rechallenge, anergic cells in which Egr-2 had been knocked down showed an increase in responsiveness, in some cases responding similarly to the previously untreated population. These data show that the presence of Egr-2 is crucial to some aspect of the anergic phenotype, at least with regard to anergy induction.

Since the primary barrier to proliferation in anergic cells is the inability to produce IL-2, we asked whether the restored ability of Egr-2-depleted anergic cells to respond to antigenic stimulation was a result of their ability to make IL-2. To see what effect Egr-2 depletion has on this signaling block, Egr-2 siRNA-treated anergic cells were stimulated with immobilized αCD3 antibody and soluble αCD28 antibody for 16 hours, at which point the culture medium was removed and stored at -20°C. For comparison, a previously untreated population of A.E7 cells, an anergic population not transfected with siRNA, and an anergic population transfected with a non-specific siRNA, were stimulated in the same manner.
Figure 14

A

\[
\begin{align*}
\text{No siRNA} & \quad \text{FITC siRNA} \\
\% \text{Cell Survival} & \\
\end{align*}
\]

B

\[
\begin{align*}
\text{No siRNA} & \quad \text{FITC siRNA} \\
\% \text{FITC+ Live Cells} & \\
\end{align*}
\]

C

D

E

F
Legend for Figure 14

Electroporation of A.E7 T cells is a highly efficient means of transfecting siRNA. A. The introduction of FITC-conjugated siRNA duplexes with a voltage of 310 V at 950 μF results in a small amount of cell toxicity. B. Flow cytometry analysis of FITC+ cells determined that over 95% of cells were successfully transfected by this method. C-F. It was determined by fluorescence microscopy that the siRNA was delivered to the cytoplasm of the cells, rather than simply sticking to the outer cell membrane. C and E are brightfield images corresponding to the fluorescence images D and F, respectively.
Figure 15

A

<table>
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Egr-2-<br>actin<br>

siRNA: None None Con Egr2-1 Egr2-2

B

Proliferation (CPMx10^{-3})

[|PCC] uM
Legend for Figure 15

Knocking down Egr-2 protein levels with siRNA restores antigenic responsiveness of anergic cells. A. Anergic cell populations were transfected with two distinct Egr-2 siRNAs, anergized with αCD3 antibody, and rested for five days. After the rest period, significantly less Egr-2 protein could be detected in those cells treated with Egr-2 siRNA, whereas Egr-2 levels were unchanged in cells transfected with a non-specific siRNA. All anergic populations expressed more Egr-2 protein than a previously untreated control population. B. Upon antigenic rechallenge, anergic A.E7 cells treated with Egr-2 siRNA display a greater specific response to pigeon cytochrome C than do anergic cells transfected with a non-specific siRNA or those not transfected. This was consistent across two different siRNAs targeting Egr-2, and the antigenic responsiveness of the cells was proportional to the efficacy of the siRNA in knocking down protein levels.
The IL-2 protein levels from the supernatants collected from these stimulation cultures were measured by sandwich ELISA. As shown in Figure 16, the Egr-2 siRNA-treated anergic cells are capable of making more IL-2 upon antibody stimulation than are untransfected anergic cells and those transfected with control siRNA.

**Egr-2 depletion in anergic cells restores ERK activation upon stimulation**

As mentioned previously, anergic cells are defective in their ability to phosphorylate ERK MAP kinases (87), and it has been proposed that this defect is at least partially responsible for an anergic cell’s inability to make mitogenic doses of IL-2. In an attempt to describe the mechanisms by which Egr-2-depleted anergic cells produce IL-2, the phosphorylation status of ERK kinases was examined. To assess for ERK activation status, A.E7 cells were transfected with Egr-2 siRNA or non-specific siRNA, or were left untransfected, rested in medium for 4-6 hours, and anergized. After removal from the anergizing stimulus, cells were rested in medium for five days, purified over a Lympholyte M gradient, and stimulated for 1 hour with immobilized αCD3 antibody and soluble αCD28 antibody. After 1 hour, cells were removed from the antibody, washed in cold PBS, and lysed in SDS buffer. The levels of total ERK and phospho-ERK were assessed by Western blotting (Figure 17). As shown, levels of total ERK do not change across the different conditions, and in the absence of subsequent stimulation, there is no active ERK present after five days of rest.

After one hour of stimulation, ERK MAP kinases were activated in the previously untreated sample and mostly inactive in the untransfected and control siRNA-transfected
Figure 16

A

![IL-2 secretion graph](image)

B

![Western blot](image)
Legend for Figure 16

Egr-2 depletion in anergic cells restores their ability to produce IL-2 upon full stimulation. Anergic AE7 T cells were transfected with siRNA targeting Egr-2 mRNA, a non-specific siRNA, or no siRNA. Four-to-six hours after transfection, cells were anergized and permitted to rest in medium for 5 days. After resting, a subpopulation of cells of each condition was restimulated for 16 hours with platebound αCD3 antibody and soluble αCD28 antibody, after which the culture supernatants were collected and IL-2 was measured by sandwich ELISA. IL-2 quantities were determined relative to a standard curve of increasing doses of recombinant mouse IL-2.
Legend for Figure 17

Egr-2 knockdown in anergic T cells restores ERK phosphorylation upon rechallenge. Anergic cells treated with no siRNA, a non-specific siRNA, or an siRNA specific to Egr-2, or previously untreated cells, were rested five days following the anergizing stimulus and rechallenged with αCD3 and αCD28 antibodies. Cells were removed from antibody by scraping after one hour, washed in cold PBS, and lysed in SDS buffer. The levels of total ERK and phospho-ERK expression were assessed by Western blotting.
anergic samples, but there was a detectable increase in ERK phosphorylation when the anergic cells were treated with Egr-2 siRNA. These data suggest that the presence of Egr-2 in anergic cells is somehow able to prevent ERK phosphorylation.

**Egr-2 depletion prevents anergy induction, but does not reverse established anergy**

Having seen that high Egr-2 protein levels correlate with the presence of the anergic phenotype, we sought to examine more closely the kinetics of Egr-2 activity. It is possible that the establishment of anergy occurs over numerous phases. The data presented thus far show the importance of Egr-2 in the induction of anergy, but the question remains as to the role of Egr-2 after anergy has been established. No study has yet described a factor active during the maintenance phase of anergy.

