A TCR TRANSGENIC MODEL OF INFECTION-INDUCED AUTOIMMUNE 
PSORIASIFORM SKIN DISEASE

by

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Psoriasiform skin diseases are a poorly understood group of disorders. Recent data has implicated the immune system with a central role in disease pathogenesis. In this thesis, various T cell populations were studied in a TCR transgenic model of psoriasiform disease, whose abnormal interactions culminate in distinctive psoriasiform pathology. The model is based upon the expression of the transgenic 2C TCR on the H-2^d^-expressing DBA/2 inbred strain (referred to as D2C mice). The 2C TCR recognizes a peptide (p2Ca) derived from the ubiquitous mitochondrial protein 2-oxoglutarate dehydrogenase, presented by the MHC class I molecule, L^d. In D2C mice, expression of the 2C TCR led to a comprehensive depletion of immature T cell progenitors, which express the 2C TCR, and a resultant lymphopenia of mature peripheral T cells. The lymphopenia of CD8^+ cytotoxic and CD4^+ helper T cells predisposes to the overgrowth of opportunistic pathogens, resulting in inflammatory skin disease restricted to the sebum rich areas of the skin, resembling the human psoriasiform disease seborrheic dermatitis. In D2C mice, there is also a deficiency in T regulatory (T_{reg}) cells as a result of slowed thymic output of mature T cells. The reduced T_{reg} function results in a lymphoproliferation of polyclonal CD4^+CD25^- cells, many of which home to the aforementioned cutaneous inflammatory sites. This expansion of “helper” cells was likely due to antigenic stimulation, presumably against immunogenic determinants of opportunistic pathogens. Reconstitution of the T_{reg} compartment by adoptive transfer abrogates the development of psoriasiform pathology and precludes the lymphoproliferation of CD4^+CD25^- cells. These data suggest that T_{reg} may downregulate the response of mature cells to ubiquitous commensal organisms as a means to maintain immunological homeostasis.

In TCR transgenic mice which express the cognate Ag of the transgenic TCR, a large population of transgenic cells exist in the peripheral lymphoid tissues. These cells are anergized
and fail to respond to stimulation with cognate ligand; however, they express a memory immunophenotype, including the intermediate affinity IL-2 receptor. The expression of this receptor enables these cells to use bystander IL-2 or IL-15 to overcome this inactivation, revealing enhanced functional properties induced by the high-affinity interaction with cognate ligand. These observations suggest that such clonally anergized cells may represent an in vivo autoimmune hazard; and, interestingly, a further consequence of the CD4^+CD25^- lymphoproliferation in D2C mice is the bystander activation of these transgenic T cells. After undergoing acute activation, 2C T cells exacerbate the cutaneous pathology in these animals, a consequence that can be abrogated by the administration of a blocking mAb against the 2C TCR.

Interestingly, the combination of immunodeficiency, Treg lymphopenia, the presence of CD4^+CD25^- cells capable of undergoing vigorous expansion, and a large population of memory phenotype 2C transgenic cells was insufficient to induce disease when D2C bone marrow was adoptively transferred to lethally irradiated syngeneic DBA/2 mice. Examination of these animals revealed that bone marrow transfer did not deplete the skin of DBA/2-derived cutaneous γδ cells. Sentinel intraepithelial γδ lymphocytes have been shown to have an important role in surveying the epithelium for signs of infection and malignancy as well as in maintaining epithelial integrity. The development of these cells is curtailed in D2C mice due to the forced expression of the 2C TCR; however, the persistence of these cells in the aforementioned bone marrow chimeras may have protected these animals from the development of the disease phenotype. While generated DBA/2 TCRδ^{-/-} mice did not develop spontaneous disease, the transfer of D2C bone marrow to lethally irradiated DBA/2 TCRδ^{-/-} recipients successfully transferred the disease phenotype, confirming the importance of these cells in protecting against the development of psoriasiform pathology. This result also demonstrated that a compromised
cutaneous barrier is necessary for disease pathogenesis, as disease does not develop when the skin is populated by sentinel intraepithelial lymphocytes.

While considerable research efforts have been focused on human psoriasiform disease, a solid understanding of disease pathophysiology is severely lacking. This limited knowledge of psoriasiform disease is highlighted by the ongoing uncertainty of whether these diseases represent primary diseases of the epithelium, or whether these diseases represent tissue specific autoimmunity occurring in normal skin as a result of dysfunctional immunity. One explanation for this failure to understand basic principles of psoriasiform disease pathophysiology can be attributed to the limited numbers of appropriate model systems to carefully study disease. The D2C model of psoriasiform disease has been shown to be an accurate model system which has demonstrated that a complex interplay between various immunocytes and epithelium culminates in psoriasiform disease. The insight that the D2C model has generated will lead to a better understanding of these poorly characterized psoriasiform conditions.
Table of Contents

Abstract  ii

Table of Contents  v

List of Tables  x

List of Figures  xi

List of Abbreviations  xiv

Acknowledgements  xx

Chapter 1: Introduction  1

1.1 The Immune System  1

1.1.1 Innate Immunity  1

1.1.2 Adaptive Immunity  2

1.2 The T Cell Receptor and T Cell Ag Recognition  3

1.3 T Cell Signalling  8

1.4 T Cell Development  12

1.5 Tolerance  22

1.6 Psoriasiform Disease  28

1.6.1 Psoriasis  29

1.6.2 Seborrheic Dermatitis  34

1.7 Thesis Goals and Approaches  41

Chapter 2: Materials and Methods  44
Chapter 4: Immunosuppression and Immune Repertoire Perturbation

4.1 Introduction

4.2 Characterization of Immune Function in D2C Mice

4.2.1 Quantification of Thymocytes and T Cell Subsets In D2C Mice

4.2.2 Assaying T Cell-Dependent Humoral Immune Function

4.2.3 Characterization of Additional Immunopathological Features Of D2C Mice

4.2.4 Further Characterization of the CD4+ T Cell Subset

4.2.4.1 Longitudinal CD4+ T Cell Quantification

4.2.4.2 Immunophenotype of Expanding CD4+ T Cells from D2C Mice

4.3 Immunological Reconstitution of D2C Mice

4.3.1 Adoptive Transfer of Syngeneic CD4+ T Cells to Pre-Diseased D2C Mice

4.3.2 Passive Immunization of Pre-Diseased D2C Mice with Opportunistic Pathogen-Specific Serum IgG

4.3.3 Analysis of T_{reg} Development and Function in D2C Mice

4.3.4 Adoptive Transfer of Purified, Syngeneic T_{reg} and CD4+CD25- "Helper" T Cells to Pre-Diseased D2C Mice

4.3.5 Comparison of D2C and FoxP3 Knockout Mice

4.4 Dexamethasone Treatment of D2C Mice

4.5 Conclusion

Chapter 5: Self-Reactive T Cells in Disease Pathophysiology

5.1 Introduction:

5.2 Self- Reactive Cells in TCR Transgenic Mice
Chapter 6: Cutaneous γδ T Lymphocytes and Their Role in Disease

Pathophysiology

6.1 Introduction

6.2 Intraepithelial Lymphocytes in 2C Mice

6.2.1 Characterization of IELs in 2C TCR Transgenic and Non-Transgenic Mice on the B6, DBA/2, and Mixed (B6xDBA/2)N1 Backgrounds

6.2.2 Cutaneous Immunoregulatory Function of 2C IELs in D2C Mice

6.2.3 Adoptive Transfer of Disease is Associated with the Replacement of Cutaneous γδ T Cells with 2C Lymphocytes

6.2.4 Adoptive Transfer of Disease to DBA/2 TCRδ−/− Mice

6.3 Conclusion

Chapter 7: Implications of the D2C Model on Contemporary Hypotheses of Psoriasiform Disease Pathogenesis

7.1 Introduction
List of Tables

Table 1. Development of Disease in Recombinant Strains ........................................ 28
Table 2: Antibodies Used .................................................................................................. 45
Table 3: Primers Used ....................................................................................................... 47
Table 4: Identification of the Fungal Isolates as *Candida guilliermondii* ......................... 77
Table 5. ITS Sequence Data of the Fungal Isolate ........................................................... 79
Table 6. Cutaneous Lymphocyte Subsets in the Various Mouse Strains Studied ............... 176
List of Figures

Figure 1. Summary of T Cell Development .............................................. 154
Figure 2. Schematic of the 2C TCR and its Antigen Interactions .................. 19
Figure 3. Gross Pathology of Psoriasis .................................................. 310
Figure 4. Histopathology of Psoriasis ................................................... 32
Figure 5. Gross Pathology of Seborrheic Dermatitis ................................ 36
Figure 6. Histopathology of Seborrheic Dermatitis .................................. 37
Figure 7. Occurrence of Seborrheic Dermatitis in the Context of HIV Infection .... 40
Figure 8. Gross Pathology of Disease .................................................... 69
Figure 9. Microscopic Pathology of Disease ............................................ 721
Figure 10. Antifungal Staining of Lesional Skin ........................................ 74
Figure 11. Isolation of Fungi from the Lesional Skin of Diseased D2C Mice ........ 76
Figure 12. PCR Amplification of the ITS Region of the Unknown Fungal Isolate ... 78
Figure 13. Fungal-Specific IgG and Relationship to Disease Activity ................ 81
Figure 14. Treatment of Diseased D2C Mice with Antifungal Medication ........... 83
Figure 15. Treatment of Pre-Diseased D2C Mice with Antimicrobial Agents ......... 84
Figure 16. Strain Susceptibility .............................................................. 88
Figure 17. Backcross Analysis of Disease Penetrance ..................................... 89
Figure 18. Adoptive Transfer of Disease with Bone Marrow ............................ 90
Figure 19. Effect of the DBA/2 C5 Mutation on the Disease Phenotype in N2C backcrosses to the DBA/2 background .................................................. 92
Figure 20. Evaluation for Lymphopenia and TCR Repertoire Skewing in D2C Mice .... 100
Figure 21. Assessment of T Cell-Dependent Humoral Immune Function in D2C Mice ... 102
Figure 22. Necropsy Observations made on D2C Mice

Figure 23. Characterization of the CD4⁺ T Cell Number and TCR Chain Usage over the Window of Disease Susceptibility

Figure 24. Immunophenotype of D2C CD4⁺ T Cells

Figure 25. Phenotypic Characteristics of Recovered D2C Mice

Figure 26. Adoptive Transfer of CD4⁺ T Cell to Pre-Diseased D2C Mice

Figure 27. Adoptive Transfer of CD4⁺ T Cells to Pre-Diseased D2C Mice (Continued)

Figure 28. Adoptive Transfer of Serum from S3 D2C Mice to Pre-Diseased D2C Animals

Figure 29. Evaluation of CD4⁺CD25⁺ T Regulatory Cells in D2C Mice

Figure 30. Effect of the Adoptive Transfer of CD4⁺CD25⁺ T_{reg} or CD4⁺CD25⁻ "Helper" T Cells to Pre-Diseased D2C Mice

Figure 31. Effect of the Adoptive Transfer of CD4⁺CD25⁺ T_{reg} or CD4⁺CD25⁻ "Helper" T Cells to Pre-Diseased D2C Mice (Continued)

Figure 32. Scurfy Mouse Cutaneous Histology

Figure 33. Additional Immunopathological Features of D2C Mice

Figure 34. Treatment of Pre-Diseased D2C Mice with the Corticosteroid Dexamethasone

Figure 35. Treatment of Pre-Diseased D2C Mice with the Corticosteroid Dexamethasone (Continued)

Figure 36. Expression of the 2C TCR in the Thymus and Peripheral Lymphoid Organs in B2C_{b} and D2C Mice

Figure 37. Immunophenotype of 2C TCR DNTC

Figure 38. Functional Characterization of 2C TCR DNTC from Disease-Resistant and Disease-Susceptible Mice
Figure 39. Determination of whether the Acutely Activated Immunophenotype and Enhanced Functional Properties of 2C DNTC are Cell-Intrinsic or Cell-Extrinsic Characteristics

Figure 40. Treatment of D2C Mice with a Blocking mAb against the 2C TCR

Figure 41. Effect of Previous Interventions on the Activation Status of 2C TCR DNTC in D2C Mice

Figure 42. Adoptive Transfer of CD69^ 2C TCR DNTC to Recipient DBA/2 Mice

Figure 43. Characterization of the DBA/2 Rag-1^ Mice

Figure 44. Characterization of D2C Rag-1^ Mice

Figure 45. Characterization of Cutaneous Intraepithelial Lymphocytes in B6, DBA/2 and Mixed (B6xDBA/2)N1 Mice With or Without the 2C TCR Transgenes

Figure 46. 2C Cells Located within Epithelial Sites

Figure 47. Microscopic Examination of Cutaneous Intraepithelial Lymphocyte Density

Figure 48. Correlation of Intraepithelial CD3^ Cell Density with Susceptibility to the Development of Cutaneous Pathology

Figure 49. Croton Oil Application to Assess Cutaneous Barrier Function

Figure 50. Susceptibility of DBA/2 Recipient Mice to Disease Following Transfer of D2C and B2C^ Bone Marrow

Figure 51. Characterization of Cutaneous Intraepithelial Lymphocytes in DBA/2 Recipients of D2C and B2C^ Bone Marrow

Figure 52. Characterization of DBA/2 TCR^ Mice

Figure 53. Characterization of DBA/2 TCR^ Recipients of D2C Bone Marrow

Figure 54. Summary of Factors Contributing to Disease Development
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>1B2</td>
<td>mAb against the 2C TCR</td>
</tr>
<tr>
<td>2C TCR</td>
<td>2C transgenic T cell receptor</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-Mercapto-ethanol</td>
</tr>
<tr>
<td>7AAD</td>
<td>7-Amino-Actinomycin D</td>
</tr>
<tr>
<td>-α</td>
<td>-alpha</td>
</tr>
<tr>
<td>α-</td>
<td>Anti-</td>
</tr>
<tr>
<td>α-gal cer</td>
<td>Alpha-galactosyl ceramide</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AIRE</td>
<td>Autoimmune regulator gene</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>β2M</td>
<td>β2 Microglobulin</td>
</tr>
<tr>
<td>B2Cᵇ</td>
<td>2C TCR-expressing B6 mouse</td>
</tr>
<tr>
<td>B2Cᵈ</td>
<td>H-2ᵈ congenic 2C TCR-expressing B6 mouse</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6 inbred mouse strain</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone marrow chimera</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
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</table>
**BXD**  | Recombinant inbred strains derived from C57BL/6 and DBA/2 crossings.
---|---
**BXD^d**  | H-2^d expressing, recombinant inbred strains derived from C57BL/6 and DBA/2 crossings

**C.**  | *Candida*
---|---
**C**  | Complement

**Ca^{2+}, Ca^{++}**  | Calcium
---|---
**CD**  | Cluster of differentiation
---|---
**CDR**  | Complementarity-determining regions
---|---
**CLIP**  | Class II-associated invariant-chain peptide
---|---
**CMI**  | Cell-mediated immunity
---|---
**ConA**  | Concanavalin A
---|---
**cpm**  | Counts per minute, as a measure of radioactivity
---|---
**d**  | Days
---|---
**D**  | Diversity T cell receptor gene segments
---|---
**D2C**  | DBA/2 2C T cell receptor transgenic mouse
---|---
**DAG**  | Diacylglycerol
---|---
**DBA/2**  | DBA/2 inbred mouse strain
---|---
**DC**  | Dendritic cell
---|---
**DETC**  | Dendritic epidermal T cell
---|---
**Dex**  | Dexamethasone
---|---
**df**  | Degrees of freedom
---|---
**DIC**  | Differential interference contrast
---|---
**DN**  | CD4^-CD8^- Double negative thymocyte
---|---
**DNA**  | Deoxyribonucleic acid
---|---
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DNTC</td>
<td>CD4⁺CD8⁻ Double negative T cell</td>
</tr>
<tr>
<td>DP</td>
<td>CD4⁺CD8⁺ Double positive thymocyte</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>F(Ab)</td>
<td>Fragments of antigen binding</td>
</tr>
<tr>
<td>FACS®</td>
<td>Fluorescence activated cell sorter – a registered trademark of BD Pharmigen</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead winged helix transcription factor</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine-nucleotide exchange factor</td>
</tr>
<tr>
<td>GMS</td>
<td>Grocott's Methenamine Silver</td>
</tr>
<tr>
<td>GPC</td>
<td>Gram positive cocci</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HEL</td>
<td>Hen egg lysozyme</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>HY TCR</td>
<td>HY transgenic T cell receptor</td>
</tr>
<tr>
<td>ICOS</td>
<td>CD278</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
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IHC  Immunohistochemistry
IL   Interleukin
IMDM Iscove's Modified Dulbecco Medium
i.p. Intraperitoneal
IP3 Inositol triphosphate
ITS Internal Transcribed Spacer
i.v. Intravenous
J  Joining T cell receptor gene segments
KGF Keratinocyte growth factor
KO Knockout (homozygous -/-)
L Ligand
l  Liter
LAT Linker of activation in T cells
M. Malassezia
M⁻¹ Inverse molarity
mAb Monoclonal antibody
MAC Membrane attack complex
MAP Mitogen activated protein
MHC Major histocompatibility complex
MICA/B Major histocompatibility complex class I chain-related A/B
min   Minutes
μl  Microliter
Mtv Mouse mammary tumor provirus
N₁₂C (C57BL/6 x DBA/2)N₁₂C mouse
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>NAPS</td>
<td>Nucleic Acid-Protein Service Unit at the University of British Columbia</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cells</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>p</td>
<td>Probability</td>
</tr>
<tr>
<td>P13K</td>
<td>Phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>p2Ca</td>
<td>Peptide LSPFPFDL (single letter amino acid code)</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen related molecular patterns</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid Schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PE-Cy5</td>
<td>Phycoerythrin-cyanin 5; also known as Tri-Color®</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phospho-inositol pyrophosphate</td>
</tr>
<tr>
<td>PKC-θ</td>
<td>Protein kinase C-θ</td>
</tr>
<tr>
<td>PLC-γ-1</td>
<td>Phospholipase C-γ-1</td>
</tr>
<tr>
<td>poly(I:C)</td>
<td>Polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>pTβ4</td>
<td>Prothymosin-β4</td>
</tr>
<tr>
<td>RAE-1</td>
<td>Retinoic acid early inducible</td>
</tr>
<tr>
<td>R-EAE</td>
<td>Relapsing experimental autoimmune encephalomyelitis</td>
</tr>
</tbody>
</table>
RBC  Red blood cell
RI   Recombinant inbred strains
S    Stage of D2C psoriasisiform disease
SA   Streptavidin
SD   Seborrheic dermatitis
SP   CD4^+CD8^- or CD4^-CD8^+ Single positive thymocyte
      species
spp.  
T3.70 mAb against the HY transgenic TCR-α chain
TAP  Transporter associated with antigen processing
Tc   Cytotoxic T cell
TCR  T cell receptor
TH   CD4^+CD25^- T helper cell
TMEV Theiler's murine encephalomyelitis virus
TNF  Tumor necrosis factor
Treg CD4^+CD25^+ T regulatory cell
TRIM TCR-interacting molecule
Tri-Color® Phycoerythrin-cyanin 5 (PE-Cy5)
TSA  Tryptic soy agar
TSLP Thymic stromal lymphopoietin
U    Units
V    Variable region of T cell receptor genes
WBC  White blood cell
YBC  Yeast biochemical card
Acknowledgements

I would like to thank my family for instilling within me the importance of learning and for helping to weather the ups and downs that invariably accompany academic pursuits. My grandparents, Molly, Joseph, and Rose, shared a similar philosophy on learning and were extremely supportive of my academic endeavours. Although I never knew my grandpa George, I have been told that he also held education in the highest regard. My parents, Walter and Joyce, demonstrated an unnatural degree of philanthropy during my impoverished student life. My brother, George, has also been extremely supportive of both my academic and personal development and has demonstrated amazing patience over the years. I am also indebted to my wonderful wife, Elisabeth, who has endured much hardship during my academic struggles and whose friendship, love and support makes me more complete.

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Chapter 1: Introduction

1.1 The Immune System

On a daily basis, somatic cells are subjected to wide variety of pathogenic microbes and other environmental insults. Diverse mechanisms have evolved to provide protection against this onslaught. Together, the innate and adaptive immune systems provide a remarkably effective defense against infectious pathogens, non-infectious foreign substances, and other sources of cell stress.

1.1.1 Innate Immunity

The innate immune response provides a general first line of defense able to act against a wide variety of pathogens in a rapid fashion by virtue of its relatively non-specific mode of action. Specific components of innate immunity include physical and chemical defenses such as epithelial barriers and high gastric acidity, respectively, which block the invasion of unwanted material. There are also “humoral” aspects of innate immunity, such as complement and other serum proteins (i.e., C reactive protein), which have an important role in early defense [1].

Another critical aspect of innate immunity are populations of circulating and non-circulating cells which neutralize invading microorganisms and foreign material by phagocytosis, the release of antibacterial and enzymatic agents as well as the liberation of important paracrine signaling molecules that facilitate the recruitment of other essential cells and mediators of the immune response [1]. The common feature shared by this diverse group of innate immune effectors is their rapid deployment, which allows for an almost-instantaneous response. This quick utilization of innate immune mechanism can be attributed to the germ-line encoded effector proteins and receptors characteristic of innate immunity as well as the perpetual renewal of large number of ready-to-use innate immune cells and effector molecules [1].
1.1.2 Adaptive Immunity

Adaptive immunity can be divided into two broad categories, humoral and cellular immunity, based upon the ontogeny of the effector cells as well as the mechanism by which the cells carry out their adaptive function. The antigen receptors of both T and B lymphocytes require the prior rearrangement of receptor gene segments by somatic recombination, resulting in novel receptors possessing a virtually unlimited ability to recognize Ag [1]. It is estimated that the mammalian immune system can discriminate approximately $10^{10}$ distinct antigenic determinants which is a testament to the diversity of adaptive antigen receptors and their unparalleled role in bodily defense [2]. Because of the vast repertoire of lymphocytes, the frequency of any one given clone is inadequate during the initial stages of a particular antigenic challenge. Therefore a “lag” period, typically 7-10 days after the initial insult [1], occurs in adaptive immunity during which lymphocytes with specific antigen-recognition properties undergo vigorous expansion, increasing in frequency by several orders of magnitude. Once sufficient levels of antigen-specific cells are attained, a focused, powerful immune response can be generated.

Humoral immunity is mediated by B lymphocytes, which arise in the bone marrow and which utilize secreted immunoglobulins as effector molecules which recognize the three-dimensional conformation of unprocessed antigens (Ag) without the aid of antigen-presenting molecules [1]. Recognition of specific antigen by the B cell receptor (BCR), which is a non-secreted, membrane-bound immunoglobulin, in association with important secondary signals, results in B cell activation and the subsequent development into antibody-secreting plasma cells [1].

The second major arm of the adaptive immune system is "cellular immunity" mediated in large part by the T lymphocyte lineage which requires the thymus for development [1]. T cells
carry out a variety of functions that are broadly divided into “helper”-based and cytotoxic functions [1]. T helper cells (T_H) are essential for the activation of B cells and macrophages by virtue the provision of the CD40L "second" activation signal to these cells [1]. The absence of this important cellular cooperation is best appreciated by the wide-spread immunodeficiency seen in common variable immunodeficiency where the failure of CD40L-CD40 signaling results in impaired immunity [3]. Cytotoxic T cells (T_C) are best known for their ability to kill viral infected cells through the release of perforin, granzyme and/or Fas/FasL signaling [4, 5]. The importance of T_C cells in anti-viral defense is made obvious by the innumerable ways that viral pathogens attempt to subvert T_C cell recognition of viral Ag [6]. Recently it has become apparent that at least some "cytotoxic" T cells have a role in the surveillance against malignant and infected cells as well, broadening the immunological function of these cells [7].

### 1.2 The T Cell Receptor and T Cell Ag Recognition

The T cell receptor (TCR) is the most specific marker of the T cell lineage [1]. The TCR of most "conventional" T cells consists of an α and β chain that form a disulfide-linked, monovalent, non-secreted heterodimer, although rarely, TCRs can consist of a homodimer of TCR β chains [8]. Both the α and β TCR chain possess an amino terminal variable region with homology to the immunoglobulin V region (V), a constant region with homology to the immunoglobulin C region (C), a short hinge region with a cysteine residue that forms the interchain disulfide bond, hydrophobic transmembrane segments, and a short cytoplasmic tail (2-7 amino acids in length) which is associated with TCR-related signalling molecules [9]. The TCR is generated from 52 variable (V) gene segments, 2 diversity (D) gene segments and 13 joining (J) gene segments for the β chain and ~70 V and 61 J gene segments for the α chain which randomly associate to form a diverse repertoire of T cell receptors [1, 9].
The regions of both the TCR α and β chains encoded by the V gene segments are found on three short, hypervariable loops, known as complementarity-determining regions (CDRs), which together form the antigen binding aspect of the TCR [9]. The junction of the V and D gene segments form the tips of the CDRs and, between these gene segments, non-germline encoded amino acids are randomly added and deleted, resulting in the generation of even greater diversity [1]. The focus of these diversity hot-spots within the area of antigen-recognition ensures the development of a large repertoire of T cells [9]. Thus, the structural diversity of TCRs is due to combinatorial and junctional diversity generated during gene rearrangement together which result in an extremely diverse repertoire of T lymphocytes.

The T cell Ag receptor is different from that of the BCR in that this heterodimeric transmembrane protein, with rare exception, recognizes short amino acid chains bound to proteinaceous antigen-presenting molecules [1]. This recognition therefore requires the prior digestion of antigenic proteins and the resultant binding of derivative peptides to Ag presenting molecules called Major Histocompatibility Complex (MHC) molecules, which are an integral component of most TCR ligands [1]. MHC was first recognized in the 1940's by George Snell who, while analysing the rejection of transplanted tumours and other tissue grafts between mice strains, discovered that tissue compatibility was controlled by several closely linked genes within the histocompatibility-2 (H-2) locus [10]. Soon after, homologous genes were found in other mammalian species and were named the major histocompatibility complex [10]. The critical importance of MHC molecules in T cell antigen recognition came with the discovery that T cells are only responsive to their specific peptide Ag when presented by self-MHC molecules, a phenomenon termed MHC-restriction [11]. Since this sentinel finding, much has been elucidated about the MHC, and recent experimental evidence is revealing fascinating insights about the immunobiology of these molecules.
MHC molecules are divided into classical and non-classical types [1]. Classical MHC are highly polymorphic molecules which are further sub-divided into class I and class II subtypes. MHC class I molecules are encoded by three highly polymorphic genes both in mice (K, D, and L) and humans (A, B, C) and are constitutively expressed on virtually all nucleated cells with further upregulation under conditions of inflammation [9]. MHC class I upregulation is induced by cytokines such as interferon-\(\alpha\), -\(\beta\), and -\(\gamma\) (IFN-\(\alpha\), -\(\beta\), -\(\gamma\)), tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and lymphotoxin [1]. The fully assembled class I complex consists of an MHC-encoded \(\alpha\) chain, a non-MHC encoded \(\beta_2\)-microglobulin (\(\beta_2\)M) sub-unit and a peptide 8-11 amino acids in length [9]. MHC class I molecules are dependent on TAP (transporter associated with antigen processing) and TAP-associated proteins which transport cytoplasmic peptides into the endoplasmic reticulum [1]. The ubiquitous expression pattern and the ability of these molecules to present cytoplasmic Ag makes MHC class I molecules critical for the presentation of viral Ag by infected cells [1]. Peptide loaded MHC class I molecules are recognized by T\(_C\) cells as the non-polymorphic \(\alpha_3\) region of MHC class I molecules is bound by the extracellular Ig domain of both the \(\alpha\)- and \(\beta\)-chains of the T\(_C\)-expressing CD8 co-receptor [1], explaining the propensity of T\(_C\) cells to defend against viral pathogens [12].