To assess whether the dampening role of Egr-2 persists throughout the course of the anergic phenotype, A.E7 cells were anergized with immobilized αCD3 antibody, rested for five days, and transfected with Egr-2 siRNA duplexes. Following transfection, the cells were rested in medium for two additional days to allow the siRNA to effectively direct the degradation of the Egr-2 message. At this point the cells were rechallenged with irradiated syngeneic splenocytes pulsed with pigeon cytochrome C. Surprisingly, knocking down Egr-2 after anergy was established did not restore antigenic responsiveness (Figure 18). In fact there was no detectable difference in antigenic responsiveness between the Egr-2 depleted anergic cells and the control populations. The absence of restored proliferation at this time-point was corroborated by the inability of
Figure 18

A

Proliferation (CPM x 10^-3)

B

IL-2 Secretion (ng/ml)

C

siRNA: None, Resting; None, Anergized; Egr-2, Anergized

Egr-2

Actin

siRNA: None, None, Con, Egr2-1
Legend for Figure 18

Egr-2 knockdown does not restore antigenic responsiveness in anergic cells after anergy has been induced. A.E7 cells were anergized with immobilized αCD3 antibody, rested for five days after removal from the anergizing stimulus, and transfected with siRNA targeting Egr-2 mRNA. A. Cells were rested for two additional days and rechallenged with irradiated syngeneic splenocytes pulsed with pigeon cytochrome C for 48 hours. After 48 hours, 1 μCi of [3H]-thymidine was added to each well and cells were incubated at 37°C for 16 hours, at which point they were harvested and read. B. IL-2 production was measured by stimulating Egr-2 knockdown anergic cells for 16 hours with immobilized αCD3 antibody and soluble αCD28 antibody, alongside anergic cells not treated with siRNA and those treated with a non-specific siRNA. Culture supernatants were collected after 16 hours and IL-2 production was measured by capture ELISA. C. A subpopulation of cells was lysed at this point for assessment of Egr-2 protein levels by Western blotting.
the Egr-2 siRNA-treated cells to produce IL-2 upon antibody stimulation, in contrast to those cells treated prior to anergy induction.

**Discussion of Chapter 3**

Anergy is very likely a complex process involving a number of genes that serve to mediate the resistance to costimulation. The data presented in this chapter show that the transcription factor Egr-2 functions in establishing the anergic state in A.E7 T cells. The expression profile of Egr-2 is consistent with the characteristics of a factor that confers anergy as predicted by Powell et al. (82) in that 1) it is initially up-regulated in both anergized and fully activated T cells before their proliferation and its expression is prevented by cyclosporine A (75); 2) its expression decreases more rapidly in proliferating cells than in anergized cells; and 3) high expression levels of Egr-2 in anergy are decreased by exposure to IL-2, which concurrently reverses the anergic state. Interestingly, high expression of Egr-2 has been found in other studies to be associated with anergy. Lechner, et. al. screened for genes expressed in primary T cells anergized in vivo, and reported the induction of Egr-2 expression in the anergized T cell population (151), but these initial findings were not expanded.

Over time there is a reversal of anergy in the A.E7 cells coincident with a loss of Egr-2 expression. It is possible that Egr-2 transactivates other anergy factors, and that the loss of Egr-2 expression contributes to the transient nature of anergy over the long term. As it was not possible to reverse anergy by depleting Egr-2 five days after anergy induction, it is possible that key transcriptional targets of Egr-2 are present by this time,
and are not down-regulated quickly enough to restore normal responsiveness in our experiments. It is possible that knocking down Egr-2 during anergy maintenance and waiting longer than two days to allow for the down-regulation of Egr-2 targets would restore responsiveness to the anergic cells.

Egr-2 may be responsible for activating a network of genes whose proteins mediate the many different aspects of the anergic phenotype, including hyporesponsiveness, differential homing, and cytokine production. Identifying the factors under the regulation of Egr-2 will provide important insight into the mechanisms involved in the maintenance of immune tolerance.
- Chapter 4-

**PD-1, a negative costimulatory receptor, has an essential role in**

**maintaining CD4\(^+\) clonal anergy**

**Chapter 4 Introduction**

The two-signal model of lymphocyte activation was first proposed by Bretscher and Cohn in 1970 (152). They showed that recognition of one determinant on an antigen resulted in paralysis of antibody production, whereas recognition of two determinants resulted in induction of antibody synthesis by B cells. It is well agreed that a similar paradigm applies to T cell signaling: a signal through the TCR results in tolerance, whereas a signal through the TCR combined with a signal through a costimulatory receptor results in expansion (78). Early studies determined that a signal through CD28 on the T cell surface would rescue the cell from tolerance, but it has been shown over time that the process of costimulation is much more complicated than this. A number of family members have been added to what has come to be known as the CD28 superfamily of receptors, and a number of B7 family members have been found to act as their ligands. The concept of costimulation has evolved from simply the presence or absence of an activating signal, to include the comprehensive balance of positive costimulatory signals, such as that through CD28, and negative costimulatory signals, the prototype of which is CTLA-4 (153). Another costimulatory receptor/ligand combination, CD40/CD154, has been shown to facilitate the rejection of allografts since blocking the interaction of these cell surface proteins in combination with DST enables
engraftment of allografts (26). The mechanisms of these processes are currently under investigation.

One of the negative costimulatory pathways has emerged recently as a topic of high interest. This receptor-ligand combination, PD1-PDL1/2 (B7-H1/B7-DC), seems to be important to peripheral tolerance, since knocking it out results in strain-specific autoimmune disorders, and studies have also shown its engagement can promote allograft survival (154). The data presented in this chapter demonstrate the importance of PD-1 in the maintenance of T cell anergy.
PD-1 knockdown at late time-points results in the reversal of T cell anergy

In the previous chapter it was demonstrated that depleting Egr-2 after anergy has been established has no effect (145). On the basis of the Affymetrix GeneChip array data, a number of genes shown to be up-regulated at the day 5 time-point were chosen as potential anergy maintenance factors. siRNA technology was again employed to determine the effects of depleting these genes. The list of the genes selected for investigation can be found in Table 1. A.E7 cells were anergized or costimulated, removed from antibody, and rested in medium for five days. At this point, anergic cells were transfected with pools of four distinct siRNA duplexes (available commercially as a SMARTPool from Dharmacon) and rested for an additional two days in growth medium, to allow the siRNA to effectively direct the degradation of the mRNA. After two days in medium, live cells were purified over a Lympholyte M gradient and restimulated with APCs pulsed with pigeon cytochrome C.

Surprisingly, depletion of many of the chosen candidates did not reverse the anergic phenotype. It has long been speculated that anergy is a complex network of differential gene expression and enzymatic activities, but despite this only one candidate exhibited a functional role in this assay. As shown in Figure 19, only PD-1 appears to contribute to anergy maintenance.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>NCBI Accession ID</th>
<th>Fold Change (Anergic/Stimulated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Growth Response 2</td>
<td>Egr2</td>
<td>NM_010118</td>
<td>1.29</td>
</tr>
<tr>
<td>Neuritin 1</td>
<td>Nrnl</td>
<td>NM_153529</td>
<td>1.45</td>
</tr>
<tr>
<td>RIKEN cDNA 2600010E01 gene</td>
<td>2600010E01Rik</td>
<td>NM_175181</td>
<td>1.07</td>
</tr>
<tr>
<td>Dual Specificity Phosphatase 2</td>
<td>Dusp2</td>
<td>NM_010090</td>
<td>1.18</td>
</tr>
<tr>
<td>Programmed Cell Death 1</td>
<td>Pdcd1</td>
<td>NM_008798</td>
<td>1.26</td>
</tr>
<tr>
<td>Beta-Site APP-Cleaving Enzyme 2</td>
<td>Bace2</td>
<td>NM_019517</td>
<td>1.05</td>
</tr>
<tr>
<td>Diacylglycerol Kinase Zeta</td>
<td>Dgkz</td>
<td>NM_138306</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>3.32</td>
<td>3.56</td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>4.59</td>
<td>2.64</td>
<td>2.58</td>
</tr>
<tr>
<td>Day 5</td>
<td>1.66</td>
<td>2.52</td>
<td>2.32</td>
</tr>
</tbody>
</table>
Legend for Table 1: Genes highly expressed five days after anergy induction.
Figure 19

Egr-2

![Graph showing Egr-2 stimulation with different concentrations of siRNA.

PD-1

![Graph showing PD-1 stimulation with different concentrations of siRNA.

Neuritin

![Graph showing Neuritin stimulation with different concentrations of siRNA.

Riken 2600010E01

![Graph showing Riken 2600010E01 stimulation with different concentrations of siRNA.

Dusp-2

![Graph showing Dusp-2 stimulation with different concentrations of siRNA.

Bace-2

![Graph showing Bace-2 stimulation with different concentrations of siRNA.

DGK-ζ

![Graph showing DGK-ζ stimulation with different concentrations of siRNA.

Legend:
- Stimulated
- Anergic Control siRNA
- Anergic Egr2 siRNA
- Anergic Pdcd1 siRNA
- Anergic Neuritin1 siRNA
- Anergic 2600010E01 siRNA
- Anergic Dusp2 siRNA
- Anergic BACE2 siRNA

Note: CPM = Counts Per Minute

Concentrations: 0, 0.5, 1.5, 2.5, 5.0 μM
Legend for Figure 19

Genes found to be differentially expressed in anergic A.E7 T cells were silenced with siRNA SMARTPools five days after anergy induction. Cells were then rechallenged with antigen-pulsed APCs to assess their antigenic responsiveness. Egr-2 was included to establish experimental continuity with our previous studies demonstrating the absence of a role for Egr-2 at late time-points. Of those selected for examination, only PD-1 knockdown restores the responsiveness of anergic cells, suggesting an important role for PD-1 in anergy maintenance. Though SMARTPools are guaranteed by Dharmaco, Inc. to effectively silence their target genes, it would be necessary to assess the protein levels of each population tested in order to confirm that the populations other than PD-1 in fact do not contribute to anergy maintenance.
PD-1 mRNA and protein levels are increased in anergic cells

To confirm that PD-1 mRNA is expressed at higher levels in anergic cells than in untreated or stimulated populations, total RNA was purified from these three populations five days after anergy induction. cDNA was synthesized from the total RNA as described in Materials and Methods and a region of PD-1 message was amplified by RT-PCR. As shown in Figure 20, the levels of PD-1 in these three populations shown by RT-PCR reflect those shown by the GeneChips. To rule out alterations in PD-1 expression by post-transcriptional and post-translational regulatory mechanisms, PD-1 levels were assessed in SDS lysates of untreated, anergic, and stimulated cells, in which PD-1 protein levels were determined to be up-regulated in the anergic populations.

PD-1 siRNA increases antigenic responsiveness in anergic A.E7 cells, but not in untreated or fully stimulated populations.

A notable idiosyncrasy of the A.E7 T cell model is the variable degree to which the cells respond to CD28 costimulation. The population of costimulated cells shows some evidence of anergy, in that they do not respond well to rechallenge with lower doses of antigen (as seen in Figure 10). In contrast, these same cells often respond as well as previously untreated cells to higher doses of antigen. This characteristic introduces a small amount of ambiguity when attempts are made to define distinctions between anergic and responsive cells.
Figure 20

A

![Bar chart showing PD-1 GeneChip Signal (x10^3) for different conditions at 12 hour, Day 2, and Day 5. Conditions include Untreated, Anergic, and Stimulated.]

B

![Bar chart showing PD-1/HPRT ratios for Untreated, Anergic, and Stimulated conditions on Day 5.]

C

![Western blot images for Untreated, Stimulated, and Anergic conditions on Day 5. Images show αPD-1 and αβ-actin bands.]

Legend for Figure 20

PD-1 is highly expressed in anergic A.E7 cells five days after anergy induction.

A. Affymetrix GeneChips show PD-1 mRNA to be increased in anergic cells (A) compared to previously untreated (U) and fully stimulated (S) cells. B. This increased PD-1 expression is confirmed by RT-PCR of samples purified five days after anergy induction. C. PD-1 protein is highly expressed in anergic cells five days after anergy induction.
For example, Egr-2 plays a role in dampening the response to antigenic rechallenge in fully stimulated cells as well as anergic cells (Figure 21). To determine if PD-1 also dampens the ability of costimulated cells to respond fully to antigenic rechallenge, PD-1 siRNA was transfected into untreated, anergic, and stimulated A.E7 cells. In contrast to the data showing the dampening effect of Egr-2 in stimulated cells, there does not appear to be a similar impact of PD-1 depletion on stimulated cells: knocking down PD-1 in stimulated populations or previously untreated populations of A.E7 cells does not appear to alter their antigenic responsiveness, despite low but detectable levels of PD-1 protein in both populations. This is shown in Figure 22.
Figure 21

[\text{[H]-Thymidine Incorporation (CPM \times 10^{-3})}]

- Control siRNA
- Egr-2 siRNA

<table>
<thead>
<tr>
<th>siRNA:</th>
<th>Control</th>
<th>Egr2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egr-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Legend for Figure 21

Despite our use of anergic and costimulated A.E7 cells as phenotypically disparate populations, the costimulated cells do not respond as well as previously untreated cells to antigenic rechallenge. This has led some to propose that the costimulated cells are partially anergic. Egr-2 protein levels can still be detected in stimulated A.E7s by Western blotting, and these cells show an increase in their antigenic responsiveness when Egr-2 is depleted with siRNA.
Figure 22

A

<table>
<thead>
<tr>
<th>Condition:</th>
<th>Untreated</th>
<th>Anergic</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA:</td>
<td>None</td>
<td>SCR</td>
<td>PD-1</td>
</tr>
<tr>
<td>anti-PD-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

[Bar graph showing [3H]-Thymidine (CPM) with PD-1 siRNA:SCR siRNA]
Legend for Figure 22

The ability of PD-1 to suppress proliferation is specific to anergic populations. A. PD-1 depletion with siRNA results in increased proliferation in anergic (A) cells, but not in a previously untreated (U) or costimulated (S) population of A.E7 cells. Cells were anergized or stimulated, rested for 5 days, transfected with PD-1 siRNA, rested for an additional 2 days, and rechallenged with irradiated APCs pulsed with cytochrome C. [$^3$H]-thymidine data are presented as a fold-increase of PD-1 siRNA-treated cells thymidine incorporation compared to control siRNA-treated cells thymidine incorporation. B. As shown by Western blotting, small amounts of PD-1 protein can be detected in previously untreated and costimulated A.E7 cell populations, but siRNA-mediated PD-1 depletion does not affect their response to antigenic challenge.
PD-1 depletion restores anergic cell responsiveness in an IL-2 dependent fashion