MHC class II molecules are highly polymorphic proteins encoded by two genes in mice (I-A and I-E) and three genes in humans (DR, DP, DQ) [9]. The fully assembled class II complex consists of MHC-encoded \(\alpha\) and \(\beta\) chains as well as a peptide, 10-30 amino acids in length. MHC class II molecules are primarily expressed on professional antigen presenting cells (APCs) such as DC, B lymphocytes, and macrophages although the expression of MHC class II molecules is also upregulated by cytokines such as IFN-\(\gamma\) [13]. In contrast to MHC class I molecules, nascent MHC class II molecules are prevented from binding peptides in the ER by the class II-associated invariant-chain peptide (CLIP) [1]. Instead, MHC class II molecules are
transported to the cell surface in vesicles that fuse with incoming endosomes containing peptide fragments from extracellular pathogens and proteins [1]. The fusion of these vesicles occurs concomitantly with the enzymatic digestion of the clip peptide, allowing derivative peptides of endocytosed proteinaceous Ag to bind the MHC class II binding groove [1]. APC with peptide loaded MHC class II molecules are bound by TH cells, as the two N-terminal Ig-like regions of the TH CD4 molecule bind to the non-polymorphic β2 domain of MHC class II [9]. The recognition of MHC class II-bearing antigenic ligands by TH cells explains why TH cells are well suited for "helping" the function of professional APC, through their delivery of a focused CD40L signal to these cells, and thereby facilitating efficient humoral and cellular immunity [1]. The importance of MHC polymorphisms in these antigen recognition events is that the presentation of identical peptides by allelic variants of MHC creates unique ligands that are recognized by different T cell clones [1]. This additional diversity in TCR ligands makes it more likely that an individual will develop an effective T cell response against a given Ag thereby eliminating potential holes in the T cell repertoire. Furthermore, from a population genetic standpoint, one advantage of maintaining these MHC polymorphisms is the insurance that at least some individuals in the population will respond to a novel infectious agent to which the majority of individuals would succumb.

While the majority of T cells in lymphoid organs express the αβ TCR heterodimer, a small population of T lymphocytes expresses a different set of T cell receptor subunits called γ and δ chains [1]. These γ and δ chains are assembled in a similar fashion as αβ TCR chains, undergoing the process of somatic recombination of V, D, and J gene segments [1]; however, there are only 92 V gene segments in γδ T cells compared to the 122 V gene segments in αβ cells, which limits the diversity of the γδ TCR [9]. While αβ and γδ cells have some common characteristics, a peculiarity of the γδ TCR is that it shares structural features with the BCR.
Specifically, the γδ TCR can recognize the three-dimensional shape of Ags as well as recognize non-proteinaceous ligands including inorganic molecules, lipids and unprocessed antigens such as the herpes simplex virus (HSV)-1 glycoproteins [14]. The unusual Ag that are recognized by γδ also includes a diverse group of unconventional Ag presenting molecules [15] which have been grouped together as MHC class Ib molecules [1]. These non-polymorphic structures, similar to MHC class I molecules, are encoded by genes located within the H-2 loci of murine chromosome 17 and may associate with β2M [16]. The MHC class IB molecules are expressed in a variety of cell types, including fibroblasts and epithelial cells and, in some cases, are induced in response to signals of cellular stress such as heat shock, making T cells responsive to these ligands sentinel lymphocytes for cellular stress [1]. While some MHC class IB molecules have been shown to present unconventional Ag such as inorganic molecules and lipids, as in the case of the α-galactosyl ceramide (α-gal cer) glycolipid presented by CD1d [17], other MHC class Ib molecules such as the MHC class I chain-related A/B (MICA/B) molecules in humans and their functional homologues in mice, H60 and RAE-1, have no Ag presenting function [18]. Instead, some MHC class IB molecules have been shown to be cognate ligands of certain clonal populations of T cells, as seen with the recognition of MICA and murine homologues by epithelial γδ T cells [15, 18]. Interestingly, the αβ TCRs of some CD4+ and CD8+ T cells as well as some double negative T cells (DNRC), which express neither co-receptor molecules, appear to recognize MHC class Ib ligands and thus this is not an exclusive property of the γδ TCR. For example, NKT cells which express the canonical Vα14 Vβ8 TCR recognize α-gal cer presented by the MHC class Ib molecule CD1d [17]. Therefore the unique synthetic and Ag “sampling” pathways, as well as the differing structures of MHC molecules, assign the immunological function of different T cell subsets.
1.3 T Cell Signalling

An important outcome of TCR Ag recognition is the nucleation of numerous signalling intermediates into a structure, referred to as the immunological synapase, which initiates numerous signal transduction pathways. One of the most proximal events in TCR signalling involves the tyrosine phosphorylation of CD3 subunits and TCRζ (CD247) which have the Src family tyrosine kinase Fyn constitutively bound [19, 20]. Upon recognition of cognate Ag, Fyn is activated by CD45 [20, 21] and Fyn in turn phosphorylates CD3 and CD247 which are then able to recruit additional molecules to the TCR complex including ZAP-70 [22]. Coordinate binding of TCR and co-receptor facilitates the association of co-receptor-bound Lck with ZAP-70, resulting in ZAP-70 phosphorylation and activation [20, 22]. ZAP-70 in turn phosphorylates the critical adaptor protein LAT (linker of activation in T cells) [23] which together with SLP-76 and GRB2 help to recruit phospholipase C-γ-1 (PLC-γ-1) [22]. The phosphorylation of PLC-γ-1 by ITK activates two distinct signalling pathways which result from the PLC-γ-1 mediated hydrolysis of phopho-inositol pyrophosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG) [22, 24]. IP3 has a critical role in activating the calcineurin pathway, as IP3 receptors cause the release of sequestered calcium (Ca2+) which can then bind to calmodulin [1, 24]. The calmodulin-Ca2+ complex activates the serine/threonine phosphatase calcineurin leading to the dephosphorylation of nuclear factor of activated T cells (NFAT), allowing this transcription factor to move into the nucleus and activate gene transcription [1, 24]. DAG formation causes the rapid recruitment and activation of protein kinase C-θ (PKC-θ) [22, 25]. Activated PKC-θ is then able to phosphorylate a number of targets which may include RasGRP, a Ras guanine-nucleotide exchange factor (GEF), thereby facilitating Ras activation [25-27]. Activated PKC-θ also phosphorylates IKK-β, promoting the formation of the IKKα/IKKβ heterodimer, known as IKK, which in turn phosphorylates the inhibitory protein IκB [27, 28].
IkB phosphorylation results in the release of NFkB and its movement into the nucleus where it can influence gene transcription [1, 27].

LAT also recruits the related linker molecules GADS and GRB2 which do not have intrinsic kinase activity but which recruit a number of important signalling molecules to the TCR complex, such as the Ras GEF SOS [23]. Once SOS has converted RAS to its active GTP-bound state [29], RAS can in turn recruit and activate proteins of the mitogen activated protein (MAP) kinase cascade which culminate in the activation of Fos [1]. TCR-induced c-jun activation increases the transcriptional activity of important genes for T cell activation by its dimerization with Ras-activated Fos to form the AP-1 transcription factor [1]. The activation of c-jun by proximal TCR signalling occurs as a result of the activation of the stress-activated protein (SAP) kinase cascade by Vav [30], following its recruitment to the immunological synapse by SLP-76 [31]. Vav’s exchange of bound GDP for GTP results in Rac activation, leading to c-Jun activation and dimerization with c-Fos [1, 30, 31].

TCR signalling also leads to the activation of the PI3K pathway [32]. Vav and the TCR-interacting molecule (TRIM) both bind PI3K [33], a signalling molecule which in turn catalyzes the transfer of a phosphate group to PIP2 to form PIP3 [34]. The recruitment of the serine/threonine kinase Akt by PIP3 results in its activation and the subsequent initiation of numerous signalling pathways including the phosphorylation of glycogen synthase kinase-3 (GSK3) resulting in decreased NFAT phosphorylation, and the activation of NFkB by an ill-defined mechanism [22, 34]. In addition to signals initiated by the TCR directly, additional cell surface receptors contribute to the formation of the immunological synapse and ultimately affect the outcome of T cell stimulation. A number of receptors are suspected to augment T cell activation following TCR engagement including CD27, TNF-α Re II (CD120b), ICOS (CD278), as well as NKG2D (CD314) [35-38]; however, most research on T cell co-stimulation has
focused on CD28 [39]. CD28 is a transmembrane homodimer consisting of a single immunoglobulin extracellular domain and a cytoplasmic tail that has no intrinsic enzymatic activity, but a number of motifs for the recruitment of signalling intermediates [39-41]. Binding of CD28 to its ligands CD80 and CD86 on mature APC results in its association with Vav, Gads, Grb2, and PI3K [39-41]. The two signal hypothesis of T cell activation postulated that CD28 activated unique signalling pathways not activated by the TCR; however, recent evidence suggests that independent CD28 signalling results in the transient expression of only a few genes [39]. Moreover, none of the CD28-induced genes were specific to this signaling pathway, but rather constituted a small subset of those implicated in TCR-signal transduction [39]. However, under physiological conditions, only a few TCRs are ligated at any given time, generating short-lasting, incomplete activation events that do not lead to cell proliferation and differentiation, but rather to T cell inactivation (anergy) or cell death [39, 42]. It is postulated that CD28 engagement strongly amplifies a weak TCR signal by either maintaining Lck in an activated state [39] and/or increasing lipid raft aggregation [43]. TCR signalling in the absence of co-stimulation results in a state of T cell inactivation called T cell anergy, and different forms of this anergy are associated with characteristic blocks in TCR signalling [42]. Although diverse signalling pathways are activated at the time of T cell activation, it is interesting to note that many of these pathways converge, regulating certain key genes involved in the proliferation and differentiation of T cells [44]. The most critical of these genes is IL-2 which has binding sequences for NKκB, NFAT-1, OCT-1 and AP-1 present in its promoter [27, 45]. Examples of this signal integration in IL-2 gene regulation are that the CD28 response element (RE) is necessary for IL-2 gene transcription and the full transcriptional activity of NFAT on the IL-2 gene requires physical association with AP-1 [22, 45-47].
Interestingly, autocrine IL-2 signalling itself is pivotal for T cell activation. While IL-2 protein and its message are undetectable in resting T cells, their induction is one of the most rapid consequences of T cell activation [44]. The upregulation of IL-2 is rapidly followed by the upregulation of the high affinity IL-2 receptor, permitting the selective expansion of effector lymphocytes activated by Ag [44]. IL-2 is a secreted glycoprotein which is produced almost exclusively by activated T cells [9, 44]. The IL-2 receptor complex consists of three distinct subunits, which are designated IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγc (CD132) [9, 48]. CD132 is constitutively expressed on all T cells while CD25 and CD122 expression are controlled by exposure to Ag [9, 49]. These receptor subunits can be assembled in different ways to create IL-2R with differing affinities for ligand [9, 48]. The expression of CD25 alone is unable to transduce any signal due to its low-affinity for IL-2 ($K_d = 10-20$ nM) and its short cytoplasm tail devoid of necessary signalling motifs, whereas the CD122/CD132 heterodimer forms an intermediate affinity receptor ($K_d = 0.5 - 2$ nM) which is capable of responding to high IL-2 concentrations [9, 50, 51]. The expression of the CD25/CD122/CD132 heterotrimeric IL-2R comprises the high-affinity IL-2 receptor ($K_d = 10-75$ pM) [9, 50, 51]. Intracellular signals from the IL-2R originate from the CD122 and CD132 subunits, whose cytoplasmic tails contain critical motifs for recruiting important signaling molecules [48, 52]. The binding of IL-2 to the IL-2R complex promotes the catalytic activation of Jak1 and Jak3 and the subsequent phosphorylation of important tyrosine residues on CD122 and CD132 [48, 52-54]. Phosphorylated tyrosine residues on CD122 recruit the adaptor protein She and the transcription factor Stat5 [54]. She recruits and activates components of the MAPK and PI3K pathways, whereas Stat5 transactivates a variety of target genes [54]. Although both She- and Stat5-dependent pathways can independently transduce a proliferative signal, these two pathways have distinct roles in regulating other aspects of T cell immunobiology [54]. The importance of IL-2
signalling in T cell activation is highlighted by the fact that deficiencies in this cytokine or its receptor are associated with defects in immunological functioning assayed in vitro such as the development of cytolytic responses and Ag-specific proliferation [55, 56]. Interestingly, insufficient amounts of this T cell growth factor during T cell activation may also be associated with the development of T cell anergy [57]. Furthermore, the addition of exogenous IL-2 can overcome the proliferative defect in some forms T cell anergy [42].

A complete understanding of IL-2 signalling requires an understanding of IL-15 immunobiology since IL-2 and IL-15 are structurally related and share two out of the three subunits of the heterotrimeric IL-2 receptor (CD122, CD132), with the specificity for ligand conferred by their different α chains [53, 58]. Although IL-15R has a broader pattern of expression than IL-2R and consequently a distinct set of biological properties [58-60], the shared usage of CD122 and CD132 by these cytokine receptors results in many of the same signal transduction pathways being activated as well as a number of shared functional characteristics [58, 60]. One feature of particular relevance to this thesis is the ability of both IL-2 and IL-15 to activate and drive the proliferation of memory phenotype T cells due to their expression of the intermediate affinity IL-2/IL-15 receptor (CD122/CD132) [60]. As some forms of anergic T cells are “Ag primed” and thereby express the IL-2/IL-15 intermediate affinity receptor [61], these cells are responsive to IL-2 and IL-15 bystander cytokine support [61, 62], which in some cases can bypass the biochemical blockade induced by T cell anergization [42, 63].

1.4 T Cell Development

The vast majority of cells developing within the thymus pass through a series of phenotypically-identifiable stages prior to their emigration from the thymus as mature T lymphocytes [1]. The critical role of the thymic microenvironment in this development is
demonstrated by the resultant immunodeficiency seen in athymic nude mice and humans with DiGeorge syndrome [64, 65]. When entering the thymus, the TCR genes of T cell precursor cells are in germ line configuration and most of the surface molecules characteristic of mature T cells are absent, including the CD4 and CD8 co-receptor molecules [1]. These double negative (DN) thymocytes are pleuripotent and can give rise to either αβ or γδ T cells [1]. DN thymocytes pass through four distinct stages during development as illustrated in Figure 1 [1, 66, 67]. In the CD44+CD25+ DN2 stage (pro-T cell), DNA recombinase machinery is initiated and recombination at the β, γ, and δ chain loci occurs [9, 68]. The factors that regulate the commitment of progenitor cells to the αβ vs. γδ lineage are not definitively known, though interactions with the thymic stroma are suspected to influence lineage commitment and a successful rearrangement of γ and δ chain gene segments before the occurrence of a productive β chain gene rearrangement seems to be critical [1, 9, 68-72].

The production of a functional β chain gene and the pairing of the β chain with the pre-T α (pTα) receptor subunit forms the pre-TCR [1, 73]. This triggers a maturation program within developing thymocytes known as “β-selection”, that prevents further recombination at the β gene locus, known as allelic exclusion [74, 75]. β-selection acts as an important checkpoint in the development of mature αβ T cells [1, 74, 75]. Failure to generate a functionally rearranged β chain or competing γ and δ chains at this stage of development results in apoptosis of the developing thymocyte [76]. Following β-selection, cells upregulate both CD4 and CD8 becoming “double positive” (DP) thymocytes [1, 9, 77], which re-express gene recombination machinery in order to rearrange TCR α- chain genes [1, 78]. The initiation of TCR α-chain gene rearrangement ultimately precludes the development along the γδ lineage as this process results
A.

Progenitor Stem Cell

γδ T Cell Development

αβ T Cell Development

DN1 → DN2 → DN3 → DN4

Mature DP CD4+CD8+ Thymocyte

Negative Selection

High Affinity

Low Affinity

Death by Neglect

Intermediate Affinity

Positive Selection

MHC class II-containing ligand

MHC class I-containing ligand

SP CD4+ Thymocyte

SP CD8+ Thymocyte

Recognition of TSLP-conditioned DC

CD4+CD25+ Treg Cell

Mature CD4+ T Cell

LEGEND

CD3

CD4

CD8

CD25

CD44

αβ TCR

γδ TCR

Steps

Unknown

(Source: Adapted from C. A. Janeway et al, [1])
### Figure 1. Summary of T Cell Development

(A) Schematic detailing the various stages of ab TCR\(^+\) and gd TCR\(^+\) thymocyte development within the thymus [1]. (B) The expression of cell surface molecules is summarized for various stages of thymocyte development [1, 81].
in the “looping out” of δ chain gene segments which are embedded within the α-chain gene loci [1, 79, 80]. The pairing of the nascent α-chains with the TCR β chain enables DP thymocytes to audition for further development through the recognition self-peptide/MHC complexes on thymic cortical epithelial cells [1, 82, 83]. A weak recognition of self-Ag on thymic cortical epithelial cells results in a slow death (death by neglect) of DP thymocytes [1, 84, 85]. However this "death by neglect" can be circumvented if additional rearrangements of TCR α chain gene segments change the Ag specificity of the TCR, enabling the cell to undergo a subsequent “auditioning” process [1, 82]. Thymocytes which recognize self-Ag on thymic cortical epithelial cells with high avidity are induced to undergo rapid cell death termed “negative selection” [1, 85]. Only DP thymocytes whose TCR recognize thymic epithelial self-Ag with intermediate strength are selected for further development into mature T cells (positive selection) [1, 83, 86]. Although the selection for moderate self-reactivity limits the maximal diversity of the TCR repertoire, this narrowing of the TCR spectrum ensures that frankly auto-reactive cells are eliminated and that only cells that will be capable of responding to an insult will be allowed to progress.

The decision of positively selected DP thymocytes to become CD4⁺ or CD8⁺ single positive (SP) thymocytes occurs as a result of the Ag-recognizing properties of the TCR [1, 77, 84]. Initially it was thought that a random downregulation of one of the co-receptors occurred independently of the TCRs ability to recognize peptide in association with class I or class II MHC [84, 87, 88]. While some evidence from MHC knockout mice supports this “stochastic” model of lineage commitment [87], evidence from TCR transgenic mice favors an “instructive” model whereby the coordinate binding of the TCR and the appropriate co-receptor molecule to the same MHC molecule commits DP cells to develop along the "correct" T cell lineage [89]. Recent evidence has suggested that the strength of TCR signaling may also modulate this lineage
commitment decision [90]. Nevertheless the Ag-recognizing properties of a given αβ TCR are paramount for the developmental decisions of DP thymocytes.

Recently it has been demonstrated that CD4 SP cells can undergo a second round of "selection" to form CD4⁺CD25⁺ regulatory T cells (T_{reg}) [91]. The development of T_{reg} involves the expression of thymic stomal lymphopoietin (TSLP) by Hassall's corpuscles which activate thymic CD11c positive DC to express high levels of CD80 and CD86 [91]. TSLP conditioned DC in turn induce the proliferation and differentiation of CD4⁺CD25⁺FoxP3⁺ T_{reg} from CD4⁺CD8⁻CD25⁻ precursor cells, with the induction of the transcription factor FoxP3 being a critical event in the ontogeny of these cells [91]. This T_{reg} induction is dependent upon the presence of MHC class II, CD80, CD86 as well as IL-2 and its receptor subunits, and appears to represent a second "positive selection" event in the development of this cell subset [91, 92].

While the details of αβ T cell development have been extensively studied, γδ development (Fig. 1) is less well understood [68]. It is known that some γδ T lymphocytes proceed along a unique developmental pathway [93, 94]. For example, unlike αβ T cell development, no pre-TCR is formed in γδ T cells although the γ chain can infrequently pair with pTα, committing to the αβ T cell lineage [68, 95]. Developing γδ T cells do not upregulate the CD4 or CD8 co-receptors, with most γδ T cells maintaining their DN immunophenotype throughout development [14]. Similarly, γδ T cells do not seem to conform to the processes of positive and negative selection [96, 97] and, although γδ T cell development occurs primarily within the thymus, a thymic-independent pathway of development may also exist [98]. An interesting feature of γδ T cell development is that subsets of these cells are formed early in development, and leave the thymus in "organ specific" waves before the initiation of conventional T cell development [93]. The first wave of these cells, occurring on days 14-17 of
murine embryogenesis, expresses the invariant Vγ3Vδ1 γδ TCR and homes to the skin where they form essentially a monoclonal population [93]. Due to their peculiar branching pattern of cellular processes reaching between adjacent keratinocytes, these cells are known as dendritic epidermal T cells (DETCs), and represent 95-100% of murine epidermal lymphocytes [99, 100]. The Ag-specificity of Vδ1 TCR-expressing epithelial lymphocytes in humans is for MICA/B MHC class Ib ligands [101] and it is assumed that murine Vγ3Vδ1 cells bind to the MICA/B functional homologues RAE-1 and A60 [102]. The rigorous thymic selection of canonical Vγ3Vδ1 cells facilitates their colonization of the skin by inducing an epidermal-homing phenotype [94]. The importance of this invariant TCR in DETC development has been demonstrated in Vγ3−/− mice using the anti-idiotypic Vγ3Vδ1 mAb 17D1 [103]. In these mice, γδ DETC utilizing substitute Vγ chains were present and, surprisingly, were still recognized by the 17D1 mAb, suggesting that a specific Ag binding specificity was being selected for [103].

Given that some of the normal dynamics of T cell development are altered in TCR transgenic mice, an in depth understanding of how normal processes are altered in these invaluable research tools is essential. In TCR transgenic mice, productively rearranged TCR chain genes are expressed under the control of the T cell specific Lck promoter [104, 105]. An example of a TCR transgenic system and one which is critical to this thesis is the 2C transgenic TCR [106, 107]. The 2C TCR consists of the Vα3.1 and Vβ8.2 TCR chains [108] which recognize the p2Ca peptide (LSPFPFDL) that is naturally processed and derived from the mitochondrial protein 2-oxoglutarate dehydrogenase [109, 110]. This MHC class I restricted TCR, as shown in Figure 2, is positively selected by an intermediate affinity interaction with p2Ca/H-2Kb (3 x 10^3 M-1) and negatively selected by a high affinity recognition of p2Ca/H-2Ld (2x10^6 M-1) [107]. On the H-2^s background, the 2C TCR does not recognize any ligand with significant affinity, resulting in the "neglect" of 2C TCR^+ DP thymocytes that are forced to
Figure 2. Schematic of the 2C TCR and its Antigen Interactions

The 2C TCR recognizes the p2Ca peptide when presented by the class I MHC molecules K\textsuperscript{b} and L\textsuperscript{d} [107, 110]. The 2C TCR consists of the V\alpha 3.1 and V\beta 8.2 chains [108]. Note the higher affinity of the 2C TCR for the p2Ca-L\textsuperscript{d} ligand [107].

(Source: Adapted from C. A. Janeway et al., [1])
rearrange endogenous TCR genes for continued development [107]. In the H-2^b background, the positive selection of 2C DP thymocytes results in the generation of a large population of 2C TCR-expressing CD8 SP thymocytes and peripheral cells [107]. In H-2^s mice, no CD8 SP thymocytes or peripheral cells expressing the 2C TCR are appreciated as 2C TCR-expressing DP thymocytes fail to be positively selected in this background [107]. Similarly, in H-2^d mice, no 2C-expressing CD8 SP thymocytes or peripheral cells are seen as 2C TCR-expressing DP cells are swiftly deleted in these animals [107]. This comprehensive deletion of DP cells has important consequences for the development of other lineages as well since DP thymocytes are the progenitors of CD4, CD8 and some specialized T cell subsets [1]. For example, the development of CD4 cells from DP thymocytes in TCR transgenic mice occurs either by the pairing of a newly rearranged TCR \( \alpha \) gene product with the \( \beta_{Tg} \) TCR chain [82], or by the pairing of newly rearranged TCR\( \alpha \) and TCR\( \beta \) gene products following the deletion of TCR transgenes [111]. Development of these CD4 cells is understandably perturbed by the massive negative selection of DP cells expressing a strongly self-reactive TCR since both of these steps leading to CD4 T cell commitment take place at the DP thymocyte level [1, 83]. Furthermore, as CD4^+CD25^+ T_{reg} cells are derived from the CD4 SP thymocyte pool [91], a dearth of DP thymocytes also severely impacts the development of this important T cell subset.

Another interesting feature of TCR transgenic mice is the production of a population of mature lymphocytes which express high levels of the transgenic TCR \( \alpha \) and \( \beta \) chains but do not express either the CD4 or CD8 co-receptor molecules, and are thus referred to as DNCTC [63, 107]. These cells appear to ignore the normal “rules” of thymic selection and are produced at the same rate in TCR Tg mice on positive, negative and non-selecting backgrounds [63, 107]. For example, in 2C mice, these cells are found in approximately the same numbers on H-2^b, H-2^d, and H-2^s backgrounds [107, 112]. One hypothesis about the ontogeny of these cells is that they...
may represent γδ cells which have been forced to express an alternative receptor, i.e., γδ T cells *incognito* [97]. The failure of most of these cells to express a γδ TCR may not exclude their commitment to this lineage since it is theorized that dedication to γδ development may occur before the expression of a functional TCR and that surrogate TCRs may support this development [97, 113]. Therefore a cell committed to the γδ lineage will not be affected by the forced expression of a Tg TCR regardless of the prevailing positive, negative, or non-selecting thymic environment [107, 112, 113]. Furthermore, such a cell will not attempt to create a new TCR with more optimal Ag recognizing properties, which is consistent with the germline configuration of TCR α chain genes in TCR Tg DNTC [97]. Additional support for the γδ theory of TCR Tg DNTC ontogeny is that circulating γδ T cells are not found in TCR Tg animals [97] and that, in some TCR transgenic mice, cutaneous T cells can co-express the Tg TCR and a γδ TCR [113]. This later finding is explained in part by the kinetics of γδ T cell development [93, 113]. Cutaneous γδ T cells develop on day 14-17 of embryogenesis, before the development of other T cell subsets [93], and presumably these cells can successfully rearrange endogenous γ and δ chain genes before the forced expression of the Tg αβ TCR [113]. In systems where the Tg TCR is “switched on” earlier, due to differences in transgene integration sites and/or construct design [114], no such cutaneous γδ T cells are observed and only TCR Tg cells with γδ-like properties are found within the skin [113]. The ability of these TCR Tg cells to home to the skin and acquire some γδ T cell attributes was likely imprinted during commitment to the γδ lineage [97, 113].

Although the evidence of TCR Tg DNTC belonging to the γδ T cell lineage is somewhat convincing [97, 113], αβ DNTC are known to exist in wildtype animals and humans and these cells represent approximately 1-5% of circulating CD3⁺ cells [115, 116]. These non-Tg DNTC
are similarly insensitive to T cell selection pressures [117, 118] and are characterized by the early rearrangement of the TCR α chain genes under the control of the E delta enhancer element [119]. This population of DNTC in non-Tg mice and humans appears to possess many of the same functional properties as DNTC in TCR Tg mice [115, 116], and thus the process of transgenesis may serve to enrich this population of cells [119].

Regardless of the ontogeny of TCR Tg DNTC, TCR transgenic mice have great utility for the study of T cell tolerance, as large numbers of mature cells in the periphery which recognize self-antigen with high affinity can be generated [107]. Many studies using the 2C system have successfully taken advantage of this population confirming the usefulness of these cells in tolerance research [61-63, 115, 120, 121].

1.5 Tolerance

The ability of the thymus to generate a diverse repertoire of T cells is critical for bodily defense; however, it also results in the production of receptors with specificity for self and thus an equally important role of the thymus is contributing to the maintenance of self tolerance [1]. A major tolerogenic mechanism ensuring tolerance to self is the intrathymic deletion of self-reactive T cells during negative selection, a process referred to as central deletion [1, 83]. The dilemma of how the thymus can control autoreactivity against “non-thymic” proteins was solved by the discovery of the “autoimmune regulator” (AIRE) transcription factor, which regulates expression of “heterotopic proteins” in the medullary epithelial cells of the thymus [122]. Central deletion is best appreciated in TCR Tg mice with a TCR specific for self-Ag [107, 123] In these animals, the comprehensive depletion of DP cells results in a very hypocellular thymus and a slow export of mature CD4⁺ and CD8⁺ T cells to the periphery [123]. A failure of this central tolerance can be seen when the AIRE gene is mutated and the expression of “peripheral proteins”
in the thymi is silenced [122]. This results in spontaneous autoimmunity involving the ovaries, testis, retina and stomach, which in humans is called the autoimmune polyglandular syndrome type 1 [122, 124].

Another mechanism by which the thymus contributes to self-tolerance is the generation of T cells with immunoregulatory function [125, 126]. Two such populations with potent immunoregulatory function and which are critical to this thesis are the CD4⁺CD25⁺ T<sub>reg</sub> and the Vy3Vδ1γδ T cells.

CD4⁺CD25⁺ T<sub>reg</sub> have been shown to be particularly important in immune regulation and abrogating the development of autoimmunity in a number of disease models [127, 128]. Both "natural" T<sub>reg</sub>, developing in the thymus after exposure to self-Ag [91], and "induced" T<sub>reg</sub>, which develop in the periphery after exposure to exogenous Ag, have been described [129, 130]. These suppressor cells have been shown to inhibit the proliferation and cytokine production of T cells through direct cell-cell interactions, although "suppressor" cytokines such as IL-10 and TGF-β may also contribute to these cells regulatory behavior [125]. FoxP3<sup>−/−</sup>, IL-2<sup>−/−</sup>, CD25<sup>−/−</sup>, and CD122<sup>−/−</sup> mice, all of which are naturally deficient in T<sub>reg</sub> cells [131-133], as well as wildtype animals which have been depleted of T<sub>reg</sub> [125, 134] develop widespread autoimmune disease including colitis, gastritis, insulin-dependent autoimmune diabetes, and thyroiditis [125, 131-134], which can be abrogated by the adoptive transfer of T<sub>reg</sub> cells from syngeneic wild-type mice [125]. FoxP3 mutations in humans are associated with a similar condition termed IPEX (immune dysfunction, polyendocrinopathy, enteropathy, x-linked) [135]. In addition to a reduced absolute number of T<sub>reg</sub>, a reduced functionality of T<sub>reg</sub> may also predispose individuals to autoimmune disease [136, 137]. For example, T<sub>reg</sub> from patients with multiple sclerosis, psoriasis, and autoimmune polyglandular syndrome type II have all been demonstrated to
possess a significantly decreased suppressor function when compared with cells from healthy donors [136-138].

Another cell type which has been shown to possess critical regulatory properties is Vδ1-expressing γδ DETC [126]. DETC have a non-redundant role in regulating cutaneous inflammation by liberating the anti-chemotactic factor prothymosin β4 (pTβ4) which inhibits the migration of inflammatory cells to the skin [139, 140]. Under normal circumstances, neutrophils are uncommon residents of the skin and it is speculated that DETC prevent neutrophil entry into the epithelium by virtue of the high basal rate of IEL pTβ4 production [141]; a hypothesis that is consistent with the spontaneous neutrophilic dermatitis and profound neutrophilic inflammatory reactions characteristic of TCR γδ knockout mice [139, 142, 143].

While the thymus plays a critical role in maintaining immunological tolerance, other thymus-independent mechanisms of this regulation exist [1, 144, 145]. For example, the sequestration of self-Ag within immunologically-privileged sites, such as the brain, eye, testis, or amniotic sac, can preclude autoimmunity despite the existence of circulating autoreactive T cells against these structures [145]. Similarly, the immune system can also remain ignorant of self-components produced at very low levels or which are not efficiently presented by MHC [146].

The induction of peripheral deletion is yet another mechanism by which self-tolerance is maintained [1, 144]. Such peripheral deletion occurs when an activated T cell upregulates the Fas receptor (CD95) rendering it susceptible to "activation induced cell death" (AICD) which occurs upon the binding of Fas to its receptor-ligand pair FasL (CD178) [147]. The importance of this mechanism of tolerance is illustrated by the lymphoproliferative disease which occurs in the absence of Fas or FasL [148, 149].

Although the exposure to self-Ag in the periphery may result in deletion, autoreactive cells may also persist indefinitely in the body in a functionally compromised state which has
been termed T cell anergy [42]. Different forms of anergy have been described which are characterized by differing pathways of induction, possibilities of reversal, associated signalling defects, and functional properties of the anergic cells [42]. Adaptive anergy represents a generalized state of unresponsiveness, with suppression of most cytokines including IL-2 [42]. This form of anergy cannot be reversed by the provision of exogenous cytokine, but the adaptive anergic state typically dissipates after the removal of cognate Ag [42]. The biochemical defect of adaptive anergy involves a profound defect in mobilizing calcium, with preserved Ras pathway function [42]. Clonally anergized cells are characterized by an impairment of IL-2 production and proliferation [42]; however, the Ag-experienced immunophenotype of these cells, including the expression of the intermediate affinity IL-2/IL-15R, enables these cells to use an exogenous source of IL-2 or IL-15 to drive their activation and proliferation [150, 151]. Despite the inability of these cells to proliferate in the absence of bystander cytokine support, these cells often have a memory-like enhancement of functional properties such as enhanced cytotoxicity and the ability to secrete IFN-γ immediately \textit{ex vivo} without a prior period of stimulation [151, 152], which led to the previous terminology “split-anergy” [153]. The biochemical defect associated with clonal anergy is the failure to activate Ras [42]; however, the finding that exogeneous cytokine support can bypass this biochemical block suggests that this mode of maintaining peripheral tolerance is imperfect and may pose an autoimmune hazard [42]. In addition to the adaptive and clonal forms of T cell anergy, multiple hybrid forms of T cell inactivation have been described [42]. For example, the T cell anergy described in the 2C model exhibits features of both of these paradigms [61, 63, 107, 120].

In H-2$^d$ 2C mice, only the selection-independent DNTRC express the clonotypic 2C TCR outside of the thymus, as 2C TCR-expressing DP thymocytes are comprehensively deleted [107]; however, the 2C TCR DNTRC which are insensitive to thymic selection pressures are found to
persist in the periphery of these animals in large numbers [63, 107]. The constant exposure of these cells to the high affinity self-Ag, p2Ca-L\textsuperscript{d}, bestows upon the 2C DNTC an Ag-experienced immunophenotype, characterized by high levels of the memory markers CD44, Ly6C, and CD122 [61-63]. These cells are also equipped with enhanced functional properties being able to synthesize IFN-\(\gamma\) and kill cognate ligand-expressing targets immediately \textit{ex vivo} without a period of prior priming [61]. The same population of cells from H-2\textsuperscript{b} 2C mice possess a naive immunophenotype and functional properties [61, 63]. Stimulation of the memory phenotype 2C DNTC from H-2\textsuperscript{d} 2C mice with Ag\textsuperscript{+} APC together with a source of exogenous cytokine resulted in the upregulation of activation markers and revealed these cells to have a higher basal level of proliferation and a reduced activation threshold relative to the equivalent population of cells from H-2\textsuperscript{b} 2C mice [120]. Though, when stimulated without a source of exogenous cytokine, the H-2\textsuperscript{d} 2C DNTC failed to make IL-2 at both the message [61, 62] and protein level [63] and were unable to proliferate even when challenged with high dose Ag [63, 120]. Interestingly, this apparent clonal anergy was not associated with defects in Ras activation, but rather with deficient CD247, ZAP-70, and LAT phosphorylation as well as impairment in calcium mobilization, typical of adaptive anergy [120]. This constellation of findings in anergic 2C DNTC has features of both clonal and adaptive anergization and thus is an example of hybrid anergy.

Consistent with the anergization of the 2C DNTC, it was found that in H-2\textsuperscript{d} 2C mice, these cells do not stain significantly for the acute activation marker CD69 [61]. Furthermore, although these animals possess enormous numbers of 2C cells which recognize self-Ag with high affinity, no obvious autoimmunity is observable in these animals [106]. Presumably the expression of cognate self-ligand by non-professional APCs, devoid of adequate co-stimulatory ligands in a non-inflamed milieu, induces this anergization. However, the significant number of
Ag-experienced self-reactive cells, capable of using bystander cytokine support, raises the concern that such cells may pose an autoimmune hazard in vivo especially since physiological levels of IL-15, released indirectly by viral infection or by injection of polyinosinic-polycytidylic acid (poly(I:C)), can cause memory phenotype T cells, with irrelevant Ag-specificity, to proliferate in vivo [154, 155]. Animal models of autoimmune disease have demonstrated that the presence of large numbers of anergized cells, can predispose to the development of severe immunopathology and frank autoimmunity [156, 157]. However these studies have also shown that a high frequency of strongly self-reactive cells together with systemic inflammation is rarely sufficient for the induction of clinically apparent autoimmune disease [156]. An important determinant in the development of autoimmune disease is the presence of “target tissue”, or localized inflammation, which is crucial for the on-going recruitment and activation of inflammatory cells to the site of immunopathology [156]. The importance of target tissue inflammation is highlighted by the finding that high concentrations of tissue cytokine can promote the development of localized autoimmunity [158-161]. [160, 161] For example, the transgenic expression of IFN-γ in the pancreas induces T cell-mediated autoimmune diabetes as well as the loss of tolerance to normal islets [158, 162] while the injection of IFN-gamma into the skin of those predisposed to psoriasis results in the development of typical psoriatic lesions [159]. Interestingly the development of target tissue inflammation and subsequent autoimmunity has been associated with a number of immune system defects [163], as the use of less effective immune tactics leads to exaggerated, prolonged immune responses with high levels of inflammation [163]. Therefore it was hypothesized that anergic cells in the 2C system could contribute to immunopathology in H-2d-expressing hosts predisposed to the development of localized inflammation.
Interestingly, Delaney et al. found that backcrossing of the 2C TCR to certain H-2d-expressing BXD recombinant inbred strains resulted in the spontaneous development of psoriasiform skin disease (Table 1) [164]. Examination of the immunophenotype of the 2C DNTC in these animals revealed these cells to be acutely activated [164], indicating that the anergization of the 2C DNTC may have failed, resulting in autoimmune pathology with resemblance to the human T-cell mediated autoimmune skin disease psoriasis [164].

Table 1. Development of Disease in Recombinant Strains

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<tr>
<td>5</td>
<td>N/A*</td>
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<td>2/17</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>0/10</td>
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* Note: Typographical errors
(Source: J.R.C. Delaney, [164])

1.6 Psoriasiform Disease

Psoriasiform diseases are a family of cutaneous disorders which share certain core histological features such as a thickened epidermal layer (acanthosis), impaired maturation (parakeratosis), elongation of the epidermal rete ridges, papillary dermal edema with ectactic...
vessels, and exocytosis of neutrophils into the epidermis [165, 166]. This constellation of histological findings represents a non-specific reaction pattern that occurs in a handful of conditions including psoriasis, seborrheic dermatitis (SD), and in chronic forms of other dermatosis [165, 166]; however, the two major diseases within this family are the related conditions psoriasis and SD.

1.6.1 Psoriasis

Psoriasis is a common relapsing inflammatory skin disease, affecting 1-3% of Caucasians [165, 167]. Early onset psoriasis occurs in teenagers and young adults (16-22 years) whereas late onset psoriasis occurs in late-middle age (57-60 years) [165, 168]. The sudden onset of psoriasis at any age or the worsening of preexisting psoriasis may suggest a severe impairment in immune function, such as AIDS where a statistically significant increase in the incidence of psoriasis is seen [169, 170].

The condition is characterized by inflamed, symmetrical, erythematous plaques with silvery white scale [165, 166, 171]. There are several morphological variants of psoriasis, the most common of which is Psoriasis vulgaris or typical “plaque psoriasis” which is characterized by chronic scaling papules and plaques over the elbows, knees, scalp, chest, nails, and lower back (Fig. 3) [165, 166, 171]. However, plaques can be widely scattered and are often found in areas subjected to frequent trauma, an observation referred to as the Koebner phenomenon [165, 166, 171]. Rarer variants of this condition include guttate psoriasis, palmoplantar pustular psoriasis and inverse psoriasis, each of which has a distinctive clinical appearance [165, 166, 171]. Extra-cutaneous features of disease are also frequently observed in psoriatic patients, with 5-40% of patients developing psoriatic arthritis [171, 172] and with 25-100% developing structural nail abnormalities [165, 171]. In addition to the typical psoriasiform changes (Fig. 4),
Figure 3. Gross Pathology of Psoriasis

Psoriatic lesions are characterized by sharply defined erythematous plaques covered with silvery scale. Typical plaque psoriasis involves the trunk (A), the knees (B), and other parts of the body subjected to pressure or trauma, such as the knuckles (C). The tendency for typical psoriatic pathology to occur at sites of trauma is called the Koebner phenomenon, which can be reproduced experimentally (D, E). Another typical feature of psoriatic lesions is the Auspitz sign which is the tendency for bleeding to occur when scales are removed (F). Plaque psoriasis has a great predilection for the head and neck region, particularly the scalp and the external auditory canal (G, H). Psoriatic lesions often have an annular configuration (I, J). Another typical feature of psoriasis is dystrophic nail changes such as pitting and onycolysis (K, L). Other morphological variants of psoriasis include napkin psoriasis (M), palmoplantar psoriasis (B, N, O), pustular psoriasis (P) and guttate psoriasis (Q).
Figure 4. Histopathology of Psoriasis

(A) Psoriatic skin is characterized by hyperparakeratosis (h), variable acanthosis (a), club-like elongations of the rete ridges (r), and dilated vessels in the edematous dermal papillae (p). (B) So called “squirting” of dermal papillae results in characteristic intraepithelial Munro microabscesses (m), which are one of the cardinal histological features of active psoriasis.

(Source: A - University of Iowa - Dermatology, 1995; B - ©DermAtlas, www.dermatlas.org)
Psoriasis is characterized by such histological features as marked thinning of the suprapapillary plates of the epidermis, loss of the granular cell layer, and neutrophilic collections within the epidermis called Munro microabscesses [166, 167].

Despite the thousands of papers written on psoriasis, the pathophysiology of psoriasis is still poorly understood. One of the first treatments for psoriasis contained the dithranol anthralin, which is still a mainstay of psoriasis treatment [173, 174]. Later treatments were based on combining coal or anthralin with UV light (Goeckerman regimen) [174, 175]. These modalities may work by reducing the hyperproliferation of keratinocytes by a variety of mechanisms [173, 176, 177], which was thought at this time to be the primary etiological factor in disease pathophysiology [178]. Later studies demonstrated that the mitotic rate of basilar keratinocytes in psoriasis is indeed markedly increased, with the keratinocyte transit time reduced from the normal 27 to 4 days [165, 179, 180]. Topical corticosteroids and systemic treatments like the folic acid antagonist methotrexate were subsequently introduced in the 1950’s, and were thought to have a similar effect upon keratinocyte hyperproliferation [181, 182]. During the 1980’s, it was discovered that immunosuppressants such as Cyclosporine were an effective treatment of psoriasis, the first indication that this condition might represent a disorder of the immune system rather than a cell-intrinsic disease of the keratinocyte [178, 181, 183]. Within the last decade, elegant experimentation has further supported the autoimmune etiology of psoriasis [184-186], leading to new “biologic” agents such as the CD2-binding, LFA-3-Ig fusion protein Alefacept, which targets activated/memory T cells, and the CD11a-binding molecule Efalizumab, which decreases T-cell activation and migration, that are dramatically effective at mitigating disease [171, 187, 188]. Nevertheless the dispute about whether psoriasis represents a T-cell mediated autoimmune disease or a primary proliferative disease of the skin still exists, as only recently,
typical psoriatic changes were induced by the deletion of epidermal JunB [189], supporting the previously held belief that epidermal alterations are sufficient to initiate psoriasis [190, 191].

There are additional factors that are believed to have a role in psoriasiform disease; however, currently there is insufficient evidence to definitively implicate them with a role in disease pathogenesis. For example, it has recently been shown that T_{reg} are functionally impaired in psoriasis patients [137] and T_{reg}-deficient mice develop psoriasiform skin changes [192]. Infection may also play a role in the pathogenesis of psoriasis; for example, the onset of guttate psoriasis is often preceded by Streptococcal pharyngitis [193]. Similarly, opportunistic pathogens such as Malassezia furfur are suspected to have a role in disease pathogenesis [194, 195]. These organisms have been implicated in triggering psoriatic scalp lesions [196], and scalp psoriasis has been reported to respond to therapy with ketoconazole [197]. Furthermore, the application of opportunistic pathogens on the skin can experimentally induce typical psoriatic plaques in some experimental systems [198]. The infectious component of psoriasiform disease pathogenesis may be linked to the recently described MICA 5.1 mutation that results in a truncated molecule incapable of alerting sentinel lymphocytes about keratinocyte infection, which has been associated with human psoriasis [199, 200]. Other poorly studied, yet suspected triggers of psoriasis, include: alcohol, sunburns, weather changes, stress, diet, allergies, mechanical trauma, and the abrupt discontinuation of medications or the initiation of new medications such as lithium or systemic corticosteroids [165, 201, 202].

1.6.2 Seborrheic Dermatitis

SD is a chronic medical condition afflicting approximately 1-5% of otherwise healthy individuals [203-205]. The prevalence of this disease is dramatically higher amongst immunocompromised patients, particularly HIV positive individuals, in whom the reported
incidence of disease ranges from 30-80% [169, 206]. While infantile SD is limited to the first year of life [207], the presentation of typical SD is rare before puberty, with disease preferentially affecting males in two age cohorts: young males with disease beginning during adolescence and late-middle aged males [165, 205]. A severe, explosive onset of disease occurring at any age may be associated with HIV infection [169, 206, 208].

The term “seborrheic dermatitis” encompasses a heterogeneous group of related disorders [165, 205]. Classical SD is a papulosquamous disorder characterized by well-defined and symmetrical patches of greasy erythematous scale affecting areas of the body richly populated with sebaceous glands (Fig. 5), such as the scalp, face, trunk, sternal region and groin [165, 202, 205]. The lesions of SD, particularly lesions involving the scalp, sometimes resemble those of psoriasis and therefore the term sebopsoriasis or seborrasis is sometimes used [165, 209]. However, significant differences in the distribution and morphology of psoriatic and SD lesions makes this an easy distinction in most cases [165]. Other variants of disease include infantile SD, which is a term used to describe a variety of skin disorders including cradle cap, diaper dermatitis, and Leiner’s disease [205, 207, 210, 211] as well as AIDS-associated SD which is a more inflammatory and recalcitrant variant of disease [169, 205, 206].

Histologically, SD is characterized by folliculocentric scale-crusts consisting of mounds of parakeratotic squames mixed with pyknotic neutrophilic debris and prominent globules of plasma situated near follicular ostia (Fig. 6) [165, 166, 212]. Intercellular edema (spongiosis) is seen in the infundibular epithelium as well as in the surface epidermis, especially in the vicinity of nascent scale-crusts [166, 212]. Papillary dermal edema and ectasia of superficial dermal vessels are other features of disease [166, 212]. Chronic SD lesions become less spongiotic and more acanthotic over time, making the histological distinction from psoriasis more difficult [166, 212].
Figure 5. Gross Pathology of Seborrheic Dermatitis

Seborrheic Dermatitis is characterized by yellowish-red, greasy scale-crusts (A) or fine white scales (B) overlying erythematous skin. SD has a very distinctive tissue distribution, occurring in areas richly populated by sebaceous glands such as the scalp, T-zone of the face, presternal area, and perigenital region (C) [165, 202, 205].
Figure 6. Histopathology of Seborrheic Dermatitis

Histologically, SD is characterized by mounds of hyperparakeratosis, overlying acanthotic epidermis in a folliculocentric distribution. Edematous dermal papillae containing widely dilated dermal blood vessels are a prominent histological feature. A perivascular lymphohistiocytic infiltrate often accompanies these other changes.
Despite its long history and the existence of a sizeable research interest, a detailed understanding of SD disease pathophysiology is lacking [205, 213]. More than a century ago, opportunistic fungi were identified in lesional SD skin, which initiated the speculation that fungal pathogens played an active role in disease [194, 203]. Shortly thereafter, it was found that these fungal organisms, the recently taxonomically revised *Malassezia* spp. [214-216], represented normal inhabitants of the skin [194, 203, 217], casting doubt upon this initial speculation and leading to the belief that SD was a hyperproliferative disease of the skin caused by an intrinsic defect of the keratinocyte [203, 205]. This view prevailed for much of the twentieth century [203, 217] and was supported by the effective treatment of SD by selenium sulfide and zinc pyrithione which were known to possess anti-proliferative properties [203, 218]. However, recognition of the intrinsic antifungal properties of these compounds [203] and the introduction of newer antifungal agents with potent activity against *Malassezia* reawakened interest in the fungal theory of SD pathophysiology [203, 217]. Similarly, treatment studies have demonstrated that disease remission is associated with a reduction in the number of these organisms on the skin and that recolonization with antifungal-resistant strains of *Malassezia* leads to treatment-resistant SD [203]. Furthermore, recently employed shave biopsy sampling techniques have demonstrated that an increased amount of *Malassezia* exists on lesional skin [219] and that a correlation exists between the number of fungi on the skin and the severity of SD [203, 220]. Furthermore, given the tropism of lipophilic *Malassezia* for oily skin [194, 203], the fungal hypothesis of disease is supported by the relative confinement of SD to areas enriched in sebaceous glands [165, 166, 203, 205]. Moreover, the onset of disease at puberty, when the sebaceous glands become active due to an increase in circulating androgens [194, 203, 205, 221], and during the neonatal period, when circulating maternal progestins stimulate neonatal sebaceous glands [221, 222], demonstrates the central role of seborrhea in this condition.
Conditions characterized by increased seborrhea, such as Parkinson's disease [223] and chronic debilitation [224], are associated with a higher incidence of SD [194, 203, 205, 225] and, moreover, disease can be treated by reducing sebaceous gland activity with retinoids [203, 225]. Although these studies seemingly support a role for opportunistic fungal pathogens in SD pathophysiology, several peculiar findings continue to perplex the understanding of this disease. For example, it is known that some asymptomatic individuals harbour enormous numbers of Malassezia on the skin and yet never develop SD [203], suggesting that the number of yeast cells on the skin is only important for those with an inherent susceptibility to disease. The high prevalence of SD amongst AIDS patients [169, 170, 206] suggests that immune deficiency can predispose to disease. SD is the most common cutaneous inflammatory disorder associated with HIV infection (Fig. 7) [169] and is an important clinical marker of AIDS [169, 170, 208]. Other immune defects have also been associated with SD, including an inherited deficiency in the 5th component of complement, which has been implicated in Leiner's disease, the inflammatory form of infantile SD [210, 211]. Presumably, these deficits impair the clearance of opportunistic fungal pathogens from the skin resulting in prolonged keratinocyte infection and subsequent pronounced cutaneous inflammation. This is supported by the observation that lesional SD skin expresses increased levels of inflammatory cytokines [226, 227] and of the stress ligand MICA [227]. Interestingly, SD is also associated with systemic immune activation and the circulation of activated immune cells [228], including NK cells which have been shown to accumulate in lesional skin [226]. The possibility that some of these cells may be activated non-specifically by bystander cytokine and that SD pathophysiology may have an autoimmune component is both intriguing and consistent with the observation that SD responds to immunosuppressive medication [181]. Nevertheless, these divergent findings in SD continue to perplex the
A

<table>
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<tr>
<th>HIV- &amp; AIDS-Associated Conditions</th>
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B

Figure 7. Occurrence of Seborrheic Dermatitis in the Context of HIV Infection
(A) The most frequent dermatological diagnoses in HIV patients are summarized. The percentage of HIV patients, in one series, afflicted with each disorder is shown [169]. (B) A graphical representation of the degree of CD4 T cell lymphopenia at which various AIDS-related cutaneous diseases occur [208].
understanding of disease pathogenesis, highlighting the need for additional studies to clarify the pathophysiology of this condition.

1.7 Thesis Goals and Approaches

The hypothesis tested in this thesis was that the D2C mouse represents an accurate animal model of human psoriasiform disease and that the interplay between a number of lymphocyte subsets culminates in psoriasiform pathology.

The primary goal of Chapter 3 was to characterize the clinical and histological aspects of disease in order to best determine the human pathological equivalent of the D2C disease phenotype. D2C mice were extensively studied with a careful evaluation of the kinetics of disease, severity of disease, and other clinical aspects of the disease phenotype. Disease was also extensively evaluated through a comprehensive histological analysis of all major organ systems. Through these initial approaches, it was determined that an overgrowth of opportunistic cutaneous pathogens was a feature of D2C disease. To further investigate this observation, various methods were employed to reveal the identity of the opportunistic pathogens involved, after which appropriate antimicrobial agents were administered to D2C mice in the attempt to treat disease. The results of these initial studies demonstrated that the disease phenotype of D2C mice most closely resembled the human psoriasiform disease seborrheic dermatitis. The last approach of this chapter was to test whether genetic mutations known to be associated with SD were also a feature of the D2C model.

The primary goal of Chapter 4 was to investigate the involvement of immunocompromise in D2C disease pathophysiology. To first address this possibility, thymocytes and peripheral lymphocyte subsets were quantitated, followed by functional assessments of normal immune function. The findings that D2C mice were severely immunocompromised and that the
phenotype in these mice resembled AIDS-related SD lead to the investigation of additional parallels between the D2C phenotype and AIDS. Various immune reconstitution experiments were then performed in an attempt to treat disease. A 2\textsuperscript{nd} goal of this chapter was to determine whether perturbations in the normal repertoire of T cell subsets co-existed with and/or contributed to immunocompromise in D2C mice. Given the superficial similarity between the cutaneous disease phenotype seen in T\textsubscript{reg} deficient FoxP3 KO mice and D2C animals, the effect of transgenesis on the development of T\textsubscript{reg} was investigated in D2C mice. Furthermore, it was sought whether the adoptive transfer of DBA/2 T\textsubscript{reg} could abrogate disease development.

The primary goal of Chapter 5 was to investigate whether a population of clonally-anergized self-specific cells in D2C mice contributed to disease pathophysiology and, if so, how this element of disease pathophysiology related to other disease susceptibility factors. To first address this goal, thymocytes and peripheral lymphocytes from D2C mice were immunophenotyped for the expression of the 2C TCR as well as for markers of Ag-experience and T cell activation. Given the presence of acutely activated, Ag-experienced 2C cells, with enhanced functional properties in D2C mice, a blocking mAb against the 2C TCR was administered to see if 2C cells were necessary for the disease phenotype. The demonstrated necessity of 2C cells in the model system led to the testing of whether these cells were sufficient for disease through adoptive transfer experiments and the generation of lymphocyte-deficient 2C mice.

The primary goal of Chapter 6 was to determine whether TCR transgenesis perturbed the development of sentinel cutaneous γδ lymphocytes and the possible effect of this upon disease pathophysiology. To first address this goal, cutaneous lymphocytes from 2C mice were immunophenotyped for the expression of the γδ TCR. The finding that the epidermis of D2C mice was deficient in γδ-cells directed subsequent studies on whether skin from 2C mice formed
a functional barrier or whether this important role was compromised in these animals. To further address this goal and to elucidate a poorly-understood aspect of the model, it was investigated whether the failure of D2C bone marrow to transfer disease to DBA/2 recipients resulted from a persistence of recipient-derived γδ cells in the chimeric epidermis. To address this possibility, DBA/2 γδ KO mice were created and, together with wild-type DBA/2 mice, these animals were used as recipients for D2C bone marrow, which confirmed the role of γδ dysfunction in the D2C model.
Chapter 2: Materials and Methods

2.1. Mice

Breeders for 2C TCR transgenic mice [107] were kindly provided by Dr. Denis Loh (then at Howard Hughes Medical Institute, Washington University, St. Louis, MO). C57BL/6 (B6), DBA/2, BALB/C, and H-2^d congeneric B6 (B6.C-H-2^d/BBY) mice, and breeders for FOXP3 hemizygous KO, Rag-1^{−/−}, and TCR δ^{−/−} mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The 2C TCR transgenes were bred from the B6 background to the H-2^d-expressing BALB/C, DBA/2, and H-2^d congeneric B6 backgrounds, and assessed for disease development at each backcross generation. The Rag-1 and TCR-δ mutations were partially backcrossed from the B6 to the DBA/2 background (6 backcross generations and 5 backcross generations, respectively). All animals were maintained in microisolator cages in the animal facility of the Department of Microbiology and Immunology at UBC and the Thier Building animal facility at the Massachusetts General Hospital, and received acidified water and autoclaved food according to guidelines set by the Canadian Council on Animal Care.