PD-1 engagement on T cell blasts has been shown to inhibit IL-2 production (155). To determine if IL-2 levels change subject to the modulation of PD-1 protein levels, anergized cells were transfected with control siRNA or PD-1 siRNA, rested in media for two days, and rechallenged with αCD3 and αCD28 antibody for 16 hours, at which point the supernatants were collected and assayed for IL-2 cytokine by IL-2 capture ELISA. Surprisingly, as shown in Figure 23A, knocking down PD-1 in anergic cells does not appear to restore their ability to make IL-2. Since the restored responsiveness seen by [3H]-thymidine incorporation was the result of APC stimulation and not antibody stimulation, this experiment was repeated by stimulating the cells with irradiated syngeneic splenocytes pulsed with cytochrome C rather than αCD3 and αCD28 antibodies. Again, that there was not a significant change in IL-2 production (shown in Figure 23B). It is possible that the kinetics of IL-2 production change in this case, and the sixteen-hour time-point is not ideal as was the case in the Egr-2 study. It is also possible that another cytokine is acting in the place of IL-2, prompting the PD-1-depleted anergic cells to proliferate. A third possibility is that the PD-1 knockdown cells are consuming IL-2 quickly enough that there is no excess in the supernatant, giving the appearance that they are making equal amounts to the anergic populations.
Figure 23

A

IL-2 Production (ng/ml)

<table>
<thead>
<tr>
<th></th>
<th>no siRNA</th>
<th>PD-1 siRNA</th>
<th>Control siRNA</th>
<th>PD-1 siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anergic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rechallenged</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unchallenged</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

IL-2 Production (pg/ml)

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Stimulated</th>
<th>Anergic no siRNA</th>
<th>Anergic Control siRNA</th>
<th>Anergic PD-1 siRNA</th>
<th>APC only</th>
</tr>
</thead>
</table>
Legend for Figure 23

Depletion of PD-1 does not permit increased IL-2 production upon rechallenge, either with A. αCD3 + αCD28 antibody or with B. APCs. AE7 cells were anergized, rested for 5 days, and transfected with control siRNA or siRNA targeting PD-1. The cells were then rested for an additional 2 days and rechallenged for 16 hours with either anti-CD3 + anti-CD28 antibody, or cytochrome C-pulsed APCs. Supernatants were collected 16 hours later and assayed for IL-2 levels by IL-2 capture ELISA.
In an attempt to consider the question from another angle, a neutralizing αIL-2 antibody (Pharmingen Clone S4B6, #554375) was added to the restimulation culture at a concentration of 10 μg/ml. An equal concentration of rat IgG2a antibody was used as a control. If another cytokine is responsible for the restorative effect of PD-1 depletion, then neutralizing IL-2 should not affect the PD-1 siRNA-treated cells’ ability to proliferate in response to antigenic stimulation. As shown in Figure 24, the restored antigenic responsiveness in the PD-1 siRNA-treated anergic cells is IL-2 dependent. Although increased levels of IL-2 production cannot be detected in cells treated with PD-1 siRNA, blocking IL-2 signaling nonetheless prevents the restoration of antigenic responsiveness. This rules out the possibility that another cytokine is acting in place of IL-2 to drive the proliferation of these cells.

**Adoptively transferred transgenic T cells tolerized with intravenous peptide up-regulate PD-1**

Adoptively transferred transgenic T cells can be tolerized *in vivo* by injecting the recipient with soluble peptide intravenously. These cells exhibit a hypo-responsiveness to antigenic stimulation similar to that of anergized A.E7 cells (72). This model is well accepted as a model of anergy *in vivo* (112).

Experiments were performed to confirm that *ex vivo*, the adoptively transferred transgenic cells were indeed hyporesponsive to their cognate antigen. It was first determined that 66.67x10^6 total lymphocytes and splenocytes need to be injected to achieve a transfer of 10^7 OVA-specific T cells. This allowed for the detection of a small
Figure 24

[Diagram showing [3H]-Thymidine Incorporation (CPM) vs. PCC (μM) for stimulated, Anergic PD-1 siRNA IgG, and Anergic PD-1 siRNA anti-IL2 treatments.]
Legend for Figure 24

PD-1 depletion restores antigenic responsiveness in an IL-2-dependent manner. Cells were anergized or stimulated, rested for five days, and a subpopulation of anergic cells was transfected with PD-1 siRNA. Cells were rested for an additional two days and rechallenged with irradiated syngeneic splenocytes pulsed with increasing doses of cytochrome C. The PD-1 siRNA-treated anergic cells were divided into two populations and their culture medium was supplemented with either 10 μg/ml of rat IgG2a antibody, or 10 μg/ml anti-IL-2 antibody. Cells were challenged for 64 hours; after 48 hours, 1 μCi of [3H]-thymidine was added to each well.
population of circulating cells at later times. Eleven days following cell transfusion, spleens and lymph nodes were harvested, a single cell suspension was made, and these cells were rechallenged with antigen-pulsed, irradiated syngeneic splenocytes to assess their proliferative capability. As seen in Figure 25, a significant increase in PD-1-expressing transgenic cells correlates with the tolerized phenotype.

**PD-1 expression in the DST + αCD154 tolerance induction protocol**

It has been proposed that anergy is one mechanism of peripheral tolerance, and there are studies that have proposed evidence for the importance of anergy in allograft transplantation (156-164). The data presented above suggest that PD-1 may be expressed specifically on anergic cells, and thus may act as a cell surface marker for hyporesponsive cells. One study has suggested a role for PD-1 in prolonging allograft survival (154). If an anergic cell population actively contributes to the peripheral tolerance induced by DST and αCD154 treatment, then it is possible that PD-1 expression would be increased on a select population of CD4+ cells in tolerized mice. To test this possibility, C57BL/6 (H2b) mice were transfused with BALB/c (H2d) splenocytes to serve as a general source of alloantigen. Half of the mice were given 500 µg of αCD154 mAb, which serves to block T cell costimulation. After 3 and 7 days, spleens were removed and homogenized to a single cell suspension, and the RBCs were lysed with Gey's solution. Total cells were then stained for CD4 and PD-1, and CD4+ cells were analyzed for PD-1 expression. It would not be surprising if a difference
Figure 25

A

[3H]-Thymidine (CPM/1x10^5 cells)

OVA_{323-339} (ug/ml)

- No Treatment
- I.V. Antigen (OVA)