2.2 Cell Lines

The TAP deficient cell line T2-L^d was created by transfection of the human (TxB) hybridoma T2 with the murine L^d MHC class I molecule [63, 229]. T2-L^d cells were a kind gift from Dr. Peter Cresswell (Howard Hughes Medical Institute, Yale University, New Haven, CT). A20 cells (TIB-208, American Type Culture Collection, Manassas, VA) are BALB/c derived, and thus H-2^d expressing, B cell lymphoma cells.
2.3 Reagents

2.3.1 Peptides
The p2Ca (LSPFPFDL) peptide [230] was synthesized at UBC’s Nucleic Acid-Protein Service (NAPS) Unit.

2.3.2 Antibodies and Staining Reagents
All antibodies used in immunohistochemistry (IHC), flow cytometry (FACS®), and enzyme-linked immunosorbent assays (ELISAs), and immunofluorescence (IF) are listed in Table 2. Unless otherwise indicated, the listed reagents were used for FACS®.

Table 2: Antibodies Used

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Source</th>
</tr>
</thead>
<tbody>
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<td>2C TCR (FACS &amp; adoptive transfer)</td>
<td>1B2</td>
<td>H. Eisen, M.I.T., Boston, MA [231]</td>
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<tr>
<td>7AAD</td>
<td>Cat # 129935</td>
<td>Calbiochem, San Diego, CA</td>
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<td>B44</td>
<td>BD Pharmingen, San Diego, CA</td>
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<td>BD Pharmingen</td>
</tr>
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<tr>
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<td>(ELISA capture mAb)</td>
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<td>(ELISA)</td>
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<td>BD Pharmingen [232]</td>
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<td>MR5-2</td>
<td>BD Pharmingen</td>
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<td>1B3.3</td>
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<td>Vβ14 TCR</td>
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<td>Vγ3 γδ TCR</td>
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<tr>
<td>Pan-γδ TCR</td>
<td>GL3</td>
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2.3.3 Primers

Primers were synthesized at either the NAPS Unit at the University of British Columbia or the DNA core facility at the Massachusetts General Hospital.

Table 3: Primers Used

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<thead>
<tr>
<th>Primer Pairs</th>
<th>Sequence</th>
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<td>Jβ 2.5: 5’-CTA ACA CGA GGA GCC GAG TGC CTG – 3’</td>
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<td>TCR 8&quot; [233]</td>
<td>IMR13-NEO Forward: 5’-CTT GGG TGG AGA GGC TAT TC-3’</td>
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<td>IMR14-NEO Reverse: 5’-AGG TGA GAT GAC AGG AGA TC-3’</td>
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<td>IMR15-TCR-8 Forward: 5’-CAA ATG TTG CTT GTC TGG TG-3’</td>
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<td>IMR16-TCR-8 Reverse: 5’-GTC AGT CGA GTG CAC AGT TT-3’</td>
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<td>Rag-1&quot; [234]</td>
<td>IMR189-NEO: 5’-TGG ATG TGG AAT GTG TGC GAG-3’</td>
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<td>IMR1746-WT: 5’-GAG GTT CCG CTA CGA CTC TG-3’</td>
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<td>IMR3104-Common WT: 5’-CCG GAC AAG TTT TTC ATC GT-3’</td>
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<td>FOXP3 [235]</td>
<td>IMR1571 WT forward: 5’–CTC AGG CCT CAA TGG ACA AG – 3’</td>
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<td>IMR1572 Mutant Forward: 5’-TCA GGC CTC AAT GGA CAA AA-3’</td>
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<td>IMR1573 Reverse Common: 5’-CAT CGG ATA AGG GTG GCA TA-3’</td>
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<td>C5 [123]</td>
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<td>Reverse: 5’– ATA ATG GGA GTC ATC TGC GTT T – 3’</td>
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<td>ITS [236]</td>
<td>ITS1: 5’– TCC GTA GGT GAA CCT GCG - 3’</td>
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<td>ITS3: 5’– GCA TCG ATG AAG AAC GCA GC-3’</td>
</tr>
<tr>
<td></td>
<td>ITS4: 5’– TCC TCC GCT TAT TGA TAT GC – 3’</td>
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</table>

2.3.4 Cytokines

Murine IL-2 was obtained from the spent media of cultured IL-2 gene-transfected X63/0 cells as previously described [237]. The concentration of IL-2 in this culture media was determined in a cytokine ELISA using the IL-2 specific mAbs JES6-1A12 (capture mAb, BD Pharmingen) and JES6-5H4 (detection mAb, BD Pharmingen) and recombinant murine IL-2 (550069, BD Pharmingen) as previously described [238, 239].
2.4 Methods

2.4.1 Genotyping of Mice

Mice were genotyped by either PCR or FACS®. PCR based genotyping was performed on ear punch DNA using the primers listed in Table 2. For the 2C TCR reaction, a 250-bp band is amplified from 2C TCR transgenic animals while amplification of non-transgenic control DNA results in a faint smear. FACS® based genotyping was performed on heparanized tail vein blood which was centrifuged over histopaque 1077 (Sigma, St. Louis, MO) for the collection of interface cells. 2C TCR-transgenic mice were genotyped using the 1B2 mAb. Identification of H-2b expressing mice was performed using the HB51 mAb. Rag-1" mice as well as D2C Rag-1" were sometimes genotyped by demonstrating a lack of circulating B-lymphocytes using the goat FITC-labeled, F(Ab')2 anti-mouse lg (1012-02).

2.4.2 Photographs

Photographs were taken using a Nikon CoolPix 990 or Canon PowerShot S400 Digital ELPH camera.

2.4.3 Disease Scoring

Disease has been arbitrarily divided into 4 stages: stage 0 (S0), no disease; stage 1 (S1), minimal peri-ocular erythema and edema; stage 2 (S2), major periocular swelling +/- lid fusion with little contiguous spread to surrounding tissue; stage 3 (S3), S2 features plus significant spread to contiguous tissues. This manner of scoring was found to be extremely reproducible, even amongst those inexperienced with the model system.
2.4.4 Ear Thickness Determination

Ear pinna thickness was measured using a Mitutoyo pocket thickness gage (Long Island Indicator Service, New York, NY).

2.4.5 Histology

Tissue was fixed in 10% buffered formalin (Fisher Scientific, Agawam, MA) and processed for permanent paraffin embedding on a Leica ASP 300 tissue processor (Leica Microsystems Inc., Bannockburn, IL). Paraffin sections were stained with Haematoxylin and Eosin (H&E) using a Leica Autostainer XL (Leica Microsystems Inc.). Grocott's Methenamine Silver (GMS) and Periodic Acid Schiff (PAS) staining was performed on a Ventana Nexes Special Stainer (Ventana Medical Systems Inc., Tucson, AZ). Unfixed tissue was cryo-embedded in optimal cutting temperature (O.C.T.) compound (Sakura Fintek Inc., Torrance, CA) for immunohistochemistry.

2.4.6 Immunohistochemistry on Frozen Tissue

Frozen sections were cut, fixed in acetone for 10 min at -20 °C and then air-dried. Immunohistochemistry was performed as per standard techniques [240]. In brief, the slides were blocked for endogenous peroxidase activity using 3% H₂O₂ in PBS for 10 min. Following removal of the H₂O₂ and PBS washing, the slides were blocked with 5% mouse serum and 5% BSA in PBS-Tween for 1 h at 37°C, before being stained with the primary mAb for 1 h at 37°C. For biotinylated primary Ab, SA-HRP (7100-05, Southern Biotech) was used as a secondary reagent. For unlabeled primary Ab, HRP-labeled goat α-rat IgG polyclonal Ab (S3050-05, Southern Biotech) was used as a secondary reagent. Secondary reagents were applied for 1 h at
37°C. The Vector® Nova Red HRP immuno-histochemistry kit (Vector Labs, Burlington, CA) was used to develop the staining for 15 min. The sections were then counterstained with haematoxylin.

2.4.7 Culture of Fungal Isolates from Lesional Skin

Swabs from the external auditory meatus or rostral skin scrapings made with a sterile #10 scalpel blade were used to inoculate Pityrosporum media (ATCC culture medium #1110 and 1072), supplemented with or without 0.4 mg/ml of cyclohexamide (Sigma) and 0.05 mg/ml chloramphenicol (Sigma). Plates were cultured for 1-2 weeks at 24°-37°C. Colonies isolated on Pityrosporum media were tested for their ability to grow on lipid-enriched as well as standard Sabouraud's dextrose agar (BD Diagnostics, Sparks, MD) under these same conditions. The most frequently isolated organisms were also tested for growth on corn meal and bird seed agar (BD Diagnostics) and were identified to species level using Vitek yeast biochemical cards (Vitek Systems, Biomerieux, France) according to manufacturer's instructions.

2.4.8 Genotyping of Fungal Isolates

DNA from two representative colonies of the most abundantly isolated fungi was obtained by zymolase digestion and used for PCR amplification with the universal fungal, internal transcribed spacer primers, ITS1 and ITS4 as well as ITS3 and ITS4, as previously described [236]. To definitively characterize the isolate, the ITS region from these isolates was sequenced with the ITS1 and ITS4 primers.

2.4.9 DNA Extraction from Paraffin-Embedded Tissue for PCR Amplification of the ITS Region

50
For PCR on paraffin-embedded samples, tissue was scraped from multiple “blank” slides with a sterile #10 scalpel blade and transferred to a microcentrifuge tube. 200-μl of xylene (Sigma) was added, mixed by inversion, heated for 15 min at 37°C, and subsequently centrifuged. The supernatant was removed, and a fresh 200-μl aliquot of xylene was added to the pelleted tissue for a second paraffin extraction. The pellet was washed twice with 1.0 ml of 100% ethanol to remove residual xylene. The ethanol was removed by centrifugation, and the pelleted tissue was air dried. DNA extraction from the pellet was performed by a standard PK digestion technique [241].

2.4.10 Fungal specific ELISA

This assay was carried out as previously described [242]. Briefly, *Candida guilliermondii* yeast grown for 2 days on Sabouraud’s Dextrose agar were disaggregated and washed in PBS. The cells were resuspended in carbonate-bicarbonate buffer at 10⁷ cells/ml and used to coat Immulon plates (Dynatech Laboratories Inc., Indianapolis, IN) for 2 hours at 37° C. After blocking with 1% BSA in PBS for 2 h at 37° C, serum samples were diluted in blocking solution and incubated for 2 h. Bound IgG was detected by incubating 100 μl of alkaline phosphatase (AP) conjugated goat-α-mouse IgG (1030-04, Southern Biotech) for 45 min at room temperature. AP activity was detected by incubation with 100 μl of p-nitrophenyl phosphate (Sigma) at a concentration of 1 mg/ml. The colorimetric assay was analyzed at 405 nm on a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA). Serum was collected from D2C mice at various stages of disease pathogenesis as well as from various control mice, by either tail vein bleeding or the collection of blood from the thoracic cavity following cervical dislocation of animals at the time of sacrifice.
2.4.11 Fungal Growth Inhibition Assays

Circular pieces of Whatman paper (Fisher Scientific, Ottawa, ON), 0.5 cm in diameter, were soaked in a 2 mg/ml solution of fluconazole (Pfizer, QC) and placed on plates of Sabouraud dextrose agar freshly inoculated with fungi.

2.4.12 Fluconazole Administration to Actively Diseased D2C Mice

Fifty-day old severely diseased D2C mice were treated once daily for 9 d with i.p. injections of fluconazole (12 mg/kg) or PBS (n=4 animals per group). Animals were considered to be convalescing at the first observation of new hair growth on rostral skin, and were photographed weekly. Following the treatment period, animals were sacrificed for necroscopic examination.

2.4.13 Fluconazole Administration to Pre-diseased D2C Mice

10-day old pre-diseased D2C mice were treated once daily for 6 weeks with i.p. injections of fluconazole (12 mg/kg) or PBS (n=4 animals per group). Animals were monitored daily for the development of disease and were photographed weekly. Following the treatment period, animals were sacrificed for necroscopic examination.

2.4.14 Bacterial Culture from D2C Skin

Rostral skin scrapings made with a sterile #10 scapel blade were used to inoculate tryptic soy agar (TSA) plates (BD Diagnostics) which were cultured at 24-37°C.
2.4.15 Gram Positive Cocci IgG ELISA

Gram positive cocci (GPC) isolated from the rostral skin of diseased D2C mice were grown on TSA plates and used as a capture reagent to detect GPC-specific serum IgG. IgG was detected as previously described for Candida guilliermondii.

2.4.16 Combined Fluconazole/Levofloxacin Administration to Pre-diseased D2C Mice

10-day old pre-diseased D2C mice were treated once daily for 6 weeks with i.p. injections of fluconazole (12 mg/kg) and levofloxacin (10g/kg) or PBS (n=4 animals per group). Animals were monitored daily for the development of disease and were photographed weekly. Following the treatment period, animals were sacrificed for necroscopic examination.

2.4.17 Adoptive Transfer of Bone Marrow

Following sacrifice, the humeri and femurs of donor mice were exposed, and bone marrow was expressed with PBS injected from a 26-gauge needle. The marrow was depleted of mature T cells using the j1j.10 mAb (ATCC) and Low-Tox-M rabbit complement (Cedarlane) according to company specifications. 1 x 10^7 T cell depleted bone marrow cells were injected by tail vein into irradiated (1150 rads) recipients ranging in age from 21 – 50 d of age. In each experiment at least 4 animals were present in each recipient group and all recipients were age matched. Use of the α-H-2^b mAb HB51 (ATCC) by FACS® was employed for some donor-recipient combinations to ensure engraftment and depletion of recipient cells.

2.4.18 Genotyping Alleles of the 5th Component of Complement (C5)

A 328-330 bp fragment of the C5 gene, containing the 2 bp deletion known to induce C5 deficiency [243] was amplified using the primers described in Table X. This mutation, occurring
in C5 deficient strains, disrupts a Hind III restriction site and, as such, digestion of the resulting amplicons with Hind III (Life Technologies, Burlington, Canada) was used to genotype the animals. C5 sufficient strains have a 211 and 119 bp band while deficient animals have a single 328 bp band. Animals heterozygous for the C5 alleles possess all three bands.

2.4.19 Isolation of Lymphoid Organs and Immunophenotyping:

Lymph nodes (LN), thymi and spleen were harvested from mice sacrificed by cervical dislocation or carbon dioxide asphyxiation. Single cell suspensions were produced by standard techniques [239]. Briefly, lymphoid organs suspended in RPMI 1640 media (GibcoBRL, Burlington, ON) supplemented with 2% Fetal Bovine Serum (FBS, Sigma) were ground through a steel sieve followed by a centrifugation step. The pellet was gently washed to resuspend lymphocytes and subsequently discarded to dispose of adherent cells. The resultant cell suspension was centrifuged and the pellet was resuspended in I media (Iscoves DMEM supplemented with 10% (v/v) heat inactivated FBS, 100 U of penicillin G/ml, 100 μg of streptomycin/ml and 5 x 10^{-5} M 2-ME). For splenic preparations in particular, a RBC lysis step was performed that consisted of resuspending the cell pellet in RBC lysis buffer (Cat # 00-4333, eBioscience) for 5 minutes prior to resuspension in I media. The concentration of the resulting cell suspensions were determined with a hemocytometer and 1-2 x 10^6 lymphocytes/well were seeded in round bottom, 96-well FACS® plates. Cells were stained with a mixture of mAb in 100 μl of FACS® buffer (PBS with 2% FBS) for 15 minutes on ice. Cells were then pelleted by centrifugation and washed with FACS® media, prior to a second incubation step with secondary staining reagents. Following a final wash, cells were resuspended in FACS® buffer and analysed with a FACScan® flow cytometer and CELLQuest software (BD Biosciences, Immunocytometry Systems, San Jose, CA).
2.4.20 Hen Egg Lysozyme (HEL) Immunization and HEL ELISA

HEL (Sigma) was diluted in PBS and emulsified with an equal volume of titermax (Sigma). 0.1 ml of the emulsified Ag (50 μg HEL) was administered i.p. After 10 days, serum was collected from immunized animals by tail vein nicking. Immulon plates were coated with 100 μl of HEL at a concentration of 0.5 μg/ml in 50 mm carbonate- buffer (pH 9.6). Plates were blocked as described previously. Serial dilutions of serum in blocking buffer, ranging from 1:50 – 1:3200 were then incubated in the blocked plate for 1 h at 37°C. Detection of bound IgG was then performed with 1030-04 (Southern Biotech) as previously described.

2.4.21 Mouse Necropsy

For most experimental animals, vital organs including kidney, liver, stomach, small and large intestine, heart, skin, thymus, spleen, LN, and lung were routinely harvested and photographed. Portions of the material were saved frozen for IHC, fixed in glutaraldehyde for EM, processed for flow cytometry and fixed in formalin for permanent parafin embedded sections. Microbiological cultures and blood samples were also procured at the time of sacrifice.
2.4.22 Assessment of Total Serum IgG

Immulon plates were coated with 100 µl of goat F(Ab')2 anti-mouse Ig (1012-01, Southern Biotech) at a concentration of 4 µg/ml in carbonate buffer. Blocking, serum sample incubations, and detection of bound IgG with 1030-04 (Southern Biotech) was performed as described above. The murine IgG1 mAb 1B2 was used to develop a standard curve.

2.4.23 CD4 Counts

An aliquot of heparinized tail vein blood was diluted in RBC lysis buffer, after which the total WBC count was determined using a hemocytometer. The remaining blood was centrifuged over histopaque (Sigma) for the collection of interface cells. Staining of interface cells with GK1.5 (BD Biosciences), and 145.2C11 (BD Pharmingen) and analysis by flow cytometry was performed to identify the percentage of WBC that were CD4+ T cells.

2.4.24 Analysis of BrdU Incorporation

Mice were fed BrdU (Sigma) in their drinking water (0.8 mg/ml) for 10 days before being sacrificed and assayed by flow cytometry for BrdU incorporation by lymphocytes as previously described [63]. Briefly, single cell preparations were generated and surface stained in the usual fashion. Cells were resuspended in 0.15M NaCl and 1.2 ml of EtOH was added slowly in a drop-wise fashion followed by a 30 min incubation. Cells were then washed with FACS buffer and subsequently incubated for 30 min in 1 ml of a 1% paraformaldehyde and 0.05% Tween 10 solution. The cells were then pelleted and incubated in 1 ml of DNAse solution, consisting of 0.15M NaCl, 4.2mM MgCl, and 100 Kunitz units/ml DNAse (Qiagen, Mississauga, ON), for 30 minutes at 25°. The cells were then transferred from FACS® tubes to a 96 well FACS® plate,
washed with FACS® media and stained with anti-BrdU-FITC (B44, BD Biosciences) for 30 min on ice. The cells were then washed and analyzed on a FACScan® flow cytometer.

2.4.25 Adoptive Transfer of Purified CD4⁺ T Cells

Single cell suspensions of DBA/2 LN cells were prepared using standard techniques. Purified CD4⁺ T cells were obtained by incubating LN cells with biotinylated GK1.5 (BD Pharmingen) and subsequently with Streptavidin-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA) before being applied to a MACS Separation Column (Miltenyi Biotec). This procedure yields > 95% purity of CD4⁺ T cells. 2 x 10^7 purified CD4⁺ T cells in 0.5 ml PBS were administered by tail vein to 25-day old pre-diseased D2C recipients. Control animals received an i.v. injection of PBS (n = 4 animals per group in each of three separate experiments). Animals were monitored daily for the development of disease and were photographed weekly. Following the treatment period, animals were sacrificed for necroscopic examination.

2.4.26 Adoptive Serum Transfer to Prediseased D2C Mice

Serum from S3 D2C mice was collected and tested for the presence of α-Candida Ab as previously described. Serum with high titers of α-fungal Ab was pooled. The resultant pooled serum had an IgG concentration of 13 mg/ml which was diluted with PBS to a concentration of 2 mg IgG/ml. 0.5 ml of this diluted serum was administered IP, twice weekly to 20-d old pre-diseased D2C recipients. Age-matched control animals received biweekly injections of the IgG1 T3.70 control Ab. Animals were monitored daily for the development of disease and were photographed weekly. Following the treatment period, animals were sacrificed for necroscopic examination.
2.4.27 T<sub>reg</sub> Suppression Assay

Suppression assays were performed as described [244]. Briefly, 2 × 10<sup>4</sup> DBA/2 CD8<sup>+</sup> T cells as responders were stimulated with 8 × 10<sup>4</sup> irradiated APC (DBA/2 splenocytes) and a 2 μg/ml concentration of Con A, in the presence of various number of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> that resulted in a 1:1, 2:1, 4:1, 8:1, and 1:0 responder:supressor ratio. Cells were cultured in U-bottom 96-well plates and grown in 200 μL of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5 × 10<sup>-5</sup> M 2-ME, 10% FCS, 100 U/ml of penicillin G and 100 μg/ml of streptomycin (all from Life Technologies). The cells were pulsed with 50 μl of (3<sup>H</sup>)thymidine (20 μCi/ml, Perkin-Elmer, Boston, MA) 8 h before the end of the 72 h assay period.

T<sub>reg</sub> cells were purified from LN by first depleting CD8<sup>+</sup> T cells, macrophages, and B lymphocytes by incubation of the cell suspension with the 53.58 (BD Pharmingen) and Mac-1 (BD Pharmingen) mAbs. Following a wash step, the cells were incubated at room temperature for 40 min in PBS:I-media (1:1) with 50 μl of M-450 sheep anti-mouse IgG Dyna beads (Dynal, Oslo, Norway) per 10<sup>7</sup> cells. The cells were then immediately applied to a magnetic column (Dynal), which resulted in the negative selection of unwanted cells. The resultant population of non-magnetized, predominantly CD4 cells was then stained with a biotinylated α-CD25 (PC61) mAb and subsequently with streptavidin-conjugated magnetic microbeads (Miltenyi Biotec) before being applied to a MACS Separation Column (Miltenyi Biotec). This procedure yields > 95% purity of CD4<sup>+</sup>CD25<sup>+</sup> T cells as assessed by FACS<sup>®</sup> analysis using the α-CD25 mAb 7D4 (BD Pharmingen) and the α-CD4 mAb GK1.5 (BD Pharmingen).

CD8 T cells were purified by incubating LN cells with biotinylated 53.67 (BD Pharmingen) and subsequently with Streptavidin-conjugated magnetic microbeads (Miltenyi Biotec) before being applied to a MACS Separation Column (Miltenyi Biotec). This procedure
yields > 95% purity of CD8+ T cells as determined by FACS® analysis using the α-CD3 mAb 145.2C11 (BD Pharmingen) and the α-CD8 mAb 53.58 (ATCC).

For the isolation of APCs, splenocyte cell suspensions were prepared in the usual fashion and red blood cells were lysed as previously described. The cells were resuspended in PBS and γ-irradiated with 2000 rads.

2.4.28 Adoptive Transfer of Purified CD4+CD25+ Treg Cells to Predisease D2C Mice

CD4+CD25+ Treg were purified as described above. 1-2 x 10^6 purified CD4+CD25+ T cells in 0.5 ml PBS were administered by tail vein to 15-day old pre-diseased D2C recipients. Age matched control animals received an i.v. injection of PBS (n = 4 animals per group in each of three separate experiments). Animals were monitored daily for the development of disease and were photographed weekly. Following the treatment period, animals were sacrificed for necroscopic examination.

2.4.29 Adoptive Transfer of Purified CD4+CD25- T Cells to Predisease D2C Mice

CD4+CD25- "helper" (T_H) T cells were purified from LN by depleting CD4+CD25+ Tregs, CD8 T cells, macrophages, and B lymphocytes by incubation of the cell suspension with the PC61 (Cedarlane), 53.58 (ATCC) and Mac-1 (BD Pharmingen) mAbs and subsequently with M-450 sheep anti-mouse IgG Dyna beads (Dynal) as previously described. The negative selection of unwanted cells routinely yielded > 95% purity of CD4+CD25- T cells as assessed by FACS® analysis using the α-CD25 mAb 7D4 (BD Pharmingen) and the α-CD4 mAb GK1.5 (BD Pharmingen). 1-2 x 10^7 purified CD4+CD25- T cells in 0.5 ml PBS were administered by tail vein to 15-day old pre-diseased D2C recipients. Age matched control animals received an i.v. injection of PBS (n = 4 animals per group in each of three separate experiments). Animals were
monitored daily for the development of disease and were photographed weekly. Following the treatment period, animals were sacrificed for necroscopic examination.

2.4.30 ELISA for Quantitating α-Double stranded DNA (dsDNA)

Immunon plates were incubated with 50 µl of a 50 µg/ml solution of polylysine in distilled water at 37°C for 1 h. Following three PBS washes, 50 µl of a 10 µg/ml solution of dsDNA (Sigma) was incubated in the plate for 1 h at 37°C. Blocking, diluted serum sample incubations, and detection of bound IgG with 1030-04 (Southern Biotech) was performed as described above.

2.4.31 Immunofluorescence Microscopy of Kidney Sections

Frozen tissue sections of D2C and control kidney were fixed in acetone for 10 min at -20°C and then air-dried. Immunofluorescence was performed as per standard techniques [239]. In brief, the slides were blocked for non-specific binding with 5% mouse serum and 5% BSA in PBS-Tween for 1 h at 37°C, before being incubated with FITC-labeled, goat F(Ab')2 anti-mouse Ig (1012-02, Southern Biotech). Following the application of anti-fade solution (VectaShield, Vector Labs), tissue sections were examined under a Zeiss fluorescence microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY).

2.4.32 Dexamethasone Administration

Pre-diseased 15 d-old D2C mice received daily i.p. injections of 10 µg dexamethasone or the PBS vehicle control for a study period of 3 weeks (n = 4 animals per group in each of three separate experiments). Animals were monitored daily for the development of disease and were
photographed weekly. Following the treatment period, animals were sacrificed for necroscopic examination.

2.4.33 7AAD Based Method for Identifying Apoptotic/Pre-Apoptotic Cells

Cell suspensions were prepared in the usual fashion and prepared for FACS®. 7AAD (1:100 dilution) together with other staining reagents, were diluted in FACS® media. The staining solution was added to pelleted cells, and incubated for 15 min on ice. The cells were washed 3 times, followed by a fixation step with 200 µl of 4% paraformaldehyde for 15 min on ice. The fixed samples were then added to 200 µl of FACS® buffer and analyzed on the flow cytometer with 7AAD measured in the FL3 channel.

2.4.34 Purification of 2C DNCTC

CD4CD82C+ DNCTC were purified from a standard LN cell suspension by depleting CD4+ T cells, CD8+ T cells, macrophages, and B lymphocytes [63]. To deplete these subsets, the cell suspension was incubated with the GK1.5 (BD Pharmingen), 53.67 (BD Pharmingen) and Mac-1 (BD Pharmingen) mAbs, and subsequently with M-450 sheep anti-mouse IgG Dyna beads (Dynal) as described above. This procedure yielded between a 70-95% pure population of 1B2+ DNCTC as assessed by staining with the α-2C TCR mAb 1B2 (Eisen, MIT). Lower yields were characteristic of actively diseased D2C mice where large numbers of contaminating non-lymphoid, Mac-1+ cells were often present.

2.4.35 Immediate Ex Vivo CTL Assay

Effector 2C TCR DNCTC were purified from the LN of various 2C mice as described above. A20 target cells (ATCC) expressing physiological levels of the 2C cognate ligand were
radio-labeled by incubating $1 \times 10^6$ pelleted cells with 0.1 ml of $^{51}$Cr-sodium chromate (1 mCi/ml, Amersham Pharmacia Biotech, Quebec, Canada) and subsequently mixed with various ratios of effectors ($1 \times 10^4$ targets/well). The cells were briefly centrifuged together and incubated in 200 µl of IMDM in 96-well, U-bottom plates. After an 18-h incubation, supernatants were collected and counted. All assays were performed in quadruplicate. Percent specific lysis was calculated as shown:

\[
\% \text{ release} = 100\% \times \left(\frac{\text{cpm (experimental well)} - \text{cpm (spontaneous release)}}{\text{cpm (maximum release)} - \text{cpm (spontaneous release)}}\right)
\]

2.4.36 IFN-γ ELISA

2C TCR$^+$ DTNC were purified from the indicated 2C mice as described above and previously. $1 \times 10^5$ 2C cells were stimulated with $1 \times 10^5$ mitomycin C-treated (Sigma) A20 cells and 20 U/ml IL-2. After 40 h of stimulation, tissue culture supernatant was collected for subsequent analysis. Immulon plates were coated with 100 µl of the IFN-γ capture antibody R4-6A2 (eBioscience) at a concentration of 4 µg/ml in 50 mM carbonate-bicarbonate buffer (pH 9.6). Various supernatant dilutions were incubated in the plate following a standard blocking step. Bound IFN-γ was detected by 100 µl of the biotinylated detection antibody XMG1.2 (1 µg/ml, eBioscience) and a subsequent incubation with 100 µl of SA-AP (1:2000, Southern Biotech) for 45 min at room temperature. AP activity was assayed by a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA) following incubation with 100 µl of a 1 mg/ml solution of p-nitrophenyl phosphate (Sigma). Recombinant IFN-γ (eBioscience) was used to develop a standard curve.

62
2.4.37 T2-L^d Proliferation Assay

2C TCR^+ DNTC were purified from the indicated 2C animals as described above. 1 x 10^4 2C cells were stimulated with 5 x 10^4 mitomycin C (Sigma) treated T2-L^d cells loaded with 0, 0.01, 0.1, 1, and 10 μM p2Ca peptide (synthesized at the UBC NAPS Unit) ± 20 U/ml IL-2, (the preparation of which was described previously [63]). The assay was performed in U-bottom 96-well plates in 200 μL of IMDM supplemented with 5 x 10^-5 M 2-ME, 10% FCS, 100 U/ml of penicillin G and 100 μg/ml of streptomycin (all from Life Technologies). The cells were pulsed with 50 μl of (^3)H)thymidine (20 μCi/ml, Perkin-Elmer, Boston, MA) 8 h before the end of the 72 h assay period. All conditions were performed in quadruplicate.

2.4.38 Treatment of D2C Mice with a Blocking α-Clonotypic mAb against the 2C TCR

Shortly before weaning, 400 μg of the α-2C TCR IgG1 mAb 1B2 (H. Eisen, MIT), was administered by tail vein to pre-diseased D2C mice. Age-matched control animals received 400 μg of the isotype-matched control mAb T3.70 (eBioscience, San Diego CA)(n = 4 animals / group in each of 3 experiments). The 0.5 ml injections were delivered i.p. every 6 d and were not associated with any sign of distress. Animals were monitored daily for the development of disease and were photographed weekly. Following the treatment period, animals were sacrificed for necroscopic examination.

2.4.39 Purification and Adoptive Transfer of 2C DNTC

DNTC were purified from the LN of 2-4 month old S2-S3 D2C donors as described above. 5x10^6 CD69^+ 2C cells in 0.5 ml PBS was administered by tail vein into irradiated (550 rads) DBA/2 recipient mice.
2.4.40 Collection of Epidermal T Cells for Flow Cytometry

Skin was shaved and treated with a depilatory cream (Nair, Church and Dwight Company Inc., Princeton, NJ). Full thickness skin was removed, and vigorously scraped to remove subcutaneous fat. The skin was then incubated dermal side down in a solution of dispase type II (6 mg/ml, Roche Diagnostics, Indianapolis, IN) and 0.001% (w/v) DNAse (Sigma) in PBS or a solution of Ca\(^{++}\)-Mg\(^{++}\)-free PBS (1 mM EDTA, 1 mM DTT) for one hour at 37°C [245]. The epidermis was then isolated using jewellers forceps and a dissecting microscope. The epidermal cells were disaggregated by vigorous pipetting in Ca\(^{++}\) and Mg\(^{++}\) free media and subsequent grinding through a steel sieve. The resulting single cell suspension was applied over a histopaque (Sigma) cushion and centrifuged to enrich for interface cutaneous IEL.

2.4.41 Collection of Epidermal Sheets for Quantitating the Density of Cutaneous IEL

Skin from the indicated animals was shaved and treated with a depilatory cream. After vigourous scraping to remove subcutaneous fat the skin was floated dermal side down in a solution of ammonium thiocynate (NH\(_4\)SCN, Sigma) for 20 minutes at 37°C. The intact epidermis was isolated using jeweller’s forceps and a dissecting microscope and transferred to a positively charged slide, dermal side up. Epidermal sheets were washed in PBS and fixed for 10 minutes in acetone at -20°C before being air-dried for one hour. Epidermal sheets from all mice were then stained with a biotinylated α-CD3 mAb (145.2C11, BD Pharmingen) by a standard immunhistochemical protocol as previously described. The average number of CD3\(^+\) cells in ten, 40x microscopic fields of the epidermal sheets was determined using an Olympus BH-2 microscope.
2.4.42 Croton Oil Administration

Fur in the lower dorsal region was trimmed with scissors as close to the skin as possible without causing epidermal injury. A cotton swab was used to liberally apply a 2% croton oil in acetone solution over a 0.75 x 0.75 cm region of “trimmed”, dorsal skin. Mice were observed daily for the development of erythema and cutaneous ulceration. Five days post-application, mice were euthanized and skin samples were collected for standard histological processing.

2.5 Data Reproducibility

For all of the data presented herein, results are representative of at least 3 individual experiments.

2.6 Statistics

Student's 2 tailed t tests were used for the statistical analysis of data.
Chapter 3: Pathological and Clinical Characterization of Cutaneous Disease

3.1 Introduction:

Given Delaney et al.'s observation that disease in D2C mice was psoriasiform in nature [164], the first efforts on this model sought to characterize the spontaneous disease in these animals and to ascertain whether the pattern of cutaneous disease was indeed psoriasiform. Furthermore, the comparison of D2C disease features to those of known human psoriasiform entities was undertaken in order to address whether this novel animal model represented a reliable model of human disease. Interestingly, disease in D2C mice was indeed psoriasiform; however, rather than resembling psoriasis as described in an earlier report [164], disease was remarkable similar to SD. The impressive similarities in the natural history of murine D2C and human SD disease, as well as gross and microscopic pathology, provided the impetus to investigate the involvement of fungi in D2C disease pathogenesis. Similar to that seen in SD, an increased burden of fungi is present in lesional D2C skin and the treatment of these animals with antifungal agents can mitigate established disease. Molecular genotyping of the most frequent fungal isolate revealed this organism to be Candida guilliermondii, against which D2C mice were found to have high titers of serum IgG. Further studies demonstrated that, in addition to C. guilliermondii, there is an increased burden of, and an elevated titer of specific serum IgG to, other organisms on the skin of D2C mice, including gram positive cocci. The addition of wide spectrum antibiotic treatment to the antifungal regimen was even more efficacious at treating disease, although the concurrent administration of these agents was unable to completely abrogate the development of disease.

The apparent susceptibility of D2C mice to infection suggested that immune defects from the DBA/2 background controlled disease penetrance. To investigate this possibility, we
determined which inbred strains were susceptible to disease and the pattern of disease inheritance in backcrosses from disease-resistant to disease-susceptible backgrounds. The result of backcrossing studies suggested that as few as one gene or a closely linked group of genes was responsible for disease penetrance. To see if this candidate factor was intrinsic to the hematopoietic system, bone marrow chimera studies were performed, the results of which suggested that the candidate susceptibility factor was not bone marrow derived as marrow from disease-resistant 2C mice could transfer disease to wild type DBA/2 recipients. Since complement factor 5 (C5) deficiency has been associated with some forms of SD [210, 211], it was hypothesized that the known deficiency of C5 in DBA/2 mice [243] was this non-hematopoietic defect. To address this possibility, a PCR/RFLP assay was used to correlate the C5 genotype with the penetrance of disease in (B6xDBA/2)N22C backcrosses. While the C5 mutation was found to modulate the disease phenotype, it was neither necessary nor sufficient for disease. Furthermore, the finding that both genetic influences as well as environmental factors, such as hygiene, influenced the severity of disease suggested that disease had a more complex mode of inheritance.

The generation and characterization of the D2C model of psoriasiform disease represents an important advancement in the field of inflammatory skin disease as few spontaneous animal models with truly representative features are available to study the pathogenesis of psoriasiform disease.

3.2 Natural History of Disease

The expression of the 2C transgenic T cell receptor on the DBA/2 genetic background (D2C) leads to the development of spontaneous inflammatory skin disease. Disease occurs around the time of sexual maturity (32-38 days) [245], 1-2 weeks after weaning, with males
tending to develop disease earlier and more severely than female siblings [123, 164]. The course of disease is chronic with periodic flares, corresponding to deteriorations in the hygienic state of the cage bedding [123]. Recovery from cutaneous disease begins at ~70 days of age after which only subtle disease, if any, persists [123]. After the establishment of remission, animals are resistant to recurrent disease [123]. Diseased mice are observed to scratch intensely at rostral skin, suggesting associated pruritus, though it has not been addressed whether this scratching is simply an epiphenomenon or whether it plays an active role in disease pathogenesis.

3.2.1 Gross Pathology of Disease

Disease in D2C mice occurs in a distinctive distribution involving primarily the ears, rostrum and perineum. Although disease is often extremely inflammatory, only rarely does it extend beyond these “seborrheic areas” to cause generalized erythrodermic dermatitis [123]. The gross appearance of lesional skin is dependent upon the chronicity of disease. Acute disease invariably begins in the periocular region with ill-defined erythema [123]. This initial blepharitis is later accompanied by prominent periocular edema that typically results in entropion and pronounced serous exudate, which forms yellow-brown crusts that can seal the lid margins [123]. Rarely, purulent conjunctivitis can occur [123]. No vesicles or pustules are obviously present; however, occasional inflammatory papules can be appreciated [123]. Although acute skin disease initially has indistinct margins, the subsequent development of lesional alopecia sharply marginates disease (Fig. 8A) [123].

Ear disease is a prominent feature of acute pathology and can precede other grossly apparent signs of disease (Fig. 8B) [123]. Ear pathology can become quite severe, resulting in large concretions of hyperkeratotic crusted debris occluding the external ear canal (Fig. 8C) [123]. Ear disease in these animals typically extends into the external auditory meatus, rather
Figure 8. Gross Pathology of Disease

(A) D2C gross pathological changes were arbitrarily divided into 4 stages: stage 0, no disease; stage 1, minimal periocular disease; stage 2, major periocular swelling ± lid fusion with little contiguous spread to surrounding tissue; and stage 3, stage 2 features plus significant spread to contiguous tissue. (B) Pre-diseased 21-day old D2C mice and age matched DBA/2 controls were assayed for ear thickness. The difference in ear thickness was found to be statistically significant (p < 0.05). (C) Ear disease in a S3 D2C mouse is shown. Note the concretion of hyperkeratotic debris occluding the external auditory meatus.
than out from it, and rarely involves the entire length of the canal [123].

In more chronic lesions, the prominent swelling and serous crusting of acute disease are replaced by thickening of the skin and the appearance of fine white scale [123]. This lichenified appearance precedes convalescence, the onset of which is marked by a further reduction of swelling, the return of dermatoglyphic skin ridges, and the regrowth of hair [123].

To facilitate objective scoring of the disease course, cutaneous signs of disease have been arbitrarily divided into 4 stages: Stage 0 (S0), no disease; Stage 1 (S1), minimal periocular erythema and edema; Stage 2 (S2), major periocular swelling ± lid fusion with little contiguous spread to surrounding tissue; and Stage 3 (S3), S2 features plus significant spread to contiguous tissue (Fig. 8A) [123]. Intriguingly, the clinical findings in D2C mice are remarkably similar to those of the human psoriasiform condition SD.

3.2.2 Microscopic Pathology of Disease

The microscopic features of D2C inflammatory skin disease are variable and dependent upon the clinical stage of disease [123]. Compared to normal murine skin (Fig. 9A), lesional tissue from acutely affected animals is characterized by: neutrophilic abscesses within the follicles and contiguous epidermis (Fig. 9B); dilated vessels within edematous dermal papilla (Fig. 9C); and, spongiosis of the follicular infundibulum, adjacent to foci of follicular inflammation (Fig. 9C) [123]. The neutrophilic abscesses coalesce into mounds of pyknotic neutrophilic debris and, together with prominent globules of eosinophilic serum and compact parakeratotic squames, form the mound-like scale-crusts that are situated near the ostia of hair follicles (Fig. 9D, E) [123]. Chronic lesions, which clinically have a lichenified appearance, demonstrate epidermal thickening from the typical 1-2 cell layer thickness in normal murine skin (Fig. 9A) to greater than 10 cell layers, with finger-like projections of acanthotic epidermis
Figure 9. Microscopic Pathology of Disease

(A) Normal epidermis (e) consists of 1-2 cell layers overlaying a non-inflamed dermis (d) containing plentiful adnexal structures (f = hair follicle; g = sebaceous gland). (B) The primary histological lesion is a neutrophilic abscess (na) in the superficial follicle (f). (C) Neutrophilic abscesses (na) are often situated adjacent to spongiotic (s) epidermis and edematous dermal papilla containing dilated blood vessels (v). (D) The neutrophilic abscesses (na) coalesce into perifollicular mounds (m) of pyknotic neutrophilic debris. (E) Sub-acute lesions are characterized by primary and secondary histological changes. Mounds of follicular debris (m) co-exist with acanthotic epidermis (a) and a multifocal coalescing inflammatory infiltrate (i) that is often concentrated around damaged follicles (*). (F) Chronically lesional skin is depleted of epidermal adnexa and has moderate acanthosis (a) coexisting with a dense dermal infiltrate (i). (F, G) Keratinaceous debris (k) released from damaged adnexa is often present in the dermis of chronic lesions and is surrounded by a dense inflammatory infiltrate (i). (H) Ear pathology has similar histological features with mounds of debris (m) situated in a follicular distribution. (I) Acetone-fixed frozen tissue sections of D2C and DBA/2 rostral skin were incubated with a rodent α-CD4 mAb (RM4-5). The HRP-labeled goat anti-mouse IgG and the Vector® Nova Red HRP immunohistochemistry kit were used to develop the staining.
extending into the dermis (Fig. 9E) [123]. A dense infiltrate of mixed inflammatory cells surrounding glands and hair follicles is another feature of chronic pathology (Fig. 9E-H). The extent of this pyogranulomatous inflammation is largely dependent upon the integrity of the adnexal structures since keratinaceous debris released from damaged glands and follicles is often at the center of such inflammatory foci (Fig. 9E-H) [123]. Immunohistochemical staining of the skin reveals that the mixed inflammatory infiltrate of lesional skin contains a large number of T lymphocytes (Fig. 9I), many of which express the CD4 co-receptor molecule. This histological pattern is most consistent with those findings seen in the human psoriasiform disorder SD which further supports the similarity between the D2C model and human SD.

3.3 Exploring the Role of Fungus in Disease Pathogenesis

3.3.1 Antifungal Staining of Diseased Skin

Overgrowth with opportunistic basidiomycetes fungi of the Malassezia genus is a feature of SD pathophysiology [219]. Given the known susceptibility of DBA/2 mice to fungal infection [246, 247] and the SD-like pathology of D2C mice, it was investigated whether fungal overgrowth is a feature of D2C disease pathogenesis. While no positively stained structures were apparent in non-lesional skin from diseased animals or from DBA/2 controls (Fig. 10A), numerous small ovoid clustered structures, with pale centers, were consistently seen within the superficial layers of keratin and within neutrophilic abscesses of D2C lesional tissue (Fig. 10B-I) [123]. GMS-stained structures between orthokeratotic bundles of keratin are sometimes seen to invade into deeper levels of the epidermis (Fig. 10E-G) [123]. However, no dermal invasion or mycelial shift was appreciated in any of the lesional skin sections [123]. Although fungal overgrowth is a feature of SD, the habitat of these opportunistic fungal pathogens is often lost during tissue processing for microscopy. Normally the direct visualization of such overgrowth
Figure 10. Antifungal Staining of Lesional Skin

(A) Non-lesional epidermis (e) does not stain with GMS; however, GMS stains dermal connective tissue (c). (B) Finely speckled staining with GMS (*) is frequently observed in lesional epidermis and surrounding follicular abscesses (na). (C) High-powered views of lesional skin demonstrate small round GMS stained structures (*) in the epidermis below a hyperkeratotic (hk) mounds and (D) within neutrophilic abscesses (na). (E) Intact GMS stained structures (*) can be found between the orthokeratotic bundles of keratin within the stratum corneum and (F, G) infrequently can be seen invading into deeper layers of the skin from this superficial local. (H, I) Small structures (*) clustered together around keratinocytes (k) from lesional skin also stain with PAS. Note the PAS positive globules of serum (g) within the neutrophilic abscess (na) and nascent scale-crusts.
involves staining skin scraping or transparent tape used to “desquamate” superficial layers of the epidermis [203]. The appreciation of this fungal overgrowth in D2C mice without these specialized techniques suggests that it is likely a very significant feature of this animal model.

3.3.2 Identification of Fungus from Lesional Tissue

To address the significance of the positive fungal staining in D2C lesional skin, skin scrapings were obtained from lesional rostral and external auditory meatal skin as well as non-involved skin from both diseased and control mice. This material was plated on Sabouraud’s dextrose agar, lipid-enriched Sabouraud’s dextrose agar, Pityrosporum media I (ATCC media #1072), and Pityrosporum media II (ATCC media #1110), both with and without inhibitors (cyclohexamide and chloramphenicol) at 25°C, 37°C and 40°C. Fungi were only routinely recoverable from S3 D2C animals and approximately 90% of the isolated colonies were soft and round with a ruffled peripheral collarette and a central dimple. These smooth, glistening white-to-tan colonies developed a yellowish discoloration over time (Fig. 11A). These colonies consisted of ellipsoidal yeast containing a single uni-polar, broad-based bud (Fig. 11B). The aforementioned isolates grew on all media described above and showed no tendency to filament in vitro (Fig. 11C, D).

Representative samples isolated from separate animals were characterized by standard morphological appearance, culture characteristics, and biochemical methods (Table 4).
Figure 11. Isolation of Fungi from the Lesional Skin of Diseased D2C Mice

Skin scrapings from D2C and DBA/2 control mice were inoculated on lipid enriched Sabouraud's dextrose agar containing cyclohexamide and chloramphenicol at 37°C. Fungi were only routinely recoverable from S3 D2C animals. (A) Approximately 90% of the isolated colonies were round with a ruffled peripheral collarette and a central dimple. These colonies developed a yellowish discoloration over time. (B- 40x) These colonies consisted of ellipsoidal yeast containing broad-based, uni-polar buds. The yeasts showed no tendency to filament in vitro. (C- 40x, D- 100x) Corresponding differential interference contrast (DIC) images of the yeasts.
Table 4: Identification of the Fungal Isolates as *Candida guilliermondii*

<table>
<thead>
<tr>
<th>Identification Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS Stain:</td>
<td>Small bottle-shaped yeast cells</td>
</tr>
<tr>
<td>Fatty Acids Required for Growth:</td>
<td>None – growth on Sabouraud’s dextrose agar</td>
</tr>
<tr>
<td>Germ Tube:</td>
<td>Negative</td>
</tr>
<tr>
<td>Growth at 25°C:</td>
<td>Positive</td>
</tr>
<tr>
<td>Growth at 37°C:</td>
<td>Positive</td>
</tr>
<tr>
<td>Growth at 40°C:</td>
<td>Positive</td>
</tr>
<tr>
<td>Cyclohexamide Resistance:</td>
<td>Positive</td>
</tr>
<tr>
<td>Corn Meal Agar:</td>
<td>No Chlamydospores; No Pseudohyphae</td>
</tr>
<tr>
<td>Bird Seed Agar:</td>
<td>Negative</td>
</tr>
<tr>
<td>Vitek Yeast Biochemical card (V1303):</td>
<td><em>Candida guilliermondii</em></td>
</tr>
</tbody>
</table>

These data were consistent with the preliminary identification of these organisms as a *Candida* species rather than *Malassezia*, as good growth was seen at 25°C and the constricted junction between the bud and mother cell is more indicative of the *Candida* genus [248]. To further classify this common-most isolated organism, PCR amplification using the universal fungal primers ITS1 and ITS4 (Fig. 12) as well as ITS3 and ITS4 [236] resulted in bands of approximately 600 bp and 380 bp, respectively. Based upon the size of these amplicons, the lipid-independent species of *Malassezia* (*M. pacyhdermatitis*) was excluded since this organism yields products of 800 bp and 330 bp with these primer pairs, respectively [236]. Based upon published amplicon sizes using both sets of these primers, the closest match of amplicon sizes were with *Candida guilliermondii* (603/378) and *Pichia anomala* (615/375). To definitively identify this isolate, the ITS region was amplified and sequenced to reveal 100% identity with *Candida guilliermondii* (Table 5).
Figure 12. PCR Amplification of the ITS Region of the Unknown Fungal Isolate

(A) The ITS1-ITS4 primer pair amplifies the intervening 5.8S rDNA sequence and the adjacent ITS1 and ITS2 regions while the ITS3-ITS4 primer pair amplifies a large portion of the 5.8S rDNA sequence and the adjacent ITS2 region [236]. (B) DNA was obtained from the commonest cutaneous isolate from D2C mice, identified as *Candida guilliermondii* by the Vitek YBC, and from a known *Malassezia pachydermatis* isolate. Purified *M. pachydermatis* DNA was also obtained from ATCC. DNA samples were amplified using the universal fungal primers ITS1 and ITS4. The sizes of the resultant amplicons were compared with the known sizes of these fragments in various fungal species [236].
Table 5. ITS Sequence Data of the Fungal Isolate

<table>
<thead>
<tr>
<th>Identification:</th>
<th>Candida guilliermondii (417/417; 100% identity)</th>
</tr>
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<tbody>
<tr>
<td>Unknown sample</td>
<td>1  atacagagaatatcttcgcaaccttacatcaacgcct 60</td>
</tr>
<tr>
<td></td>
<td>5  atacagagaatatcttcgcaaccttacatcaacgcct</td>
</tr>
<tr>
<td>Known Candida guilliermondii</td>
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</tr>
<tr>
<td></td>
<td>458  tagacagcactatctagtactacccatgccaatactttttccag</td>
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<tr>
<td></td>
<td>12 1 taagagctcctggaataocaggaggggcaatgtgcgttcaaagattcgatgattcacgaaah 398</td>
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<td></td>
<td>158  aattgcgttttgttaaaccctctggcccacccatatctccggcacaaccacgcaagcga 102</td>
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Candida spp. have long been considered opportunistic pathogens and C. guilliermondii has been implicated in several human diseases [249-252], including cutaneous candidiasis [253]. Furthermore, C. guilliermondii has been shown to cause cutaneous candidiasis in veterinary populations [254]. Moreover, some forms of SD may also be attributable to the overgrowth of Candida spp. and thus these organisms may have relevance for human psoriasiform disease [205, 255, 256]. Interestingly, Malassezia spp. have not previously been isolated from rodents [257],
and thus the failure to isolate these commensal organisms from the skin of D2C mice may reflect their genuine inability to colonize murine skin. Given the possible failure of *Malassezia* spp. to colonize rodent skin, it is possible that other organisms may contribute to the induction of psoriasiform pathology in rodentia. Nevertheless, the unique distribution of disease and the clinical and histological similarities to *Malassezia*-associated conditions suggested that the commonly isolated *Candida guilliermondii* may not have been the pathogenic organism. To ascertain whether other fungal species were present on the skin, an attempt was made to amplify the ITS sequence from paraffin-embedded lesional tissue; however, no fungal-specific sequences were amplified by this methodology (data not shown). Although this data is consistent with the absence of other fungal pathogens on the skin, the failure to amplify the *C. guilliermondii* ITS sequence argues that technical difficulties with this assay precluded the investigation of this question. Future studies using this assay to amplify the ITS sequence from fresh unprocessed tissue could help to clarify this question further; and, therefore, the current results have not completely ruled out the possibility that *Malassezia* fungi are present in these animals and have a role in disease pathophysiology.

To assess the significance of the overgrowth of *C. guilliermondii* seen in D2C mice and to see if this opportunistic pathogen overgrowth had elicited an immune response, D2C mice at different stages of disease were assayed for fungal-specific serum IgG using a standard ELISA protocol with fungal cells used as the capture reagent [242]. These data demonstrated that severely diseased (S3) D2C mice had a 4,100-fold elevation in the level of fungal specific IgG relative to age matched DBA/2 control mice (Fig. 13A). Furthermore, analysis of fungal Ab titers in D2C mice at various stages of disease revealed that the titer of fungal-specific serum IgG positively correlated with the degree of cutaneous pathology (Fig. 13B).
Figure 13. Fungal-Specific IgG and Relationship to Disease Activity

(A) Serum was collected from tail vein blood from the indicated animals. Immulon ELISA plates were coated with a 1x10^7 cells/ml concentration of pure Candida guilliermondii suspended in carbonate buffer. Serum dilutions were incubated and bound-IgG was detected with alkaline phosphatase (AP) conjugated, goat α-mouse IgG and the AP substrate p-nitrophenyl phosphate (n = 10 animals per group). (B) Candida guilliermondii-specific serum IgG was detected as described above for D2C mice at various stages of disease pathogenesis.
3.3.3 Effect of Antifungal Treatment on Established Cutaneous Pathology

The presence of fungal material in the primary histological lesion of D2C histopathology suggested that fungi might be playing an active role in disease pathogenesis. Imidazole antifungal agents have excellent activity in the skin [258] and fluconazole has been shown to be highly effective in the treatment of SD and related *Malassezia* associated conditions [203, 204, 217], as well as murine Candidiasis [259]. Moreover, pure cultures of *C. guilliermondii* isolated from the lesional skin of D2C mice were inhibited with discs impregnated with a 2 mg/ml solution of fluconazole (data not shown). To see whether the D2C disease phenotype could be mitigated by the administration of fluconazole, severely diseased D2C mice were randomly divided into two groups for treatment with either fluconazole or a vehicle control. Using an established dosing strategy for azole-responsive murine fungal infections [259], the majority of clinical disease in S3 animals was reversed after a nine day course of fluconazole (Fig. 14A, B) [123]. The condition of PBS-treated animals remained unchanged or deteriorated (Fig. 14A, B) [123]. Importantly, the clinical resolution of disease in fluconazole-treated animals was associated with the mitigation of the typical D2C histological changes and a reduction of PAS staining in tissue sections taken from previously lesional skin (Fig. 14C) [123].

3.3.4 Effect of Antimicrobial Treatment on the Suppression of Cutaneous Pathology

To see whether antifungal therapy could abrogate the development of the D2C disease phenotype, fluconazole was administered to prediseased D2C mice beginning at 10 days of age (Fig. 15A). Interestingly, while disease appeared to be kinetically-impaired and less inflammatory than disease in PBS-treated D2C mice, some fluconazole treated animals still developed the full disease phenotype (S0 = 20%; S1 = 30%; S2 = 30%; S3 = 20%). Rostral skin from these animals appeared to be less inflamed and had less alopecia than littermate PBS-
Figure 14. Treatment of Diseased D2C Mice with Antifungal Medication

(A) Acutely ill (S3) D2C mice with severe pathological changes were treated for 10 days with PBS or fluconazole (azole). (B) Recovery of mice was monitored over the 10-day treatment period. Mice were considered to be disease-free when hair regrowth began over lesional rostral skin. (C) Representative histological sections stained with H&E and PAS are shown. Note the punctuate epidermal PAS staining (*) in the PBS control skin, which underlies a large mound of debris (m) that also stains prominently with PAS due to the inclusion of serum.
Figure 15. Treatment of Pre-Diseased D2C Mice with Antimicrobial Agents

(A) Pre-diseased 10-day old D2C mice received daily i.p. injections of PBS, fluconazole (azole) or a combination of fluconazole and levofloxacin for a period of 3 weeks. Mice were monitored during the treatment period for the development of disease. Representative photographs and histological sections stained with H&E are shown. (B) The effectiveness of the treatments at preventing disease was quantified by observing and grading resultant pathology (n=5 animals per treatment group). (C) Immulon ELISA plates were coated with gram positive cocci (GPC) isolated from lesional D2C skin. Serum dilutions from the indicated mice were incubated and bound IgG was detected with alkaline phosphatase (AP) conjugated, goat α-mouse IgG and the AP substrate p-nitrophenyl phosphate (n=5 animals per group).
control animals (Fig. 15A, B), suggesting that the elimination of fungi was important for halting
the progression to a more severe disease phenotype. Current dogma about cutaneous fungi in SD
holds that inflammation directed against the causative microorganism is most severe after the
initiation of dermatitis [204]. Consistent with this current understanding, the highest titers of
Candida-specific IgG in D2C mice occurs during peak symptomatology (S3) (Fig. 13B). The
reduced titer of fungal specific serum IgG in convalescent D2C animals (Fig. 13B), likely
reflected the ability of an intact epithelial barrier to preclude the invasion of opportunistic
pathogens, and the subsequent immunization with microbial Ag.