B

% PD1^+ / Ki67^+ Cells

No Treatment I.V. Antigen (OVA)

p < 0.0001
Legend for Figure 25

PD-1 expression increases in CD4+ T cells tolerized in vivo. A. DO11.10 OVA transgenic CD4+ T cells were adoptively transferred into BALB/c recipients and tolerized 24 hours later with an intravenous injection of 300 μg of OVA peptide. As a control, transgenic cells were injected without the tolerizing peptide injection. Eleven days after adoptive transfer, lymph node and spleen cells were taken from the mice and rechallenged with irradiated BALB/c splenocytes pulsed with OVA peptide. Interestingly, the peptide-induced tolerance can be reversed by including 15 U/ml rmIL-2 in the restimulation culture, in addition to the 4 ug/ml OVA peptide. B. A population of cells was also stained for PD-1 and the clonotypic T cell receptor, showing that cells tolerized in vivo up-regulate PD-1.
between the groups was undetectable, since the percentage of total CD4$^+$ cells responding to alloantigen is likely small, but as seen in Figure 26, there is a significant increase in PD-1 expression on the CD4$^+$ population from the mice that received DST only. Interestingly, there is significantly less PD-1 expression in the mice given DST and treated with αCD154 mAb.
Figure 26

Day 3

% PD1+ / Total CD4+

p = 0.0031

No Treatment
DST
DST + MR1

Day 7

% PD1+ / Total CD4+

No Treatment
DST
DST + MR1

Treatment
Legend for Figure 26

Treatment with αCD154 mAb down-regulates PD-1 in DST-treated mice. C57BL/6 mice were transfused with $10^7$ BALB/c splenocytes in RMPI 1640. Half of these were concurrently given an intraperitoneal injection of 500 μg of αCD154 mAb (MR1). As a control, a group of C57BL/6 mice were not injected with cells or antibody. Spleens were removed on day 3 or 7 and stained for CD4 and PD-1. The mice whose spleens were examined on day 7 were given another injection of αCD154 on day 3.
Chapter 4 Discussion

Programmed Death-1 was named as the result of its appearance in a subtractive hybridization screen comparing T cells undergoing apoptosis with live T cells (66). Later studies from the same group detected increased PD-1 expression on mouse thymocytes and splenocytes stimulated with αCD3 or concanavalin A, and on spleen B cells stimulated with anti-IgM antibody (165). Subsequent studies in human Jurkat T cells confirmed this up-regulation by stimulation with TPA, and showed that maximal expression took place at 48 hours. This study also definitively showed that PD-1 is expressed on activated cells and not on cells undergoing apoptosis (166). Since PD-1 is a member of the immunoglobulin superfamily, it was first speculated that it might play a role in T cell activation, but when the gene was targeted in the C57BL/6 mouse strain the result was autoimmunity rather than lymphopenia (167). This phenotype was an indication that PD-1 may play a role in maintaining peripheral tolerance. Since the amino acid sequence of PD-1 contains a transmembrane region and PD-1 is a member of the CD28 superfamily, the natural question that arises is, to what ligands does PD-1 bind? PD-1 does not contain the MYPPPY motif necessary for binding to B7-1 and B7-2 (168). This distinguishes it from both CD28 and CTLA-4, who bind these two B7 family members in order to carry out their respective functions. Since PD-1 and CTLA-4 share a 23% identity, investigators examined other B7 family members in attempts to identify ligands for PD-1 (169). The result of these studies was the discovery of a novel B7 family member, B7-H1 (PD-L1), shown to specifically bind PD-1-expressing CHO cells. These studies also examined the expression of PD-L1, B7-1, and B7-2 on APCs, and found that
PD-1 is not expressed by unstimulated monocytes, but is rapidly up-regulated when the cells are stimulated, such as with IFN-γ. This up-regulation displayed very similar kinetics to B7-1 expression on monocytes. When the authors examined PD-L1 expression on dendritic cells, they found PD-L1 expression increased dramatically over 16-20 hours after stimulation with LPS and IFN-γ.

Using a PD-L1.Ig fusion protein, the authors of these studies extended their findings to examine the effect of PD-1:PD-L1 interaction on T cell stimulation, comparing wild-type mice with PD-1 knockout mice. Adding PD-L1.Ig to the stimulating culture inhibited wild type T cell proliferation in a dose-dependent manner. This was not the case with the PD1/-/- T cells, which did not show a different response between αCD3 stimulation and αCD3 + PD-L1.Ig stimulation. Interestingly, PD-1 engagement by PD-L1 on APCs prevented IFN-γ and IL-10 production by purified CD4+ T cells, suggesting that PD-1 plays an inhibitory role in both Th1 and Th2 subsets. To characterize this anti-proliferative effect further, cells were stimulated with αCD3, αCD28, and PD-L1.Ig in different combinations. These experiments showed that strong signaling through CD28 is able to override the effect of PD-L1.Ig on T cell stimulation.

Shortly following these studies, another ligand for PD-1 was discovered and named PD-L2 (B7-DC) (170). Studying these ligands together, the authors found that ligation of PD-1 on T cells arrested them in G0/G1 phase, but did result in cell death. Interestingly, the authors also showed that ligation of PD1 and TCR resulted in rapid phosphorylation of SHP-2, suggesting that increased phosphatase activity is a possible mechanism by which PD-1 exerts its anti-proliferative effects.
PD-1 and Peripheral Tolerance

A number of studies have implicated PD-1 in peripheral tolerance. The expression of PD-1 has been detected on a greater variety of cell types than that of CTLA-4. It has been speculated that PD-1 may be acting in concert with CTLA-4 to globally suppress unsolicited immune reactions (171).

Studies in cardiac allografts have suggested a possible role for PD-1 in regulating peripheral tolerance. In a fully-MHC-mismatched cardiac graft, PD-1, PD-L1, and PD-L2 can be seen to be up-regulated between days 2 and 5 with their expression remaining high until end-stage rejection (172). This was not the case with isografts. These experiments utilized total RNA isolated from heterotopic cardiac grafts, and therefore it is not known which cell populations were specifically expressing these transcripts. Interestingly, the use of αCD154 mAb therapy, with or without concomitant DST, decreased the intra-graft mRNA expression of PD-1 and PD-L2, and significantly decreased the expression of PD-L1. This result is somewhat counterintuitive, given that this therapy is known to prolong graft survival by inhibiting the immune response, which is presumably also what PD-1 does. Heart transplant recipients treated with cyclosporine A or rapamycin to prolong engraftment also showed decreased expression of PD-1, PD-L1, and PD-L2. In order to further dissect the role of PD-1 in allograft tolerance, the authors employed PD-L1.Ig fusion protein in combination with CSA or RPM to attempt to prolong graft survival and found that PD-L1.Ig + rapamycin therapy enabled long-term engraftment. These studies demonstrate that the modulation of PD-1 signaling may be
utilized in other models of transplantation to establish or maintain a state of peripheral tolerance.