Another explanation for the failure of antifungal medication to completely abrogate the
development of disease is the development of fluconazole-resistance. Consistent with this
 possibility, PAS stained histological sections from long-term fluconazole treated animals
sometimes reveals PAS-positive structures within lesional tissue, suggesting that animals were
re-colonized with antifungal-resistant strains or new fungal organisms with intrinsic resistance
(data not shown). The emergence of azole-resistant Candida spp. after long-term treatment is a
significant problem in clinical medicine and fluconazole has been shown to increase resistance in
Candida spp. by a variety of mechanisms [260, 261]. The addition of further anti-fungal agents
to fluconazole treated mice may therefore have an additive effect on suppressing the disease
phenotype. It is also known that there is a marked increase of Staphylococci spp. in lesional skin
of SD patients [207, 242, 262] and that Staphylococci species have a synergistic relationship with
cutaneous fungi [263, 264]. Consistent with these findings in SD patients, lesional skin from
D2C mice was found to have a greater burden of clustered gram-positive cocci, presumably of
the Staphylococci spp. (data not shown), and a greatly increased titer of serum IgG against these
organisms (Fig. 15C) relative to non-transgenic DBA/2 control mice.
To address whether a reduced burden of both cutaneous fungi and bacteria would be adequate to protect against the development of spontaneous disease, both fluconazole and levofloxacin were administered to pre-diseased D2C animals (Fig. 15A, B). Levofloxacin, the optically active form of ofloxacin, is a broad spectrum fluoroquinolone antibiotic with activity against most gram positive and gram negative bacteria, and is not associated with the emergence of resistant staphylococci organisms as seen with other fluoroquinolones [265]. Relative to animals treated with fluconazole alone, these mice had a slightly less severe disease phenotype (S0 = 20%; S1 = 40%; S2 = 40%; S3 = 0%) but were still capable of developing moderate pathology (Fig. 15A, B). However, the failure to eliminate all organisms may explain the inability of combined fluconazole/levofloxacin therapy to suppress the development of disease. For example, the addition of levofloxacin to the fluconazole treatment regimen does not address the development of azole-resistant fungal pathogens. Moreover, while the combination of fluconazole and levofloxacin cover a wide spectrum of microorganisms, the addition of further broad-spectrum agents, to both target “uncovered” organisms and reduce the development of resistance, may be sufficient to abrogate the development of disease. Therefore, while the possibility that microorganisms initiate disease cannot be completely excluded by this data, the development of germ-free D2C mice would unequivocally address the role of microorganisms in disease initiation.

3.4 Exploring Genetic Influences on Disease Penetrance

3.4.1 Pattern of Inheritance and Strain Susceptibility

To investigate the genetic susceptibility factors that predispose to disease, the 2C TCR transgenes were backcrossed to various H-2^b- and H-2^d-expressing inbred strains. B6^b 2C (B2C^b), H-2^d congenic B6^d 2C (B2C^d), (B6^bxDBA/2)N;2C^bd, and (B6^dxDBA/2)N;2C^d animals
were found to be resistant to disease while BALB/c 2C (H-2^d) mice developed a mitigated disease phenotype (< 50% of BALB/c 2C mice have clinical disease, n=10) (Fig. 16) [123]. Previously, the 2C transgenes were backcrossed to the H-2^d-expressing B6xDBA/2 (BXD) recombinant inbred (RI) strains [164]. It was found that BXD RI strains 1, 5, 6, 9, 11, 16, 18, 22, 24, 25, 30, and 31 developed disease whereas strains 12, 27, and 30 were resistant to transgene-mediated pathology (Table 1) [164]. To further evaluate the contribution of the DBA/2 genetic background to disease development, the frequency and severity of disease development in successive backcrosses from the B6 to the DBA/2 background was determined. While B6^b and (B6^bxDBA/2)N12C^bd (N12C) mice are resistant to disease (n > 100), approximately 50% of N22C (n = 50) animals, and 100% of N32C (n = 25) and further DBA/2 2C backcrosses, develop variable degrees of spontaneous cutaneous pathology (Fig. 17) [123]. While only 2C TCR-expressing animals develop disease, there was no difference in the incidence or severity of disease in H-2^bd and H-2^dd N2 DBA/2 2C backcrosses, indicating that an increased dose of the cognate Ag is not a factor in disease pathogenesis (data not shown) [123]. The extent of disease in successive backcrosses to the DBA/2 genetic background became progressively worse, up to approximately the 4th backcross generation at which point the typical D2C pattern of disease penetrance was established (Fig. 17) [123]. This pattern of inheritance is consistent with as few as one susceptibility factor, or a group of closely linked genes, controlling disease penetrance.

3.4.2 Adoptive Transfer of Disease

To determine whether the DBA/2 susceptibility factor(s) were of hematopoietic origin, T cell depleted bone marrow from D2C mice was adoptively transferred to lethally irradiated non-transgenic B6^d recipients. Interestingly, none of these recipient mice developed gross pathological changes or histological evidence of disease (Fig. 18A, B) [123]. To ensure that the
Figure 16. Strain Susceptibility

B6 2C (B2Cb), B6 H-2d congenic 2C (B2Cd), and (B6xDBA/2)N12Cd (N12C) mice are resistant to disease. BALB/c 2C mice develop a mitigated form of disease with low penetrance.
Figure 17. Backcross Analysis of Disease Penetrance

Disease incidence and severity was monitored in successive backcrosses of the 2C TCR transgenes from the B6 background (B2C<sup>b</sup>) to the DBA/2 background (D2C).
Figure 18. Adoptive Transfer of Disease with Bone Marrow

(A) Bone marrow from pre-diseased D2C animals was adoptively transferred to lethally irradiated, non-transgenic B6<sup>d</sup> recipients while bone marrow from B2C<sup>d</sup> animals was adoptively transferred to lethally irradiated non-transgenic DBA/2 recipients. Representative gross and microscopic anatomy of the rostral skin is shown. Data is representative of 20 animals analyzed per group. (B) Graphical demonstration of disease development in the resultant bone marrow chimeras (BMC).
recipient's hematopoietic systems were in fact donor-derived, (B6\textsuperscript{b}\times DBA/2)N\textsubscript{1}\textsuperscript{b/d} mice were also used as recipients. Upon reconstitution, (B6\textsuperscript{b}\times DBA/2)N\textsubscript{1}\textsuperscript{b/d} recipients circulating peripheral blood lymphocytes fail to stain with the anti-H-2\textsuperscript{b} mAb HB51 indicating that the recipient hematopoietic system had been replaced by the donor graft [123]. These results indicated that the DBA/2 defect(s) could be non-hematopoietic and that disease might be transferred to non-transgenic DBA/2 recipients with marrow from disease-resistant B2C\textsuperscript{d} mice. Strikingly 100% of the DBA/2 recipients of B2C\textsuperscript{d} bone marrow developed the disease phenotype with gross and microscopic features indistinguishable to those of D2C mice (Fig. 18A, B) [123]. However, the penetrance and severity of disease in these bone marrow chimeras (BMC) was slightly less (S0 = 0%; S1 = 40%; S2 = 35%; S3 = 25%) than in unmanipulated D2C mice, indicating that some DBA/2 hematopoietic factors may have a role in pathogenesis [123]. However, the increased age of the bone marrow recipients may have also modulated the degree of disease severity since the age of disease development in these animals was greater than the age that natural disease occurs in "wild-type" D2C mice.

3.4.3 DBA/2 Complement Factor 5 Defects and Disease

The striking resemblance between D2C and human SD pathology [123] and the known role of complement deficiency in some forms of SD suggested that the natural deficiency in the fifth component of complement (C5) in DBA/2 mice [243], which is known to predispose to fungal infections [246, 247], might play a role in D2C disease pathogenesis. The mitigated phenotype observed in C5-sufficient BALB/c 2C (H-2\textsuperscript{d}) mice [123] provided further support for this hypothesis. To test this possibility, a PCR-RFLP based assay was used to determine if the segregation pattern of the deficient DBA/2 C5 allele was similar to the pattern of disease inheritance in (B6xDBA/2)N\textsubscript{2}2C backcrosses (Fig. 19A). This analysis revealed that C5-
Figure 19. Effect of the DBA/2 C5 Mutation on the Disease Phenotype in N₂2C backcrosses to the DBA/2 background

(A) A 328-330 bp sequence of the C5 (5th component of complement) gene encompassing a polymorphic HIND III restriction site was amplified from B6 (C5⁺/⁺), (B6xDBA/2)N₁ (C5⁺/⁻), and DBA/2 mice (C5⁻/⁻) and subsequently digested with HIND III. C5⁺/⁺ mice have a 211 and a 119 bp band while C5⁻/⁻ mice have a single 328 bp band. C5⁺/⁻ mice possess all three bands. (B) N₂2C backcrosses to the DBA/2 background were genotyped by the above mentioned approach for C5 sufficiency. These results were compared to the extent of clinical disease in these animals to ascertain whether C5 deficiency was associated with a worse disease phenotype in N₂2C backcrosses.
deficient (C5−/−) N2C animals had a slightly worse phenotype relative to C5-sufficient animals, suggesting that C5 deficiency may modulate disease expression (Fig. 19B) [123]. However, several C5 sufficient N2C mice developed advanced pathology and many N2C mice homozygous for the defective DBA/2 copy of the C5 gene were completely asymptomatic (Fig. 19B), demonstrating that this defect is neither necessary nor sufficient for disease [123].

3.5 Conclusion

The histological and gross pathological findings in D2C mice are strikingly similar to those observed in human seborrheic dermatitis but not human psoriasis [123, 165]. Although the initial work on this model suggested that one, or a closely linked group of non-hematopoietic genes, may control disease penetrance, it became clear with further experimentation that multiple genetic and environmental influences probably play a role in disease pathogenesis. Similarly, while the bone marrow transfer experiments isolated the suspected DBA/2 defect to the non-hematopoietic compartment, the reduced severity of disease seen in B2Cd → DBA/2 BMC may indicate that some of the DBA/2 factors affecting disease penetrance may in fact be hematopoietic.

Multiple candidate gene loci were investigated in an attempt to understand the pathophysiology of disease. The finding that the known DBA/2 deficiency in C5 was a factor contributing to disease pathogenesis was not unexpected as defects in human C5 are associated with a severe inflammatory form of SD termed Leiner’s disease [210, 211]. Furthermore, studies on lesional skin from SD patients have demonstrated the deposition of complement around Malassezia [219]. Complement is known to be important in the defense against fungi as both the classical and alternate pathways are activated to opsonize fungal pathogens [1, 266]. Serum antibody and mannan-binding lectin, which is directed against fungal cell wall determinants, can
both initiate the classical complement cascade [1, 266]. The absence of sialic acid on fungal cell wall zymosan also promotes the precipitation of spontaneously formed C3b which initiates the alternate complement cascade [1, 267-272]. These pathways of complement activation converge at the production of C3b, which promotes opsonization and serves as a nidus for further complement activation, namely formation of the C5 convertase complex [1]. These pathways also result in the generation of the anaphylatoxins C3a and C5a [1] and it has been shown that fungi in the stratum corneum increase vascular permeability, as well as promote the trans-epidermal chemotaxis, adherence and degranulation of leukocytes by generating C5a [266, 273]. The activation of complement also results in the generation of the MAC which forms transmembrane channels on the target membrane, resulting in target cell lysis [1]. Not surprisingly, deficiencies in complement predispose towards fungal infections, explaining the infectious susceptibility of C5-deficient DBA/2 mice to fungal pathogens [246, 247]. While the DBA/2 defect in complement was found only to modulate disease severity, it is likely that this defect contributed to the overgrowth of cutaneous fungal pathogens which is a component of disease pathophysiology.

Several other candidate genes were also explored but were not found to predispose to disease. For example, comparison of the pattern of disease penetrance in 2C-expressing BXD\textsuperscript{d} RI strains with the approximately 1,650 chromosomal markers that are fully genotyped between the BXD strains [274, 275] revealed a candidate locus controlling disease penetrance at position 16.4 cM of chromosome 17. Situated within this chromosomal region is the gene for the anti-apoptotic serine threonine kinase, Pim-1 [275, 276]. Interestingly, the phenotype of Pim-1 KO mice [277] is similar to the known phenotypic abnormalities of the DBA/2 inbred strain [278], including microcytic anemia and no grossly apparent immune defects. Moreover, restriction-fragment length polymorphisms exist between Pim-1\textsuperscript{d} B6 mice and Pim-1\textsuperscript{b} BALB/c and DBA/2
mice [276], and DBA/2 CD8 cells were found to have a severe impairment of cell survival relative to B6 cells following TCR stimulation (data not shown). However, no difference in the level of the Pim-1 protein or the downstream anti-apoptotic molecule bcl2 was identified between B6 and DBA/2 CD8 cells in this assay (data not shown), thereby casting doubt upon the possible involvement of this factor in disease pathogenesis.

Another candidate locus that was investigated for possible involvement in the D2C model is the known defects within portions of the DBA/2 "NK complex" on murine chromosome 6 [279, 280] which encodes numerous receptors involved in "tuning" the activation threshold of NK cells and T lymphocytes [281]. Given Delaney's observation that in certain BXD 2C mice the self-reactive, 2C TCR-expressing DNTC were acutely activated [164], it was explored whether defects in these NK receptors resulted in the reactivation of these anergized cells thereby contributing to disease pathogenesis. 2C TCR+ cells in cognate Ag-expressing backgrounds upregulate inhibitory receptors (data not shown), presumably to adjust their activation status following chronic exposure to self-antigen, and therefore deficiencies of these inhibitory receptors might predispose to T cell mediated autoimmune disease. Nevertheless, the penetrance of disease in (B6xDBA/2)N22C backcrosses to the DBA/2 background did not correlate with the loss of expression of these receptors as assayed by flow cytometry using the α-NK1.1 mAb PK136, demonstrating that the deletion of the NK complex was neither necessary nor sufficient for the full disease phenotype (data not shown).

The data presented herein clearly demonstrates that opportunistic pathogens play a significant role in propagating disease pathogenesis and that the deficiency in C5 (Fig. 19) and other unknown genetic factors, which likely have a role in microbial defence, increases the susceptibility of D2C mice to disease. These findings are consistent with the observation that less sanitary conditions are correlated with more severe disease in these animals [123]. While the
data thus far suggests that the involvement of opportunistic pathogens in disease pathogenesis occurs after the disruption of the cutaneous barrier, the development of massive titers of α-fungal Ab preceding disease convalescence (Fig. 13) may indicate that the development of protective humoral immunity may contribute to disease remission. It is known that the inflammatory SD occurring in the context of Leiner’s disease happens in the 2nd to 4th month of life [210, 211], during the physiological window of hypogammaglobulinemia when maternal IgG levels drop below a critical protective level [282, 283]. Remission in these children, which occurs as a result of maturation of humoral immunity [210], is analogous to the development of pathogen specific humoral immunity in D2C mice (Fig. 13). Moreover cell-mediated immunity has a known role in the defense against superficial fungal infections [284, 285] and the high incidence of SD in HIV patients [169, 170, 206] suggests that T cell adaptive immunity maintains opportunistic pathogens in a commensal state. Although the frequency of opportunistic pathogen-specific T cells has not yet been determined in D2C mice, the finding of massive numbers of CD4 T cells in the dermis of lesional skin (Fig. 9) suggests that cell-mediated immunity against opportunistic pathogens is important in this model system. It was therefore hypothesized that pre-diseased D2C mice possess deficits in immune function that, together with environmental and additional genetic factors, predispose to opportunistic infection and the D2C phenotype. Furthermore, the onset of convalescence in these animals may be associated with the maturation of immune function or the induction of compensatory immune mechanisms. These possibilities provided the impetus to study whether disease pathophysiology of the D2C mouse involved profound immunodeficiency including immunocompromising effects of TCR transgenesis.
Chapter 4: Immunosuppression and Immune Repertoire Perturbation

4.1 Introduction

Based on the established role of infection in D2C pathophysiology, it was hypothesized that immunocompromise induced by the strongly negatively-selecting 2C system synergized with DBA/2 genetic defects and environmental conditions to cause the D2C phenotype. To first address this hypothesis, we gauged the extent of immunocompromise in pre-diseased D2C mice by quantifying T cell subsets and by vaccinating with a T cell-dependent antigen. These results demonstrated that D2C mice are remarkably immunocompromised with a severe lymphopenia and major repertoire skewing of T cells [123]. A profound humoral immunocompromise was found to coexist with dysregulated B cell functioning in these mice [123], despite a quantitatively normal B cell compartment [164]. These immunological impairments are similar to changes seen in human AIDS and, interestingly, D2C mice were found to possess additional pathological changes [123] frequently observed in AIDS such as lymphoid organomegaly, gastrointestinal dysfunction, and hypergammaglobulinemia [286, 287]. Serial T cell immunophenotyping of D2C mice revealed that active disease is associated with a massive expansion of CD4 T cells and disease convalescence was found to spontaneously occur when the concentration of CD4 T cells reached a critical threshold level [123]. This threshold level of circulating CD4 T cells is remarkably similar to the CD4 count at which AIDS patients become susceptible to SD [208]. It was therefore hypothesized that restoration of the CD4 compartment would protect D2C mice from disease. Consistent with this belief, D2C recipients of syngeneic DBA/2 CD4+ T cells were found to be resistant to development of disease and fully capable of mounting a humoral immune response [123]. To see if the provision of T cell help to make protective antibody was the mechanism of disease protection, pre-diseased D2C mice were passively immunized with serum from severely diseased D2C animals that are known to contain
high titers of opportunistic pathogen-specific antibodies. However, this treatment was found to provide only marginal protection, and therefore it was hypothesized that the mechanism by which transferred CD4$^+$ cells protected against disease was the restoration of T cell immunoregulation by the provision of CD4$^+$ CD25$^+$ T$_{reg}$ cells. This hypothesis was substantiated by fractionated CD4$^+$ T cell transfer experiments where purified T$_{reg}$ were found to abrogate the disease phenotype without restoring humoral immune function. Whereas, while purified CD4$^+$CD25$^-$ cells were able to correct humoral dysfunction, these cells actually exacerbated the disease phenotype. The obvious similarities between the D2C model and FoxP3-deficient mice warranted a further comparison of these model systems, which revealed that the cutaneous pathology in FoxP3-deficient mice was psoriasiform in nature and that D2C mice shared additional phenotypic changes with FoxP3-deficient mice, including the presence of circulating auto-antibodies and glomerulonephritis.

Given the important role of dysregulated CD4$^+$CD25$^-$ T cell expansion resulting from a lymphopenia of T$_{reg}$ in disease pathogenesis, it was theorized that the treatment with corticosteroids would abrogate disease due to the effect of these drugs on inhibiting CD4$^+$CD25$^-$ T cell signalling and cytokine production [288, 289] while augmenting T$_{reg}$ functioning [289]. Not surprisingly, it was found that treatment with the corticosteroid dexamethasone (Dex) had essentially the same effect as the transfer of purified T$_{reg}$, further supporting the hypothesis that the lymphopenia of this specialized T cell population has a paramount role in D2C disease pathogenesis.
4.2 Characterization of Immune Function in D2C Mice

4.2.1 Quantification of Thymocytes and T Cell Subsets In D2C Mice

The expression of the 2C TCR in the H-2D^d-expressing DBA/2 background induces a massive central deletion that results in a 10-fold reduction in the total number of thymocytes (3.8x10^6 vs. 3.8x10^7, p < .05) and a 500-fold reduction in DP thymocytes (6.1x10^4 vs. 3.2x10^7, p < .05) compared to DBA/2 controls (Fig. 20A) [123]. The D2C thymus was also characterized by a marked reduction of CD8^+ and CD4^+ SP cells relative to non-transgenic controls (Fig. 20A) [123]. A peripheral T cell lymphopenia in D2C mice reflects the negatively selecting thymic environment with a 5-30-fold reduction in total lymphoid CD4^+ T cells accompanying a 10-20-fold reduction in total lymphoid CD8^+ T cells (Fig. 20B) [123]. These peripheral T cell anomalies seem to reflect a reduced thymic output of mature T cells as this lymphopenia is most pronounced in younger mice [123].

To see if the forced expression of the 2C TCR transgenes affected the T cell diversity in these animals, D2C and age-matched DBA/2 mice were assayed for TCR β chain usage. Although all developing T lymphocytes are forced to express the α_{TG} and β_{TG} chains in TCR Tg mice, recombination of endogenous TCR chain genes results in the development of non-Tg T cells. DP thymocytes expressing the Tg TCR may give rise to non-Tg T cells either by the rearrangement of endogenous TCR α chain genes, forming an endogenous TCR α chain that pairs with the β_{TG} chain [82], or following the deletion of both the α_{TG} and β_{TG} TCR transgenes and the subsequent rearrangement of endogenous TCR chain genes [111]. The small population of DP thymocytes in which this later phenomenon occurs is unrestricted in its capacity to use TCR α and β chain gene segments. Therefore CD4^+ cells in 2C mice would be predicted to be both positive and negative for the 2C TCR Vβ chain (Vβ8.2). Not surprisingly, significant numbers of CD4^+ cells utilizing the Vβ2, Vβ4, Vβ8.2, Vβ8.3, Vβ10.5, Vβ13, and Vβ14 TCRβ
Figure 20. Evaluation for Lymphopenia and TCR Repertoire Skewing in D2C Mice

(A, B) Thymi and lymph nodes were collected from the indicated animals. Single cell preps, made from the harvested organs, were stained as shown and analyzed by flow cytometry. Dot plots are gated on live cells. (C) The spleen was collected from 10-day old D2C and DBA/2 mice and evaluated for Vβ TCR chain expression by CD4\(^+\) T cells using FACS\(^\text{®}\). The percentage of the total CD4\(^+\) T cell count expressing each of the seven Vβ TCR chains utilized by DBA/2 mice is shown.
chains, which are not clonally eliminated due to the proviral inserts of mouse mammary tumor virus (Mtv) strains in the DBA/2 genome [290, 291], were identified in DBA/2 mice of all ages, including 10-day old pups (Fig. 20C). However, the Vβ analysis of CD4+ cells from 10-day old D2C mice detected the expression of few Vβ chains with 88% ± 13% of cells found to be Vβ8.2+, indicating significant repertoire skewing (Fig. 20C). These data clearly demonstrate that T cell lymphopenia and significant TCR repertoire skewing occur as a result of TCR transgenesis in D2C mice. To see if these effects had important consequences, the physiological significance of these changes became the next focus of this work.

4.2.2 Assaying T Cell-Dependent Humoral Immune Function

The colonization of D2C mice by an increased density of opportunistic pathogens (Fig. 10) suggested that the aforementioned T cell lymphopenia and repertoire skewing had important effects on immune function. As B cell development in D2C mice is not obviously perturbed [164], immunization with T cell-dependent Ag assays for the frequency of Ag-specific T lymphocytes. To address whether the T cell lymphopenia and repertoire skewing observed in D2C mice resulted in a physiologically important impairment in immune function, the T cell-dependent Ag hen egg lysozyme (HEL) was used to immunize 40-day old D2C and syngeneic, age-matched DBA/2 littermates. Following immunization, DBA/2 but not D2C mice mounted a strong humoral response (Fig. 21) [123]. These data, together with the known role of infection in D2C disease pathogenesis, suggested that a compromised ability to make protective antiserum predisposed to infection.
Figure 21. Assessment of T Cell-Dependent Humoral Immune Function in D2C Mice

Forty-day old, diseased D2C and age-matched DBA/2 control mice were immunized with the T cell-dependent Ag hen egg lysozyme (HEL). Ten days post-immunization, tail vein blood was collected and assayed for HEL-specific IgG by ELISA.
4.2.3 Characterization of Additional Immunopathological Features Of D2C Mice

Given the immunodeficiency of D2C mice demonstrated by the HEL immunization studies, additional evidence of immunodeficiency was sought in these animals. These studies revealed that D2C mice possessed a number of pathological changes similar to those observed in human AIDS, intimating that profound immunocompromise may exist in D2C mice. For example, D2C mice develop variable degrees of lymphoid organomegaly which is positively correlated with the extent of cutaneous disease (Fig. 22A, B) [123]. Relative to control animals (Fig. 22C), D2C splenic parenchyma is characterized by a disorganized pattern of follicular hyperplasia and an increased number of multinucleated giant cells and tingible body macrophages, forming a prominent “starry sky” pattern (Fig. 22D). Although D2C lymphadenopathy occurs in a generalized distribution, enlargement of the cervical and upper extremity nodes which drain diseased skin tends to be most pronounced. Such enlargement typically occurs secondary to an expansion of the medullary cords, consisting primarily of lymphocytes with plasmacytoid morphology (Fig. 22E-G), or an infiltration of epitheloid histiocytes, and Langhans giant cells which efface the normal lymphoid architecture (Fig. 22E, H-J). D2C mice also develop intermittent diarrhea (Fig. 22K) associated with a non-specific pattern of intestinal inflammation, characterized by increased numbers of lymphocytes in the lamina propria and prominent lymphoid aggregates (Fig. 22L). The presence of massive hypergammaglobulinemia (Fig. 22M) in D2C mice indicates that B-cell hyperactivity is also a feature of disease [123]. The coexistence of this B-cell hyperactivity with humoral immune dysfunction most likely indicates a dysregulation of B cells. This constellation of pathological findings in D2C mice, including susceptibility to the development of psoriasiform skin disease, is similar to the pathology of human AIDS [169, 170, 208, 286, 287]. Given the similarities between D2C pathology and human AIDS, further experiments were directed towards examining
Figure 22. Necropsy Observations made on D2C Mice

A

D2C

B

D2C

C

f

D

DBA/2

E

mc

his

F

mc

G

mc

H

his

I

LGC

J

ehis

K

L

la

c

M

Serum IgG (mg/ml)

DBA/2  S3  D2C

104
Figure 22. Necropsy Observations made on D2C Mice

(A) Lymph nodes and (B) spleens were harvested from the indicated animals. Representative photographs are shown. (C) Control DBA/2 spleen with normal lymphoid follicles (f). (D) D2C splenic parenchyma is characterized by a disorganized pattern of follicular hyperplasia. (E, F) D2C LN enlargement occurs secondary to expansion of the medullary cords (mc) which consist primarily of lymphocytes with plasmacytoid morphology (G). (E, H, I, J) Enlargement of the LN can also be secondary to a diffuse infiltration of histocytes (his) which may take the form of Langhans giant cells (LGC) or diffuse sheets of epitheloid histocytes (ehis). (K) Photograph of loose stool which occurs intermittently in diseased D2C mice. (L) The histopathology of D2C intestine is characterized by a pronounced increase in the cellularity (c) of the lamina propria, and an increased number of interepithelial lymphocytes. Prominent lymphoid aggregates (la) are also a commonly observed feature of the D2C small intestine. (M) Serum from the indicated animals was collected at 40-days of age for the determination of serum IgG concentration by ELISA (n=10 animals per group).
the CD4 T cell compartment in D2C mice as a disruption of these cells is a critical feature of HIV infection.

4.2.4 Further Characterization of the CD4⁺ T Cell Subset

4.2.4.1 Longitudinal CD4⁺ T Cell Quantification

The significant lymphopenia of CD4⁺ cells in D2C mice, accompanied by the development of inflammatory SD-like disease, is seemingly analogous to the occurrence of SD in AIDS when the CD4⁺ T cell count falls below 400-500 cells/mm³ [208]. To see if a similar relationship between the CD4⁺ T cell level and susceptibility to disease existed in D2C mice, peripheral blood from age-matched D2C and DBA/2 mice was obtained for CD4⁺ quantification. At 20 days of age, shortly before the onset of pubescence [245], when animals become susceptible to disease, D2C mice had nearly 30-fold lower levels of peripheral blood CD4⁺ T cells than age-matched controls (66 ± 35 vs. 1724 ± 443 cells/mm³; Fig. 23A) [123], and were found to have TCR repertoire skewing (data not shown) similar to that observed in 10-day old D2C mice (Fig. 20C). Interestingly, examination of older D2C mice revealed variable increases in the number of circulating CD4⁺ cells and it was hypothesized that spontaneous disease convalescence in D2C mice might occur as a result of the acquisition of a protective number of CD4⁺ T cells. Consistent with this possibility, serial CD4⁺ cell quantification in D2C and age-matched DBA/2 mice revealed that over the window of disease susceptibility, between 20- and 75-days of age, peripheral blood CD4⁺ T cells in D2C mice had expanded over 400% (Fig. 23B) while the CD4⁺ T cells from DBA/2 control animals had expanded a meager 28% over this same time period (347 ± 71 vs. 2205 ± 568 cells/mm³, respectively) [123]. Complete disease remission occurred when CD4⁺ T cells had accumulated to between 400-1000 cells/mm³ which occurred shortly after the onset of convalescence (Fig. 23C).
Figure 23. Characterization of the CD4+ T Cell Number and TCR Chain Usage over the Window of Disease Susceptibility

(A) Tail vein blood was collected from the indicated 20-day old mice. The CD4 cell count was determined by performing a complete blood count using a hemocytometer and by immunophenotyping peripheral blood leukocytes by FACS®. (B) Tail vein blood was collected from the indicated mice at 20- and 75-days of age. The CD4 count was performed as previously described. The percent change in the concentration of circulating CD4 cells is plotted. (C) CD4 counts on convalescent D2C and age-matched DBA/2 mice were obtained as previously described. (D) LN were harvested from 50-day old, S3 D2C mice and age-matched DBA/2 controls who were administered a 10-day pulse of the nucleoside analogue BrdU. Cells were analyzed by flow cytometry for the incorporation of BrdU. Histogram plots are gated on live CD4+ T cells. (E) LN were collected from 80-day old D2C and DBA/2 mice and evaluated for Vβ TCR chain expression of CD4+ T cells by FACS®. The percentage of the total CD4+ T cell count expressing each of the seven Vβ TCR chains is shown. (F) Spleen (10-day old mice) and LN (80-day old mice) cells from DBA/2 and D2C mice were stained with an α-CD4 mAb and a mAb recognizing the representative TCR Vβ chain Vβ 14. Dot plots are gated on live cells.
To better demonstrate the extent of CD4$^+$ T cell peripheral expansion in D2C mice, a 10-day course of BrdU was administered to 40-day old D2C mice and age-matched DBA/2 controls. Consistent with previous studies [292], this short administration of BrdU labeled negligible numbers of peripheral CD4$^+$ T cells from non-transgenic mice while 30% of the CD4$^+$ T cells from D2C mice incorporated this marker, indicating that a massive peripheral T cell expansion was occurring (Fig. 23D) [123].

To see if this expansion had an effect on the CD4$^+$ T cell TCR repertoire skewing observed in young D2C mice, LN cells from 80-day old D2C mice and age-matched DBA/2 controls were obtained to examine CD4$^+$ V$\beta$ TCR chain usage. In these convalescent D2C animals, a more balanced expression pattern of V$\beta$ chains was observed with no statistically significant difference in the percentage of total CD4 cells expressing any of the non-V$\beta$8.2 TCR V$\beta$ chains (Fig. 23E). Nevertheless, the percentage of CD4 cells expressing the TCR Tg V$\beta$8.2 chain was still significantly greater in convalescent D2C mice relative to age-matched DBA/2 controls (Fig. 23E), perhaps reflecting the ongoing thymic emigration of primarily V$\beta$8.2$^+$ CD4 cells. This relative reduction in V$\beta$8.2$^+$ CD4 cells with age likely reflects the peripheral expansion of CD4$^+$ cells which had deleted both TCR Tg during development since the thymic contribution of CD4 cells to the periphery consists of almost entirely V$\beta$8.2$^+$ CD4 cells (Fig. 20C). Nevertheless, these data show that the profound CD4 lymphopenia and highly skewed TCR repertoire normalizes over time and that this change is associated with the induction of disease convalescence in D2C mice.

4.2.4.2 Immunophenotype of Expanding CD4$^+$ T Cells from D2C Mice

It has been shown that in the absence of thymic output, mature T cells can divide spontaneously in the periphery of lymphopenic hosts; a phenomenon termed homeostatic
proliferation [293]. Cells undergoing homeostatic proliferation are known to masquerade as memory T cells expressing markers typically associated with Ag experience; however, such cells are actually in a state of partial activation possessing an immunophenotype distinct from naïve, activated, and memory T cells characterized by high levels of the memory markers CD44 and CD122 without down-regulation of CD62L or the upregulation of the acute activation marker CD69 [294].

To address the possibility that the massive CD4+ expansion in D2C mice was occurring as a result of homeostatic expansion, LN cells from 50-day old DBA/2 and D2C mice were assayed by flow cytometry for the expression of CD44, CD62L and CD69. As anticipated, the vast majority of CD4+ T cells from DBA/2 mice expressed an immunophenotype typical of naïve cells being CD44lo, CD69-, and CD62Lhi. In S3 D2C mice, the majority of CD4+ T cells were CD44hi, CD69+, and CD62Llo (Fig. 24A) indicating that the proliferation of these cells was Ag-driven rather than from homeostatic expansion to fill a lymphopenic environment [123]. Presumably this Ag-driven expansion was driven in part by opportunistic pathogens and that the acquisition of adequate numbers of Ag specific T cells is an important milestone preceding disease convalescence. Consistent with this interpretation, examination of CD4+ T cells from recovered D2C animals (Fig. 24B) demonstrated that these cells possessed an Ag-experienced but non-acutely activated immunophenotype (CD44high, CD62Llo, CD69-). These data suggest that the CD4 population was no longer undergoing Ag-driven expansion, presumably due to the acquisition of a protective frequency of Ag-specific cells. The finding that these convalescent mice, whose cutaneous and other immunopathological features of disease were in remission (Fig. 25A-C), still failed to respond to HEL immunization (Fig. 25D) indicated that despite the significant reconstitution of CD4 T cell numbers (Fig. 23C), and the apparent normalization of TCR diversity (Fig. 23E), these animals had persistent severe immunodeficiency with numerous
Figure 24. Immunophenotype of D2C CD4$^+$ T Cells

(A) LN were harvested from 50-day old, S3 D2C mice and age-matched DBA/2 controls. Single cell preps were stained with the designated markers and analyzed by flow cytometry. Histogram plots are gated on live CD4$^+$ T cells. (B) LN were harvested from 80-day old recovered D2C mice and age-matched DBA/2 controls. Single cell preps were stained with the designated markers and analyzed by flow cytometry. Histogram plots are gated on live CD4$^+$ T cells.
(A) Gross photograph from a recovered D2C mouse and representative histology from previously lesional rostral skin are shown. (B) Representative photographs of recovered D2C and age-matched DBA/2 lymphoid organs. (C) Total serum IgG is shown for DBA/2 and recovered D2C mice (the difference in serum IgG concentration between these groups was not found to be statistically significant). (D) Recovered D2C and age-matched DBA/2 mice were immunized with HEL and assayed for HEL-specific IgG as previously described.
holes in their immunological repertoire. This finding argued against a slow but diverse thymic reconstitution of the periphery, resulting in an adequate frequency of all T cell clones, including those recognizing unusual foreign Ag such as HEL. Rather this data strongly supported the conclusion that the CD4$^+$ T cell accumulation in D2C mice occurred as a result of the oligoclonal expansion of Ag-specific T cells against opportunistic pathogens. Since these mice are not likely exposed to avian lysozyme (HEL) before immunization, it is doubtful that the exceedingly infrequent population of HEL-specific T cells would have participated in the CD4$^+$ T cell expansion.

These data strengthen the parallel between disease in D2C mice and AIDS-related SD where an inadequate CD4$^+$ T cell concentration renders HIV patients susceptible to opportunistic infections, and treatment with CD4$^+$ cell-boosting HAART therapy cures them of opportunistic infections [295]. Based upon these similarities, it was hypothesized that the provision of an adequate population of CD4$^+$ T cells would protect D2C mice from disease.

4.3 Immunological Reconstitution of D2C Mice

4.3.1 Adoptive Transfer of Syngeneic CD4$^+$ T Cells to Pre-Diseased D2C Mice

To determine if the observed expansion of CD4$^+$ T cells was responsible for D2C disease convalescence, syngeneic DBA/2 CD4$^+$ T cells were adoptively transferred to pre-diseased D2C mice in attempt to abrogate disease development. CD4$^+$ T cell recipient mice were completely resistant to the development of disease (100% SO, n = 12) while D2C recipients of PBS developed typical pathological changes (Fig. 26A, B) [123]. Sections taken from the skin of CD4$^+$T cell recipients were devoid of any microscopic evidence of disease and were indistinguishable from DBA/2 skin sections (Fig. 26C) [123]. Other phenotypic abnormalities such as the development of lymphadenopathy and splenomegaly were also ameliorated by this
Figure 26. Adoptive Transfer of CD4+ T Cell to Pre-Diseased D2C Mice

(A) 25-day old, pre-diseased D2C mice received 2x10^7 syngeneic DBA/2 CD4+ T cells or PBS. Representative photographs are shown. (B) The effectiveness of the cell transfer was quantitated by observing for and grading resultant disease. (C) Rostral skin sections from CD4+ T cell and PBS-treated D2C mice are shown.
cell transfer (Fig. 27A) [123]. Although the transfer reconstituted D2C recipients with a functional humoral immune system enabling these animals to respond to immunization with HEL (Fig. 27B), this maneuver only partially corrected the hypergammaglobulinemia typical of D2C mice (Fig. 27C) [123]. While this reduction in serum IgG was found to be statistically significant (p < 0.05) relative to PBS-treated D2C animals, the concentration of serum IgG in D2C recipients of CD4\(^+\) T cells was markedly higher than in age-matched DBA/2 controls [123]. The complete abrogation of this hypergammaglobulinemia defect may have necessitated an earlier transfer of CD4\(^+\) cells because B cell dysregulation in these animals likely begins at an earlier age while the transfer of the CD4\(^+\) T cells occurred at 25-days of life [123]. These data confirm the hypothesized role of CD4 lymphopenia in the development of D2C psoriasiform pathology and lend further support for the proposed parallels drawn between D2C mice and AIDS related SD. Given the results of earlier experimentation, we hypothesized that the mechanism by which the restored CD4 T cell compartment protected against disease was by reconstituting cell-mediated immunity (CMI) and the provision of T cell help for the generation of protective antiserum.

4.3.2 Passive Immunization of Pre-Diseased D2C Mice with Opportunistic Pathogen-Specific Serum IgG

Given the high titer of pathogen-specific IgG in S3 D2C mice immediately preceding disease convalescence (Fig. 13), a possible mechanism by which D2C mice become resistant to disease is the generation of pathogen-specific, protective humoral immunity. This protective antiserum likely reduces the antigenic burden of opportunistic pathogens on the skin, thereby reducing the concentration of microbial Ag to which opportunistic pathogen-specific T cells can respond. The findings that convalescent D2C mice have less PAS positive material on the skin
Figure 27. Adoptive Transfer of CD4+ T Cells to Pre-Diseased D2C Mice (Continued)

(A) Representative pictures of lymphoid organs harvested from D2C recipients of syngeneic CD4 T cells and PBS. (B) D2C recipients of syngeneic CD4 T cells and PBS, together with DBA/2 control animals, were immunized with the T cell-dependent Ag HEL. Ten days post-immunization, serum was collected and assayed for the presence of anti-HEL specific IgG by ELISA. (C) Serum was collected from DBA/2 controls as well as D2C recipients of syngeneic CD4 T cells and PBS. The concentration of serum IgG was determined by ELISA.
(data not shown) and that the CD4⁺ cells in these animals are no longer acutely activated (Fig. 24B) are consistent with this interpretation. As D2C recipients of syngeneic CD4⁺ cells were fully capable of responding to a HEL immunization (Fig. 27B) [123], these animals would be expected to efficiently mount pathogen specific protective antiserum in response to microbial invasion, explaining their resistance to disease.

To test this hypothesis, serum from S3 D2C mice containing high titers of pathogen-specific IgG, was administered to pre-diseased D2C mice. Interestingly, despite the enormous amount of serum IgG transferred, this supraphysiologic level of IgG (up to 4 mg IgG/week) resulted in only a moderate mitigation of the disease phenotype (Fig. 28). The failure of passive immunization to provide a level of protection equal to that of the syngeneic CD4⁺ T cell transfer could be explained by the inability to "cover" microbial agents to which the donor animals had not been exposed. However, this possibility is not likely since the donor serum was pooled over many months from dozens of diseased animals, all of which were housed in conditions identical to that of the recipient mice. Instead, these data suggest that the protective effect of the syngeneic cell transfer may have resulted from the provision of critical CD4⁺ regulatory cells.

4.3.3 Analysis of T_{reg} Development and Function in D2C Mice

An absence of T_{reg} in FoxP3 KO mice results in massive CD4⁺ T cell expansion, as well as severe skin disease [296, 297]. Furthermore the transfer of purified CD4⁺CD45RB^{hi} cells to severe combined immunodeficient (SCID) mice, a technique to create severe T_{reg} lymphopenia in recipient animals, results in psoriasiform skin disease [192]. These data suggest that T_{reg} lymphopenia or dysfunction could predispose to psoriasiform disease and, based upon the low thymic output of mature CD4 cells from the D2C thymus, it was anticipated that T_{reg} development would be proportionally reduced in these animals. However, it was also plausible
Adoptive Transfer of Serum from S3 D2C Mice to Pre-Diseased D2C Animals

(A) Serum from S3 D2C mice, shown to have high titers of opportunistic pathogen-specific IgG, was pooled and injected into pre-diseased D2C mice. Twice-weekly, mice received 0.5-1.0 ml of diluted serum containing 2 mg/ml of IgG. Age-matched control D2C animals received twice-weekly injections of the T3.70 control mAb. Representative photographs are shown. (B) The extent of clinical disease was monitored over the three week study period and the summarized results are shown. (C) Representative histological sections are shown of rostral skin harvested from serum-treated mice and T3.70-treated control mice.
that D2C mice were totally deficient in T\textsubscript{reg} cells as a result of TCR transgenesis interrupting T\textsubscript{reg} ontogeny. Examination of LN from young D2C mice revealed the presence of a normal frequency of typical CD4\textsuperscript{dim}CD25\textsuperscript{bright} T\textsubscript{reg} relative to DBA/2 mice but, given the severe lymphopenia of CD4\textsuperscript{+} T cells, the absolute number of T\textsubscript{reg} cells was extremely low (data not shown). However, when D2C T\textsubscript{reg} were purified and used in a well-described suppression assay [131], T\textsubscript{reg} from D2C mice performed identically to those from age-matched DBA/2 animals at suppressing the proliferation of conventional CD8 cells (Fig. 29A). These data argued against the abrogated development of T\textsubscript{reg} predisposing to disease; however, it was reasoned that a lymphopenia of T\textsubscript{reg} resulting from the slow thymic production of mature CD4\textsuperscript{+} cells, would be insufficient to control the massive peripheral expansion of conventional CD4\textsuperscript{+} "helper" T cells whose development was similarly hampered by severe thymic negative selection.

Examination of convalescent D2C mice for the presence of T\textsubscript{reg} revealed that relative to age-matched DBA/2 control mice, T\textsubscript{reg} constituted a higher proportion of total CD4\textsuperscript{+} cells (Fig. 29B), although the absolute number of T\textsubscript{reg} was still reduced (data not shown). Increased frequencies of T\textsubscript{reg} have been observed in a variety of inflammatory diseases including atopic dermatitis [298], where an expansion of these cells occurs presumably to dampen inflammatory processes. Therefore the relative increase of T\textsubscript{reg} in convalescent D2C mice may have helped to abrogate the further expansion of conventional CD4\textsuperscript{+} cells and thereby may have induced disease remission. It was therefore reasoned that the selective restoration of the T\textsubscript{reg} compartment by a fractionated CD4\textsuperscript{+} T cell transfer would be sufficient to protect pre-diseased D2C mice from the development of the D2C disease phenotype.
Figure 29. Evaluation of CD4⁺CD25⁺ T Regulatory Cells in D2C Mice

(A) Responder DBA/2 CD8⁺ T cells were stimulated for 72 hours with ConA in the presence of irradiated antigen presenting cells. Varying numbers of DBA/2 or D2C CD4⁺CD25⁺ T<sub>reg</sub> were also present at the indicated ratios. Cells were pulsed with tritiated thymidine for the final 8 hours of culture. (B) LN were collected from the indicated animals and prepared for FACS®. Cells were stained as shown (note: D2C LN cells were enriched for CD4⁺ T cells by first depleting B lymphocytes and macrophages). Dot plots are gated on live cells.
4.3.4 Adoptive Transfer of Purified, Syngeneic T\textsubscript{reg} and CD4\textsuperscript{+}CD25\textsuperscript{-} "Helper" T Cells to Pre-Diseased D2C Mice

The increased frequency of T\textsubscript{reg} in convalescent D2C mice suggested that these cells may have played an important role in inducing disease remission. Furthermore, this data was also consistent with the possibility that the effectiveness of adoptively transferred DBA/2 CD4\textsuperscript{+} cells at abrogating disease (Fig. 26, 27) was attributable to the restoration of the T\textsubscript{reg} population. Alternatively, the provision of a balanced repertoire of CD4\textsuperscript{+} cells by this CD4\textsuperscript{+} T cell transfer, and therefore the transfer of normal cellular immunity to pre-diseased D2C mice, was also a possible explanation for the disease resistance of these recipients. To dissect out which possible mechanism protected recipient D2C mice from disease, syngeneic DBA/2 CD4\textsuperscript{+} T cells were fractionated into CD4\textsuperscript{+}CD25\textsuperscript{-} “helper” T cells and CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{reg} (Fig. 30A) and transferred to separate groups of pre-diseased D2C mice (Fig. 30B, C). The recipients of purified CD4\textsuperscript{+}CD25\textsuperscript{-} cells received 1-2 x 10\textsuperscript{7} cells, approximately the same number of cells that were transferred in the unfractionated CD4\textsuperscript{+} transfer experiment (Fig. 26, 27). Despite having a balanced CD4\textsuperscript{+} repertoire with diverse V\textbeta chain usage, these CD4\textsuperscript{+}CD25\textsuperscript{-} cell recipients experienced an accentuation of the disease phenotype and an acceleration of disease pathogenesis relative to the control recipients of PBS (Fig. 30B, C). Additionally, these recipients were noted to have a more pronounced cutaneous CD4\textsuperscript{+} T cell infiltration and a greater degree of polyclonal gammopathy (Fig. 31A-C). Consistent with previous results which discounted the role of protective Ab as a prime mechanism of disease protection (Fig. 28), the restored ability of these CD4\textsuperscript{+}CD25\textsuperscript{-} cell recipients to mount a T cell dependent humoral immune response did not correlate with disease resistance (Fig. 31D). These data supported the hypothesis that the provision of T\textsubscript{reg} and not a large population of diverse “helper” cells was the mechanism by which unfractionated CD4\textsuperscript{+} cells protected from disease. Consistent with this suspicion, T\textsubscript{reg} recipients experienced a
Figure 30. Effect of the Adoptive Transfer of CD4\(^+\)CD25\(^+\) \(T_{\text{reg}}\) or CD4\(^+\)CD25\(^-\) "Helper" T Cells to Pre-Diseased D2C Mice

(A) Purified CD4\(^+\) T cells from DBA/2 mice were fractionated into CD4\(^+\)CD25\(^+\) T regulatory (T_{\text{reg}}) and CD4\(^+\)CD25\(^-\) "helper" T cell populations. (B) A graphical representation of disease activity in D2C recipients of PBS, CD4\(^+\)CD25\(^-\) T_{\text{reg}}, and CD4\(^+\)CD25\(^-\) "helper" T cells is shown. (C) Representative photographs of gross pathology are shown.
Figure 31. Effect of the Adoptive Transfer of CD4⁺CD25⁺ T_{reg} or CD4⁺CD25⁻ "Helper" T Cells to Pre-Diseased D2C Mice (Continued)

(A) Representative photomicrographs of H&E-stained sections of rostral skin from mice in the specified experimental groups are shown. (B) Acetone-fixed, frozen tissue sections from the rostral skin of mice in the indicated experimental groups were incubated with a rat α-CD4 mAb. HRP-labeled, goat α-rat IgG and the Vector® Nova Red HRP immunohistochemistry kit were used to develop the staining. Representative photomicrographs are shown. (C) The concentration of serum IgG in the indicated experimental groups was determined by ELISA. (D) PBS, CD4⁺CD25⁺ T_{reg}, and CD4⁺CD25⁻ "helper" T cell recipients were immunized with HEL and assayed for HEL-specific IgG by ELISA.
complete abrogation of the cutaneous disease phenotype (Fig. 30B, C), with normal cutaneous histological findings devoid of the prolific cutaneous CD4^+ cell infiltration characteristic of disease (Fig. 31A, B). T_{reg} recipients, which received the cell transfer earlier than the unfractionated CD4^+ recipients described previously (Fig. 26, 27), had levels of serum IgG equivalent to DBA/2 animals, indicating that the transfer of T_{reg} precluded the B cell dysregulation normally seen in D2C animals (Fig. 31C). While the provision of T_{reg} prevented polyclonal gammopathy, the recipients of these cells were completely unresponsive to HEL immunization (Fig. 31D), providing further evidence against a protective role of humoral immunity in this condition, and demonstrating unequivocally that the restoration of humoral responsiveness (Fig. 27B) was not the mechanism by which the unfractionated CD4^+ cell transfer protected against disease. These data suggested that the FoxP3-deficient and D2C model systems might share a related immunophysiology, which necessitated a closer comparison of these animals.

4.3.5 Comparison of D2C and FoxP3 Knockout Mice

With the knowledge that T_{reg} lymphopenia predisposes D2C mice to cutaneous disease, these animals were studied further to detect abnormalities known to occur in other T_{reg}-deficient model systems. A complete T_{reg} deficiency occurs in FoxP3 KO mice and these animals are known to develop scales and crusts on the eyelids, ears, and tail in association with a number of other inflammatory changes [299, 300]. Histopathological examination of the skin from FoxP3 KO mice demonstrated that the cutaneous pathology in these animals was clearly psoriasiform (Fig. 32) suggesting that T_{reg} deficiency may be a key factor in psoriasiform pathophysiology. In addition to cutaneous disease, FoxP3 KO mice are known to develop chronic gastrointestinal disease, lymphoid organomegaly, anemia, and marked polyclonal B cell activation resulting in
Figure 32. Scurfy Mouse Cutaneous Histology

Photomicrographs of representative H&E and PAS-stained histological sections from affected FoxP3 hemizygous male mice (Scurfy) and age- and sex-matched B6 control mice are shown. Note the punctate PAS-positivity (PAS+) underlying the perifollicular mounds (m) of parakeratotic debris.
Figure 33. Additional Immunopathological Features of D2C Mice

(A) Polylysine-treated, Immunon ELISA plates were coated with 50 µl of a 10 µg/ml solution of dsDNA. Various dilutions of serum from the indicated animals were incubated in the plate and bound IgG was detected with AP-conjugated, goat α-mouse IgG. (B) Acetone-fixed frozen sections of D2C and DBA/2 kidney were incubated with FITC-labeled, goat F(Ab’)_2 α-mouse Ig. Tissue sections were examined under a Zeiss fluorescence microscope. Representative glomeruli are shown.
hypergammaglobulinemia [299, 301], conditions from which D2C mice are also known to suffer (Fig. 22) [123]. Given that T\textsubscript{reg} have also been shown to protect against glomerulonephritis [302], it was tested whether the lymphopenia of these cells in D2C mice was associated with circulating immune complexes and glomerulonephritis. These studies revealed that D2C mice, but not age-matched DBA/2 control animals, had large titers of anti-dsDNA antibodies (Fig. 33A), and that D2C kidneys demonstrated membranous glomerular deposits which were not apparent in control sections (Fig. 33B). Therefore while differences between D2C and FoxP3 KO mice exist, such as the distribution and severity of cutaneous disease, many pathological changes are shared by these model systems, highlighting the critical role of CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{reg} cells in D2C disease pathophysiology.

### 4.4 Dexamethasone Treatment of D2C Mice

Corticosteroids are powerful immunosuppressant medications which dampen immunological functioning by multiple mechanisms which include inhibiting the release or synthesis of: cytokines; complement components; platelet activating factor; and eicosanoids, as well as directly antagonizing T cell signalling pathways [288, 303-305]. Interestingly, while corticosteroids have been shown to preclude the survival [306] and expansion of naive “helper” CD4 T cells by inhibiting TCR signal transduction [288], these agents do not antagonize the survival or functioning of T\textsubscript{reg} cells [289]. In fact the relative frequency of T\textsubscript{reg} is increased in steroid-treated animals due to the reduced susceptibility of T\textsubscript{reg} to glucocorticoid-induced apoptosis [289]. Therefore another mechanism by which corticosteroids dampen immunoresponsiveness is by increasing the relative frequency of T\textsubscript{reg}. It was therefore hypothesized that treatment with the corticosteroid dexamethasone (Dex) might have a favorable outcome similar to that observed in D2C recipients of purified T\textsubscript{reg}. To address this possibility,
this possibility, pre-diseased D2C mice were treated with a previously demonstrated effective dose of Dex [307]. Dex-treated animals were protected against the development of the disease phenotype while PBS-treated control D2C mice developed typical gross and histological manifestations of disease (Fig 34). This protection from disease was associated with a massive reduction in the total number of CD4$^+$ cells as well as considerable atrophy of the lymphoid organs in Dex-treated, but not PBS-treated, D2C mice (Fig 35A, B). Dex treatment had the added effect of precluding the polyclonal gammopathy and generation of auto-antibodies that are typical of D2C mice (Fig. 35C, D). These findings are consistent with the known efficacy of corticosteroids in the treatment of human psoriasiform disease [171, 204] and therefore it is tempting to speculate that the effectiveness of corticosteroids in the treatment of psoriasiform disease may in part be the result of shifting the balance in the ratio of T$_{reg}$ to "helper T cell" [289].

4.5 Conclusion

Current dogma states that the adaptive immune system maintains opportunistic fungal pathogens in a commensal state and that waning immunity predisposes to the induction of SD [169, 206]. This premise is supported by the known role of cell-mediated immunity in the defense against superficial fungal infections [284, 285] and that impairments in cell-mediated immunity, as occurs in HIV [169, 206, 208], predispose to the development of SD. However, most studies of cellular immunity in SD have either demonstrated an increased reactivity to Malassezia relative to control patients or no difference between patients and controls [194]. Furthermore, while impaired helper T cell function would result in functional defects in humoral immunity, studies have not demonstrated significant differences in pathogen-specific antibody levels between SD patients and controls [194, 308]. In fact, studies have shown that antibodies
Figure 34. Treatment of Pre-Diseased D2C Mice with the Corticosteroid Dexamethasone

(A) Pre-diseased D2C mice received daily i.p. injections of 10 μg of dexamethasone or the PBS vehicle control for a study period of 3 weeks (n=4 animals per group). Representative gross photographs are shown. (B) The effectiveness of the drug treatment was quantified by observing and grading resulting disease. (C) Representative H&E-stained histological sections of the skin are shown.
Figure 35. Treatment of Pre-Diseased D2C Mice with the Corticosteroid Dexamethasone (Continued)

(A) LN were collected from dexamethasone- and PBS-treated D2C mice. Total CD4⁺ T cell counts were determined using a hemocytometer and T cell immunophenotyping as previously described. (B) Representative lymphoid organs from dexamethasone- and PBS-treated D2C mice and from an age-matched DBA/2 control animal are shown. (C, D) The concentration of total serum IgG and the concentration of α-dsDNA in the dexamethasone- and PBS-treated animals was determined by ELISA as previously described.
against these organisms are ubiquitously found in both those with and without a past history of skin disease [309]. Moreover, while some authors report that abnormally high levels of Malassezia can exist on the skin of AIDS patients (Schechtman) [205, 206], others report that an overgrowth of Malassezia in AIDS is found in only a minority of cases [205]. These findings appear inconsistent with a simple “susceptibility to infection” model, likely reflecting a more complex disease pathophysiology.