The role of PD-1 has been examined in other models of transplantation. In islet transplantation experiments, stimulating PD-1 with PD-L1.Ig while blocking costimulation with αCD154 mAb prolonged the survival of islet allografts (154). Neither agent used alone extended survival significantly. In parallel studies, the authors stimulated splenocytes with αCD3 ex vivo and found that low doses of PD-L1.Ig and αCD154 antibody completely abolished T cell proliferation. In contrast to the previously discussed study, these findings suggest that it may be possible to exploit the combination of PD-1 signaling and costimulation blockade with αCD154 antibody to facilitate allograft tolerance over the long term.

PD-1 has been shown to play a role in the development of autoimmune diabetes in the NOD diabetic mouse model (173). The use of blocking antibodies against PD-1 and PD-L1 rapidly precipitated diabetes in 10-week-old female NOD mice, whereas antibody against PD-L2 had no effect. In 4-week-old mice, αPD-L1 antibody induces diabetes in mice 20 days following the initiation of treatment, while PD-1 antibody has a less profound effect, precipitating diabetes in just over a third of mice tested at this age. Again, PD-L2 blocking antibody had no effect in this case. Despite the fact that few male NOD mice develop overt diabetes at 10 weeks of age, blocking PD-1-PD-L1 interaction had a similar effect to that seen in females of this age: PD-L1 antibody precipitated diabetes in 100% of the male mice treated, and PD-1 antibody did so in 80%. Again, PD-L2 antibody had no effect. Interestingly, at 4 weeks of age, PD-1 antibody
had no effect, whereas PD-L1 antibody induced diabetes in 75% of treated mice. Hopefully future studies will clarify this seeming discrepancy. It has been shown in tumor models that PD-L1 may drive T cell apoptosis through a receptor other than PD-1 (174), which may explain the greater activity of the PD-L1 blocking antibody than that of anti-PD-1 in these experiments.

**Mechanisms of PD-1 signaling**

The full details of what signals PD-1 is delivering to the cell are not known, but a number of studies have revealed important clues as to how PD-1 may dampen a T cell response. Ligation of PD-1 on T cells has been shown to decrease IL-2 production in response to stimulation with αCD3 antibody and PD-L1.Fc conjugated beads (175). Stimulation with αCD3 antibody + PD-L1.Fc-conjugated beads also resulted in fewer cellular divisions than αCD3-bound beads alone. Interestingly, when CD28 was added to these stimulation cultures, sufficient IL-2 was produced to drive cell proliferation, suggesting a capability of CD28 to override negative signals delivered by PD-1 in some contexts. These data suggest that CD28 and PD-1 signals are balanced against each other when T cells are stimulated; when a strong PD-1 signal is delivered, IL-2 production is inhibited and the cell is incapable of proliferation. When CD28 signals are present in sufficient strength, they override the negative signal delivered by PD-1 and are able to drive clonal expansion.

PD-1 has no secondary or tertiary structural features suggesting it has any inherent enzymatic activity that might be acting to directly inhibit TCR signaling.
Rather, there are two tyrosines whose surrounding residues classify them as an immunoreceptor tyrosine inhibitory motif (ITIM) and an immunoreceptor tyrosine switch motif (ITSM). These domains are known to bind phosphatases such as SHP-1 and SHP-2 (176), which are believed to mediate the negative regulatory functions for the receptors they bind. In a series of studies demonstrating the importance of these domains in PD-1 signaling, the cytoplasmic tail of PD-1 was mutated at each of the domains to inactivate them. The studies found that while mutation of the ITIM had no effect on the ability of PD-1 to inhibit IL-2 production, mutation of the ITSM completely abrogated the ability of PD-1 to dampen IL-2 production and proliferation (177). These studies also showed, interestingly, that SHP-2 binds the cytoplasmic tail of PD-1 upon TCR stimulation, even in the absence of any interaction between PD-1 and its ligands, but the ability of PD-1 to inhibit cellular proliferation is dependent on receptor ligation. This rules out reasonably well the possibility that PD-1 is able to act in a ligand-independent manner, such as has been shown with CTLA-4 (63).

Studies have investigated the relationship between other costimulatory pathways and PD-1. Purified CD4+ T cells stimulated by αCD3 antibody and ICOS-ligand.Fc-coated beads proliferate more vigorously than those stimulated with anti-CD3-coated beads alone. The addition of PD-L1.Fc to the stimulation beads abrogates this effect. This suggests that costimulation through ICOS does not produce enough IL-2 to override the negative signals delivered through PD-1 (178). These studies show that exogenous IL-2 can rescue CD4+ T cells from PD-1-mediated inhibition, and they also found that exogenous IL-7 and IL-15 are able restore T cell proliferation, possibly through their
activity at the $\gamma_c$ subunit of the IL-2 receptor. Interestingly, IL-4 and IL-21, which also deliver signals through $\gamma_c$ cannot overcome PD-1-mediated inhibition. The authors attempt to reconcile this distinction by speculating that it is the activation of STAT5, which IL-2, IL-7, and IL-15 are known to do (179), that enables the cell to overcome PD-1 signals, whereas IL-4 and IL-21 act predominantly through other members of the STAT family.

Attempts to define the biochemical mechanisms of PD-1 signaling examined a number of enzymes known to be necessary for productive T cell signaling. One study showed that ERK and PKC-$\theta$ phosphorylation are impaired upon PD-1 ligation, suggesting the intervention of PD-1 in two important branches of the IL-2 production pathway (155). The authors extend their findings to show that in Jurkat T cells, CD3-$\zeta$ and ZAP-70 phosphorylation are impaired by treatment with PD-L1.Fc conjugated beads. These data identify a number of points in the IL-2 production pathway at which the effects of PD-1 signaling can be found.

There is more work that needs to be done to describe to relationship between the PD-1 negative costimulatory pathway and IL-2 production. Increasing our knowledge of a signaling pathway that biochemically intervenes in the IL-2 production process has a great deal of therapeutic potential. Finding ways to selectively activate the PD-1 pathway at specific times during an immune response or on specific populations of T cells may enable the precise tailoring of an immune response, possibly leading to more advanced transplantation protocols.
Chapter 5 Introduction

The biochemical picture of T cell anergy is constantly being revised and improved, and there are still many details to be learned. Given the results shown here, it is possible to develop a model assimilating what has previously been shown about the signaling mechanisms in anergy, and these new findings. With each new study, it becomes more evident that anergy depends on a complicated network of signals under careful regulation. It is possible to divide the anergic phenotype into three major phases: early induction, late induction, and maintenance/rechallenge (Figure 27). Within this paradigm, it is possible to assign known anergy factors to each the phase, constructing a progression of events that explain the phenotype of an anergic cell at each stage of the process.

Proposed Phases of Anergy Induction and Maintenance

The TCR is able to signal by two pathways, one pathway transducing an activation signal to the cell and the other establishing a hyporesponsive phenotype. It is likely that the branching point at which these two diverge is reached a short time after
Figure 27

Proposed Phases of Clonal Anergy

<table>
<thead>
<tr>
<th>0-12 hours</th>
<th>12 hours - 5 days</th>
<th>5-12 days</th>
</tr>
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<tbody>
<tr>
<td>Early Anergy Induction</td>
<td>Late Anergy Induction</td>
<td>Anergy Maintenance/Rechallenge</td>
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</table>
Legend for Figure 27

Anergy is established by a progression of biochemical events, with different phases being orchestrated by different types of factors. Shown here are the three proposed phases of clonal anergy.
TCR engagement, within 4-16 hours (72). The activity of E3 ligases is probably most crucial during this time, particularly that of GRAIL whose expression has been shown to be up-regulated in anergic cells at 6 hours following ionomycin treatment, returning to baseline by 18 hours (133). This timeframe can be thought of as the early induction phase of anergy. It has been proposed that the expression of ubiquitin ligases early in the induction of anergy serves to degrade key TCR-dependent signaling molecules such as PLC-γ1 and PKC-0, both of which are necessary for the production of IL-2 (136).

The lack of initial IL-2 production after a signal through the TCR is the primary determinant shunting T cells into anergy rather than productive stimulation, and it is likely that it is the major role of E3 ligases in anergy is to prevent this initial IL-2 production. A more extensive study of the kinetics of E3 ligase expression and the specific timing of target degradation would be required to further flesh out this idea.

Egr-2 and PD-1 in different phases of anergy

We have shown that Egr-2, a zinc-finger transcription factor, is important for anergy induction, and that its expression is quickly decreased by the treatment of anergic cells with IL-2. Despite the expression of Egr-2 at very early times in both anergic and costimulated T cell clones, it is likely that the actions of Egr-2 are most important at later times, such as 48-72 hours following removal from the anergizing stimulus. During this window, there is a marked distinction between Egr-2 expression levels in anergic and costimulated cells; it is apparent that by this time the costimulated cells have proceeded
to down-regulate Egr-2 as part of the process of progressing to cell cycle entry and proliferation.

The process by which proliferating cells down-regulate Egr-2 is not known. One possibility is that Egr-2 may be diluted as a consequence of cell division thus reducing their levels to below some threshold level required for their activity. Another possibility is that there is an active degradation process in place to override the anergic signaling pathway in order to proceed to an activated status. It is most likely that the latter is the case, given data showing that cells that receive a signal through the IL-2 receptor but do not proliferate due to the presence of hydroxyurea can still break anergy and are responsive to antigenic stimulation following removal of the hydroxyurea (82).

The secondary and tertiary protein structure of Egr-2 reveal little other than its ability to bind DNA with a C$_2$H$_2$ zinc-finger binding domain (180). This and numerous other studies demonstrating the necessity of Egr-2 for expression of various proteins suggest that its primary role in anergy is to up-regulate genes responsible for preventing IL-2 production in anergic cells. This hypothesis is supported by the data shown here: the knockdown of Egr-2 in anergic cells is effective in restoring proliferative capabilities when Egr-2 siRNA is transfected into cells prior to the induction of anergy, but this restoration does not occur when Egr-2 is depleted following the establishment of the anergic phenotype.

Perhaps the best way to determine the targets of Egr-2 transactivation would be to constitutively express Egr-2 in a population of T cells, stimulate the cells, and compare
the transcriptome and proteome of the Egr-2-expressing cells with those of normally anergized, fully stimulated, and untreated T cells.

The phase that includes the activity of those putative genes driven by Egr-2 can be thought of as the maintenance phase of anergy. This is the phase in which the phenotype of anergy actually manifests, resulting in the biochemical blocks that prevent IL-2 production. Since a number of biochemical blocks in the TCR transduction pathways have been observed in anergic cells, it is not unreasonable to think that anergy maintenance is implemented by numerous factors acting in concert. The possibility remains, however, that anergy is maintained by a single factor, whose functions can prevent IL-2 production in anergic cells. The data presented here show that PD-1 depletion during the maintenance phase of anergy results in a complete restoration of antigenic responsiveness when compared with that of costimulated cells, suggesting that PD-1 is capable of suppressing IL-2 production at least to a degree that lowers IL-2 levels below the threshold required for entry into the cell cycle. It has been proposed that one consequence of PD-1 engagement in T cells is the suppression of IL-2 production, although the means by which it does this have not been definitively determined.

A summary of how these factors fit into the phases of anergy can be found in Figure 28. If a small number of factors can be found whose actions are responsible for preventing the proliferation of an anergic cell, the chances for effective therapeutic intervention are greater than if an extensive and redundant biochemical network was in place to suppress IL-2 production. Mapping out the active factors in each phase of
### Figure 28: Anergy Induction and Maintenance

<table>
<thead>
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<th>0-12 hours</th>
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<tbody>
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<td><strong>Late Anergy Induction</strong></td>
<td><strong>Anergy Maintenance/Rechallenge</strong></td>
</tr>
<tr>
<td>Upregulation of E3 Ubiquitin Ligases/ Degradation of PLCγ1, PKCθ</td>
<td>Expression of Egr-2</td>
<td>Expression of PD-1</td>
</tr>
<tr>
<td></td>
<td>Transcriptional Activation of Anergy Maintenance Genes</td>
<td>Institution of signal transduction blockades preventing IL-2 transcription/production</td>
</tr>
</tbody>
</table>
Legend for Figure 28

The activities of factors discovered in our laboratory and others correspond to the different phases of anergy.
anergy offers the opportunity to intervene prior to anergy induction, or to try to reverse anergy in a specific population of cells after anergy has been established. As there is good evidence that anergy plays an important role in numerous instances of peripheral tolerance, it may be possible to exploit anergy regulatory points to prevent an undesired immune response.

A comprehensive model of clonal anergy is shown in Figure 29. It is possible that by refining our knowledge of the steps detailed in this diagram, we may be able to identify means by which to harness the anergic phenotype in select populations of T cells. A means of inducing a hypo-responsive state in allo-reactive T cells could lead to safer, less invasive ways to enable allo-transplantation while minimizing the risks and long-term harm to patients.
Legend for Figure 29

Early signaling events determine whether a T cell enters an anergic state or proceeds to productive clonal expansion. A. T cells that receive costimulation in addition to a TCR signal do not express E3 ligases, the absence of which permits sufficient IL-2 production to drive cell cycle progression. This leads to the down-regulation of Egr-2 and PD-1 leaving the cell subsequently capable of responding appropriately to antigenic stimulation. B. The presence of E3 ligases early in an anergic cell leads to the degradation of proteins crucial to IL-2 production, facilitating the full induction of anergy, at least in part by the transactivating capabilities of Egr-2. It is likely that Egr-2 directs the up-regulation of proteins whose activities are capable of rendering the cell unresponsive to full antigenic stimulation. Whether PD-1 expression is regulated directly by Egr-2 or not, its high expression in anergic cells on day 5 after anergy induction is able to prevent IL-2 production in anergic cells.
Discussion of *In Vivo* Anergy PD-1 Expression

The data produced by the DO11.10 adoptive transfer experiments offer support to the hypothesis that PD-1 is up-regulated on tolerant populations of CD4⁺ T cells, but there is a caveat to the data shown. In the absence of a control group showing a break in peptide-induced tolerance (achievable by the use of any of a number of adjuvants) it is difficult to emphatically claim that PD-1 expression is specific to tolerized cells. Despite the correlation seen here, it is possible that an immunized population of cells, having received peptide in conjunction with adjuvant, could express PD-1 at similar levels as the peptide-tolerized cells, despite showing an ability to respond to antigen. The initial attempts to include this control here resulted in an inability to prevent tolerance, despite the inclusion of 25 μg of lipopolysaccharide with the intravenously administered peptide. This has been shown to be an effective way of breaking peptide-induced tolerance (112), although in my hands, the cells treated with LPS and peptide were equally as hyporesponsive as the peptide-tolerized cells, and did not show a significant decrease in PD-1 expression levels (data not shown). It is possible that increasing the amount of LPS administered could result in a successful reversal of peptide-induced tolerance, or it may be necessary to substitute another adjuvant for LPS to successfully reverse tolerance in this case.
Implications of PD-1 Expression in Costimulatory Blockade Protocols

It has not yet been clearly shown what peripheral tolerance mechanisms are working when tolerance is induced with αCD154 mAb and transfusions of donor splenocytes, although there is good evidence that deletion of CD8+ cells (181) and immune suppression by CD4+CD25+ T cells (182) are processes essential to allograft tolerance. It is also possible that a population of donor-specific T cells is anergic, preventing them from responding to a graft. Identifying the antigen-specific population of CD4+ cells to determine their anergic status is difficult, although recently developed protocols using CD4+ cells from TCR transgenic mice have the potential to enable this type of experiment.

When total CD4+ cells are examined shortly after the administration of DST and αCD154 mAb, it appears either that there is a block in PD-1 expression or PD-1 is being actively down-regulated. It has not been shown that costimulation is required for PD-1 expression; in fact, in the A.E7 T cell model PD-1 is up-regulated strongly by TCR engagement alone. The data shown here confirm data shown previously that αCD154 down regulates PD-1 expression in an allograft, even at 7 days post-transplantation (172), although taken together these findings are difficult to reconcile with those of studies showing the ability of PD-1 engagement to prolong allograft survival in conjunction with other protocols (154). It is possible that the effects shown in total CD4+ cells in DST and anti-CD154–treated mice do not represent the subset of CD4+ cells actually responding to alloantigen, and that PD-1 is being down-regulated on another population of cells. Perhaps CD4+CD25+ cells actually need to expand in order to facilitate tolerance to an
allograft, and are able to do this by down-regulating PD-1. Little has been published about the surface expression of PD-1 on CD4\(^+\)CD25\(^+\) T cells, although one study has identified PD-1 on purified CD4\(^+\)CD25\(^+\) populations (183). This study did not compare PD-1 levels on regulatory T cells to those of other cell populations, so the finding is not entirely informative with regard to predicting what may be happening in the DST + αCD154 protocol. If anergic cells are in fact employed in this tolerance protocol, it is possible that they are important at times later than 7 days. There is sufficient evidence that PD-1 may play a significant role in peripheral tolerance, and it is very possible that adding a means by which to activate PD-1 negative regulatory pathways to avoid a rejection response (perhaps both acute and chronic) could augment the graft survival currently seen with established costimulatory blockade protocols. Understanding the means by which PD-1 is able to negatively regulate alloimmune responses could provide insight into new methods of therapeutic induction of graft tolerance, keeping in mind the end goal of avoiding systemic immune suppression altogether.

**Future Questions**

The data presented in this thesis show two factors that are required for the induction and maintenance of anergy. There are many questions left to answer. For example, it will be useful to know whether Egr-2 is directly up-regulating PD-1, or if the ability of Egr-2 to manage anergy induction is dependent on the up-regulation of other gene products. Another interesting unknown is the mechanisms of how PD-1 is preventing anergic cells from proliferating, and more specifically how it is preventing IL-
2 production. Preliminary experiments not shown here suggest that PD-1 is not preventing ERK, Akt, NFkB, or PKCδ activation, thus it will be necessary to examine other important components of the IL-2 production pathways such as TCRζ, ZAP-70, and PLCγ. Given that the cytoplasmic tail of PD-1 is known to bind phosphatases, it is possible that anergic cells express sufficient levels of PD-1 to recruit high levels of phosphatases to important signaling microdomains and thereby block the activation of key signaling molecules. If this is the case, it may be possible to induce anergy therapeutically by identifying agents that deactivate these same important signaling molecules even in the absence of high levels of PD-1.

**Possible Roles for Anergy in Transplantation Tolerance**

The role that anergy has in transplantation tolerance remains open to speculation. It is tempting to contemplate a number of possibilities. Since CD8+ cells depend on IL-2 produced by CD4+ cells, it is possible that rendering CD4+ cells anergic could prevent the expansion of CD8+ cells by decreasing the amount of IL-2 help. Since CD8+ T cells are vital to graft rejection, anergizing the CD4+ cells on which they depend could block this aspect of the rejection process. It is also possible that B cell activation could be impaired when CD4+ T cells are rendered anergic and unable to make sufficient cytokines to drive B cell activation.

In addition to the implications of anergy in provision of IL-2 help, the process of anergizing graft-specific CD4+ T cells could generate a subpopulation of regulatory T cells that can prevent the activation of other CD4+ and CD8+ T cells. More specific
markers for these populations need to be identified before good characterization of a phenomenon like this would be possible. Anergic T cells may also play a role in establishing a tolerizing environment. The Affymetrix mRNA profiles generated early in this project showed an increase in CCL-1 production (data not shown), a chemokine known to induce chemotaxis in CD4^+CD25^+ regulatory cells (184). If anergic T cells and regulatory T cells are in fact distinct populations, it is possible they act in concert to attenuate T cell responses at the site of a graft, with anergic cells migrating to a potential inflammatory locus first and then establishing a CCL-1 gradient to recruit regulatory T cells. These are only a few possible ways that anergic cells could play an important role in facilitating peripheral tolerance.

**Final Conclusion**

Furthering our understanding of the mechanisms of peripheral tolerance will enable the grafting of tissues and organs across HLA-incompatible environments, without the use of systemic immunosuppression. It is possible that the induction of T cell anergy is a means by which T cells can be trained not to mount an immune response against a specific set of antigens. The knowledge that Egr-2 is a primary regulator of anergy induction and PD-1 is responsible for anergy maintenance could inform attempts to do this with greater precision. Further investigation into the downstream targets of Egr-2, and the biochemical means by which PD-1 negatively regulates T cell responses could provide additional targets with the potential for therapeutic intervention. Given the current state of research on negative costimulation, and the data presented here, it is
possible that therapeutic agents tailored to impose tolerance by these pathways could progress toward clinical applications in the near future.
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