One concept which has, until recently [137], been conspicuously absent from current models of psoriasiform pathophysiology is the role of immunoregulation. The data described herein, which helps to reconcile some of the inconsistencies of psoriasiform pathophysiology, suggests that a relative deficiency of T_{reg} may tip the balance between immunotolerance and the generation of Ag-specific immunity. While immunocompromise is not sufficient on its own to result in spontaneous psoriasiform disease, a deficiency of T_{reg}, in a setting of T cell lymphopenia appears to strongly preclude to this cutaneous reaction pattern. The mechanism by which these factors synergistically create psoriasiform pathology likely involves the expansion of conventional helper cells against opportunistic organisms following their release from T_{reg} mediated regulation. It is not surprising that such a combination of factors would arise in the setting of HIV infection as both helper T cells [310] and T_{reg} [311] are targets for the HIV virus. However, given the expression of additional HIV co-receptors (CCR5) by T_{reg} [311], these cells are exquisitely vulnerable to HIV-mediated attack [311, 312] and thus HIV infection likely results in an immunodeficient state with a relative deficiency of T_{reg}. Results from emerging studies support this hypothesis, and indicate that T_{reg} function is impaired in AIDS [313] and that these cells are more comprehensively depleted by the HIV virus [311, 312]. This interpretation also predicts that the effective mitigation of AIDS immunopathology through HAART [314] may work by inducing a rapid recovery in T_{reg} number rather than a correction of the overall
CD4 count. Therefore, in AIDS, the routine monitoring of \( T_{reg} \) may be equally, if not more, important than the total CD4 count for following disease course and assessing a patient’s susceptibility to immunopathology. While these data have obvious implications for the treatment of AIDS-related illnesses, an understanding of the complex relationship between \( T_{reg} \) and conventional CD4\(^+\) "helper" T cells may illuminate the mechanism by which the body maintains tolerance to the normal microbial flora. Such insights into the mechanism of commensalism would have a broad impact on a number of diverse human disease processes.

Having proposed that opportunistic pathogen-specific CD4\(^+\) "helper" T cells were important in disease pathogenesis, the hypothesis that a significant contribution of the CD4\(^+\) T cell expansion was directed against \( C. \) guilliermondii was tested. To address this question, the MHC class II- and H-2\(^d\)-expressing A20 B cell line was pulsed with a sonicate of \( C. \) guilliermondii and used to stimulate CD4\(^+\) cells from DBA/2 and D2C mice. Under these stimulation conditions D2C-derived CD4\(^+\) cells consistently incorporated more tritiated thymidine and had a higher frequency of “blasting” cells relative to DBA/2-derived cells. Although the results of these experiments were not statistically significant, in part due to the high standard deviation of the results, the data was encouraging. It is anticipated that the use of more appropriate APC, such as mature DC, will show that convalescing D2C mice have a significantly increased number of T cells specific for opportunistic pathogens relative to age-matched DBA/2 mice.

While the dysregulated expansion of T cells was in part the result of impaired \( T_{reg} \) functioning, the mechanism by which the serum hypergammaglobulinemia occurred was not actively investigated; though, the finding of auto-antibodies in D2C mice suggested that this represented a polyclonal B cell activation. It is possible that the severely lymphopenic CD8 compartment in D2C mice predisposed to viral pathogens, which are known to cause polyclonal
B cell stimulation [315]; however, the failure of serology to identify any viral infection in D2C mice to date (data not shown) and the full penetrance of the phenotypic abnormalities in D2C mice (Fig. 17) makes this less likely. Furthermore, at the time of disease convalescence, when serum Ig levels are dramatically falling (Fig. 23), the CD8 cell compartment remains severely lymphopenic, having expanded only minimally over the window of disease susceptibility (data not shown). Alternatively, this B cell dysregulation may also have resulted from a lymphopenia of CD4⁺ cells, since the polyclonal B cell activation of human AIDS has been shown to be inversely proportional to the CD4 count [316]. In murine AIDS (MAIDS), hypergammaglobulinemia is known to result from aberrant interactions between B cells and CD4⁺ T cells [317], and thus dysregulation of rapidly expanding D2C CD4⁺ cells may also contribute to this polyclonal gammopathy. Nevertheless, a role for CD4 T cell dysfunction in this B cell abnormality is supported by a temporal association between the normalization of the CD4 T cell compartment and the return of near-normal serum IgG levels.

Although the pathology resulting from T reg deficiency in D2C transgenic mice was found to be similar to that of T reg-deficient FoxP3 KO mice, clear differences in the cutaneous phenotype exist between these two model systems. In D2C mice disease occurs in the "seborrheic" areas of the skin and is characterized by erythematous and edematous skin predominantly over the rostrum that invariably results in lesional alopecia; whereas in FoxP3 KO mice, skin from all body regions becomes thickened and taut, with crusted areas predominantly located over the tail and ears which not uncommonly results in auto-amputation of these structures [299]. The unique tissue distribution of disease in D2C mice is suspected to occur as a result of DBA/2 susceptibility factors predisposing to overgrowth with opportunistic pathogens, including lipophilic fungi which have a tropism for the seborrheic areas of the skin. FoxP3 KO mice, which are on a B6 genetic background, do not share these DBA/2 factors, and therefore it
is not surprising that their disease does not conform to the “D2C” pattern. Furthermore FoxP3 KO mice are not lymphopenic of CD4 helper cells as thymic development of conventional T cells is not perturbed in these animals [318]. A further distinction between the D2C and FoxP3 KO model systems is that D2C mice possess a large population of clonally anergized self-reactive cells responsive to bystander IL-2 and IL-15 [61, 63, 120]. Delaney showed that these cells were acutely activated in disease-susceptible, 2C TCR-expressing BXD<sup>6</sup> RI strains [164] and therefore the possibility that these self-reactive cells are involved in disease pathogenesis is conceivable since studies have shown that the bystander activation of cells with an irrelevant Ag specificity can be an autoimmune hazard \textit{in vivo} [156, 157, 319]. These previous studies also highlighted the necessity of target tissue inflammation in bystander activation mediated localized autoimmune disease [156]. Given the marked inflammation of the rostral skin in D2C mice (Fig. 9) and the evidence of smoldering infection with opportunistic pathogens (Fig. 10), it is likely that sufficient target tissue inflammation exists in the skin of D2C mice to activate the self-reactive cells. Intriguingly, novel treatments of psoriasis which non-specifically target memory phenotype cells have been shown to successfully mitigate disease [185]. Similarly, the non-specific recruitment of large numbers of NK lymphocytes to the skin is a frequent finding in SD [226]. Thus, the recruitment and bystander activation of memory phenotype lymphocytes may represent an important step in the pathophysiology of psoriasiform disease. Therefore, the possible involvement of clonally anergized, self-reactive 2C cells in D2C disease pathophysiology was studied further.
Chapter 5: Self-Reactive T Cells in Disease Pathophysiology

5.1 Introduction:

Previous work on the D2C model clearly demonstrated that T_{reg} lymphopenia and a resultant dysregulated expansion of "helper" T cells was necessary for disease pathogenesis. While the results of this previous work were important for the understanding of psoriasiform disease, these data did not definitively explain the mechanism by which disease develops in D2C mice. An interesting feature of TCR transgenic mice, which has not yet been studied in D2C disease pathogenesis, is the development of a large number of potentially self-reactive cells which are insensitive to negative selection. These cells become clonally anergized when they develop in a background expressing the cognate ligand for the Tg TCR, and thus TCR Tg mice have proven to be excellent tools for the study of T cell anergy [63, 120]. However the reversibility of this inactivation by exogenous cytokine [61, 120] suggests that these cells may pose an autoimmune hazard \textit{in vivo}. Therefore, it is tempting to speculate that the 2C cells in D2C mice may be contributing to disease following their bystander activation.

To first investigate the role of self-reactive 2C cells in D2C pathophysiology, the thymus and lymphoid organs from D2C mice were assayed by flow cytometry to determine whether the pattern of expression of the 2C TCR in D2C mice was similar to that of previously characterized H-2^{d}-expressing 2C animals [63, 107]. These data demonstrated that, similar to that seen for other H-2^{d}-expressing 2C mice, the 2C TCR is expressed only upon a population of selection-independent CD4^{+}CD8^{-} DNCTC in the thymus and peripheral lymphoid organs. The 2C cells in the D2C peripheral lymphoid organs expressed a similar level of the memory markers CD122 and CD44 as 2C cells from previously described H-2^{d}-expressing 2C mice [61-63]; however, in contrast to the 2C cells from these other animals, D2C 2C cells were also found to be acutely activated [164] with vastly enhanced functional properties. The extent of the acute activation and
enhanced functional properties were positively correlated with the severity of clinical disease. Proliferation assays and the results of cell transfer experiments revealed that this acute activation of the self-reactive cells was a cell extrinsic process influenced by undefined recipient factors. To see whether 2C TCR-expressing cells were necessary for disease development, a blocking monoclonal antibody directed against the transgenic 2C TCR was administered to pre-diseased D2C mice. The abrogation of all but infrequent S1 disease by this treatment confirmed the hypothesis that 2C cells have an important role in disease pathogenesis. Given this new understanding of D2C pathophysiology, 2C cells in D2C recipients of T_{reg} were assayed for evidence of acute activation, which demonstrated that the activation of 2C cells occurs downstream of the dysregulated expansion of CD4 cells. Since the acute activation of 2C cells is a terminal event in disease pathogenesis, CD69-expressing 2C cells from S3 D2C mice were transferred to DBA/2 recipients to determine whether activated 2C cells were sufficient for disease pathogenesis. The failure of 2C cells to cause disease in these immunocompetent recipient mice with diverse T cell repertoires demonstrated that the CD69^{+} 2C cells were insufficient for disease. To eliminate the protective effect of this diverse T cell repertoire, the Rag-1^{−/−} mutation was backcrossed to the DBA/2 background, to create DBA/2 Rag-1^{−/−} mice. The disease resistance of DBA/2 Rag-1^{−/−} mice demonstrated that severe immunocompromise coexisting with DBA/2 susceptibility factors was insufficient for disease. Furthermore, the subsequent generation of D2C Rag^{−/−} mice, which were resistant to disease, supported the premise that 2C cells are not sufficient for disease. These data suggest that the previously described dysregulated CD4 cells likely interact with the self-reactive 2C cells in D2C mice, resulting in the subsequent autoimmune exacerbation of cutaneous disease.
5.2 Self-Reactive Cells in TCR Transgenic Mice

5.2.1 The Expression of the 2C TCR in D2C Mice:

To first explore the role of 2C cells in disease pathogenesis the thymus and peripheral lymphoid organs were assayed for cells expressing the 2C transgenic receptor using the anti-2C idiotypic mAb 1B2. In H-2\textsuperscript{b} 2C mice, large populations of 2C TCR expressing DP and mature CD8\textsuperscript{+} SP thymocytes are present in the thymus (Fig. 36); however, in the thymus of the H-2\textsuperscript{d} 2C mice, the 2C receptor is expressed only upon DNTC [107]. These thymic immunophenotypes reflect the positive selection of the 2C TCR by physiological levels of p2Ca-K\textsuperscript{b} and the negative selection of this receptor by p2Ca-L\textsuperscript{d} [107, 320]. The pattern of 2C TCR expression in the D2C thymus (Fig. 36) was similar to that of other H-2\textsuperscript{d} expressing backgrounds such as B2C\textsuperscript{d} and (B6xDBA/2)N\textsubscript{1}2C mice [63, 107]. There was no significant difference in the thymic cellularity between young, age-matched D2C and (B6xDBA/2)N\textsubscript{1}2C mice indicating that a greater dose of cognate ligand and DBA/2 genetic factors did not influence thymic selection (data not shown). In diseased D2C animals, the thymic cellularity was approximately half that of age-matched (B6xDBA/2)N\textsubscript{1}2C mice (3.2 x 10\textsuperscript{6} ± 0.66 cells total vs. 5.5 x 10\textsuperscript{6} ± 1.3 cells total, respectively; n=4 animals per group), most likely reflecting the sensitivity of thymocytes to stress hormones [321].

Next, the expression of the 2C TCR in the peripheral lymphoid organs was assayed. In positively selecting 2C mice (H-2\textsuperscript{b}), expression of the 2C TCR in peripheral T cells is divided between the positively selected CD8\textsuperscript{+} T cells and the selection-independent DNTC [63, 107] whereas, in H-2\textsuperscript{d} expressing 2C mice, the peripheral expression of the 2C TCR is restricted to the selection-independent DNTC [63, 107], reflecting the negative selection of 2C TCR-bearing DP thymocytes (Fig. 36). The pattern of 2C TCR expression in the periphery of D2C mice (Fig. 36) was similar to that described for other H-2\textsuperscript{d}-expressing 2C mice [63, 107], and no significant
Thymi and LN were collected from the indicated animals. Single cell preps were stained with the α-2C TCR mAb 1B2 and with the CD8-specific mAb 53.67 and analyzed by FACS®. Representative dot plots, gated on live cells, are shown.

Figure 36. Expression of the 2C TCR in the Thymus and Peripheral Lymphoid Organs in B2C<sup>b</sup> and D2C Mice

Thymi and LN were collected from the indicated animals. Single cell preps were stained with the α-2C TCR mAb 1B2 and with the CD8-specific mAb 53.67 and analyzed by FACS®. Representative dot plots, gated on live cells, are shown.
difference was seen in the number or distribution of 2C cells between young age-matched D2C and B2C\textsuperscript{d} or (B6xDBA/2)N\textsubscript{1}2C mice (data not shown). However, in diseased D2C mice, a significant reduction in the number of peripheral 2C cells was observed relative to age-matched (B6xDBA/2)N\textsubscript{1}2C animals (2.4x10\textsuperscript{6} vs. 1.0 x 10\textsuperscript{7} respectively, 10 df, p<0.001). This reduction of 2C cells was associated with a significant, 60% increase in the percentage of pre-apoptotic and apoptotic (7AAD\textsuperscript{+}) 2C cell in the periphery of S3 D2C mice relative to (B6xDBA/2)N\textsubscript{1}2C animals (46.3\% vs. 29.2\% respectively, 6 df, p<0.02).

5.2.2 Characterization of Peripheral 2C Cells in D2C Mice:

Previously it was found that peripheral 2C cells in some 2C TCR transgenic BXD\textsuperscript{d} recombinant inbred strains were acutely activated, as evidenced by the expression of CD69 \cite{164}. To see whether 2C cells were acutely activated in D2C mice and to further characterize their immunophenotype, LN cells from D2C, B2C\textsuperscript{d}, B2C\textsuperscript{b}, and (B6xDBA/2)N\textsubscript{1}2C animals were assayed by flow cytometry. The chronic exposure of 2C cells to high affinity Ag in H-2\textsuperscript{d}-expressing backgrounds bestows upon these cells a memory-like immunophenotype, characterized by the expression of high levels of the memory markers CD44 and CD122 \cite{61-63} as well as a slightly increased expression of the CD43 effector glycoform \cite{61} as relative to 2C cells in low affinity Ag-expressing B2C\textsuperscript{b} mice (Fig. 37). 2C cells in D2C mice have similar elevations of the memory markers CD44 and CD122, but a much higher level of the CD43 effector glycoform relative to the same cells in B2C\textsuperscript{d} and (B6xDBA/2)N\textsubscript{1}2C mice (Fig. 37). Consistent with the results from BXD\textsuperscript{d} recombinant inbred 2C mice \cite{164}, variable numbers of 2C cells in D2C mice also express the acute activation marker CD69 (Fig. 37). While the memory immunophenotype of D2C 2C cells had no relationship with disease activity, the expression of high levels of CD69 and the CD43 effector glycoform were strongly correlated
Figure 37. Immunophenotype of 2C TCR DNTC

LN were collected from the indicated animals, prepared for FACS® and stained with the indicated markers. Representative histograms are gated on live 2C TCR DNTC.
with disease severity, being most highly expressed on cells from S3 D2C animals (data not shown). Moreover, in pre-diseased and recovered D2C mice, CD69-expressing 2C cells are rare, and the expression of the CD43 effector glycoform on 2C cells from these animals is equivalent to that seen in (B6xDBA/2)N12C mice (data not shown).

Previously, the Ag-experienced 2C cells from (B6xDBA/2)N12C mice were demonstrated to possess enhanced functional properties relative to the same population of cells from low affinity Ag-expressing B2C\textsuperscript{b} animals [61, 120]. Acutely activated 2C cells from D2C mice were tested to see if their activated immunophenotype was associated with even greater enhancements of these functional properties. The modest increase in the expression of the CD43 effector glycoform in 2C cells from (B6xDBA/2)N12C mice relative to 2C cells from B2C\textsuperscript{b} animals (Fig. 37) is associated with a slightly increased ability to kill L\textsuperscript{d} transfected cell lines pulsed with high concentration of the p2Ca peptide [61]. However, when these same effector cells were tested for their ability to kill BALB/c-derived A20 cells expressing physiological levels of the cognate ligand p2Ca-L\textsuperscript{d}, no significant difference in killing was appreciated (data not shown). When 2C cells from S3 D2C mice, which express high levels of the CD43 effector glycoform, were used as effector cells in this assay, they were shown to have a 3-4-fold increased ability to kill A20 cells relative to 2C cells from either B2C\textsuperscript{b} or (B6xDBA/2)N12C mice (Fig. 38A). The increased capability of 2C cells from S3 D2C mice to kill A20 target cells may be attributable to an increased level of NKG2D found on these cells (Fig. 38B). NKG2D is a known activating/cytotoxic receptor that has a specificity for stress ligands [322, 323] which the A20 cell line is known to express [324, 325].

In a prior report, 2C cells from (B6xDBA/2)N12C mice were shown by intracellular FACS\textsuperscript{®} to have an enhanced ability to secrete IFN-\(\gamma\) immediately \textit{ex vivo} relative to 2C cells from B2C\textsuperscript{b} mice, following brief TCR stimulation [61]. Although this supraphysiological
Figure 38. Functional Characterization of 2C TCR DN T cells from Disease-Resistant and Disease-Susceptible Mice

(A) 2C TCR DN T cells were purified by negative selection using a magnetic column (Dynel). Purified 2C TCR DN T cells from diseased D2C and disease-resistant B2C<sup>d</sup> mice were incubated with <sup>51</sup>Cr-labeled A20 target cells at the indicated effector:target (E:T) ratios. Data is representative of 5 individual experiments where each data point was done in quadruplicate. (B) LN were collected from the indicated animals, prepared for FACS<sup>®</sup> and stained with an α-NKG2D mAb. Representative histograms gated on live 2C TCR<sup>+</sup> DN T are shown. (C) Purified 2C TCR DN T from diseased D2C and disease-resistant B2C<sup>d</sup> mice were stimulated <em>in vitro</em> with A20 cells for 40 hours, after which culture supernatant was collected for IFN-γ ELISA. Culture supernatant IFN-γ was detected by ELISA using the IFN-γ-specific capture and detections mAbs R4-6A2 and XMG1.2, respectively. Recombinant IFN-γ was used to generate a standard curve and shown data is representative of 4 individual experiments. (D) Purified 2C TCR DN T cells from B2C<sup>d</sup> and diseased D2C mice were stimulated with T2-L<sup>d</sup> cells, 20 U/ml IL-2, and the indicated concentrations of the p2Ca peptide in a standard proliferation assay. The cells were pulsed with 50 µl of (³H)thymidine (20 µCi/ml) eight hours before the end of the 72-hour assay period. Data is representative of 5 individual experiments where each data point is done in quadruplicate.
stimulation with p2Ca-pulsed, L<sup>d</sup>-transfected cells demonstrated a greater propensity of Ag experienced 2C cells to liberate this inflammatory cytokine, it is unlikely that a TCR signal of this magnitude would ever be encountered in vivo, and thus this functional attribute is of questionable biological significance. Therefore, 2C cells from B2C<sup>b</sup> mice, (B6xDBA/2)<sub>N</sub>2C mice, and S3 D2C mice were stimulated immediately ex vivo with A20 cells to see whether functional differences in this effector function were appreciable after more physiologic doses of stimulation. Consistent with the killing data, no significant difference was observed between 2C cells from B2C<sup>b</sup> and (B6xDBA/2)<sub>N</sub>2C mice (data not shown); however, 2C cells from S3 D2C mice produced a 10-20-fold greater amount of IFN-γ relative to the 2C cells from these other strains (Fig. 38C).

Next, standard proliferation assays were used to test the hypothesis that the activated immunophenotype and enhanced functional properties of 2C cells from D2C mice resulted from a failure of clonal anergy and/or an increased self-reactivity of these cells. When 2C cells from S3 D2C mice were stimulated with p2Ca-L<sup>d</sup>-expressing APC, it was found that these cells were still dependent on bystander cytokine support for their proliferation, as they failed to proliferate in the absence of supplemental cytokine (data not shown). While this data indicated that the anergization to self-Ag had been preserved, Ag stimulation of these cells in the presence of IL-2 or IL-15 was performed to determine whether these cells exhibited enhanced self-reactivity. Previously, clonally anergized 2C cells from (B6xDBA/2)<sub>N</sub>2C mice were shown to have a reduced threshold of activation and greater proliferative potential relative to 2C cells from B2C<sup>b</sup> mice when exogenous IL-2 or IL-15 was supplied to the culture medium [61, 63, 120]. When 2C cells from S3 D2C mice were stimulated under these same conditions, these cells were found to have an even greater proliferative potential with a higher basal proliferation rate and reduced activation threshold relative to 2C cells from (B6xDBA/2)<sub>N</sub>2C mice (Fig. 38D).
The enhanced effector functions of 2C cells from S3 D2C mice were observed to correlate with the extent of cellular activation as measured by the percentage of 2C cells expressing the acute activation marker CD69. 2C cells derived from pre-diseased or convalescent D2C mice rarely expressed CD69 and were found to perform equivalently to 2C cells from (B6xDBA/2)N12C animals in the aforementioned assays of T cell function (data not shown). This data argued against a cell intrinsic increase in self-reactivity in 2C cells and therefore it was concluded that the enhanced effector functioning of 2C cells from diseased D2C mice was attributable to a heightened activation state induced by cell extrinsic factors.

Furthermore, it was hypothesized that, regardless of donor strain, 2C DNTC adoptively transferred to the DBA/2 background would assume the immunophenotype and functional attributes typical of 2C cells developing naturally in D2C mice. To test this hypothesis, T cell-depleted bone marrow from D2C and (B6xDBA/2)N12C mice was adoptively transferred to (B6xDBA/2)N1 and DBA/2 recipients respectively. H-2d congenic B2Cd donor mice and B6d recipient animals were also employed, in lieu of (B6xDBA/2)N12C donors and (B6xDBA/2)N1 recipients respectively, to eliminate the effect of H-2 incompatibility; however, this substitution had no effect upon the outcome of the studies. B6d or (B6xDBA/2)N1 recipients of D2C marrow (Fig. 18) were found to have a thymus and peripheral lymphoid compartment equivalent to (B6xDBA/2)N12C mice (data not shown). Furthermore the D2C-derived 2C cells in these animals were indistinguishable from 2C cells derived from (B6xDBA/2)N12C mice, with respect to their CD44+CD122+CD69- immunophenotype (Fig. 39A), and their reduced ability to kill targets bearing physiological levels of p2Ca-Ld (Fig. 39B) or liberate IFN-γ following Ag stimulation (Fig. 39C). Presumably B6-derived, protective factors in these chimeras precluded the acute activation of the 2C cells by providing a less-inflammatory milieu for their development.
Figure 39. Determination of whether the Acutely Activated Immunophenotype and Enhanced Functional Properties of 2C DN T cells are Cell-Intrinsic or Cell-Extrinsic Characteristics

(A) D2C bone marrow was adoptively transferred to lethally irradiated, non-transgenic B6<sup>d</sup> recipients while bone marrow from B2C<sup>d</sup> animals was adoptively transferred to lethally irradiated, non-transgenic DBA/2 recipients (n=10 animals per group). LN collected from the bone marrow chimeras (BMC) were processed for flow cytometry and stained with mAbs against CD69 and the 2C TCR (1B2). 2C TCR DN T cells from B2C<sup>d</sup> mice were used as a negative control. Representative histograms are gated on live 1B2<sup>+</sup> cells. (B) Purified 2C TCR DN T cells from the BMC and from wild-type D2C and B2C<sup>d</sup> mice were incubated with 51Cr-labeled A20 target cells at the indicated effector:target (E:T) ratios. Data is representative of 3 experiments where each data point was done in quadruplicate. (C) Purified 2C DN T cells from the indicated animals were stimulated with A20 cells and subsequently culture supernatant was assayed for IFN-γ by ELISA as previously described. Data is representative of 3 experiments where each data point was done in quadruplicate.
2C cells from diseased DBA/2 recipients of (B6xDBA/2)N12C or B2C\textsuperscript{d} bone marrow (Fig. 18) possessed an acutely activated CD69\textsuperscript{+} immunophenotype (Fig. 39A), and had enhanced effector functions as evidenced by enhanced cytolytic activity (Fig. 39B), and an increased ability to produce IFN-\(\gamma\) (Fig. 39C). These cells were indistinguishable from 2C cells derived from unmanipulated D2C mice, supporting the hypothesis that the enhanced effector functioning of 2C cells in unmanipulated D2C mice is not a cell-intrinsic phenomenon but rather a reflection of the ambient inflammatory milieu to which the cells are exposed.

5.3 Involvement of 2C cells in Disease Pathogenesis

5.3.1 2C Cells are Necessary for Disease Development

The acute activation and enhanced functional properties of the 2C cells in D2C mice were consistent with the hypothesis that the presence of anergized cells may pose an autoimmune hazard \textit{in vivo} and that these cells have an active role in disease pathogenesis. However, it was equally plausible that the bystander activation of the CD122-expressing 2C cells might represent an inconsequential epiphenomenon. Therefore, more definitive evidence for the direct involvement of the 2C cells in disease pathogenesis was sought.

To determine whether the 2C TCR\textsuperscript{+} T cells were responsible for mediating the immunopathological changes in the D2C mouse, disease-susceptible mice were treated with the anti-clonotypic mAb 1B2 which blocks the 2C TCR recognition of cognate Ag [326]. 1B2-treated D2C mice were resistant to all but infrequent S1 disease, characterized histologically by focal areas of moderate acanthosis, while animals treated with the isotype-matched control mAb T3.70 [89] developed typical pathological changes (Fig. 40A-C). In order to understand the mechanism of this mAb suppression, 2C TCR T cells recovered from treated animals were immunophenotyped. Since a saturating concentration of the anti-clonotypic 1B2 mAb was
Figure 40. Treatment of D2C Mice with a Blocking mAb against the 2C TCR

(A) Pre-diseased D2C mice were treated weekly with 400 µg of the blocking anti-2C-TCR mAb 1B2 or the isotype-matched control mAb T3.70. Representative photographs are shown (n=4 animals per group in each of 3 experiments). (B) Rostral skin from D2C mice, treated with either the 1B2 or T3.70 mAb, and from a DBA/2 control animal was collected and processed routinely for histological analysis. Representative H&E-stained photomicrographs are shown. (C) Treatment effectiveness was quantified by observing and grading resulting disease. (D) To identify 2C cells, an α-CD122 mAb (TM-β1) and a mAb against the β chain of the 2C TCR (F23.1, pan-Vβ 8 mAb) were employed due to saturation of the 2C TCR with the 1B2 mAb. LN cells were stained with an α-CD69 mAb (H1.2F3) to evaluate 2C cell acute activation. Histograms are gated on live CD122⁺ F23.1⁺ cells. (E) CD122 vs. F23.1 dot plots of LN cells from 1B2-treated and T3.70-treated mice are shown. Dot plots are gated on live cells. Note the downregulation of the TCR β chain (F23.1) in 1B2-treated mice.
administered, it was not possible to identify 2C TCR\(^+\) cells with this reagent. Instead, 2C cells were quantified by staining LN cells for the expression of CD122 (TM,\(\beta\)1) and the 2C TCR V\(\beta\)8.2 chain (F23.1) [327] since bound 1B2 mAb does not inhibit binding of the V\(\beta\)8.2-specific mAb F23.1 [328] and virtually all V\(\beta\)8.2\(^+\)CD122\(^+\) cells in H-2\(^d\)2C mice express the 2C TCR (data not shown). The 1B2 mAb treatment precluded the acute activation of the F23.1\(^+\)CD122\(^+\) cells in D2C mice as measured by FACS\(^\circledR\)-based CD69 staining (Fig. 40D) and prevented their depletion, which typically occurs during disease pathogenesis (9.0\(\times\)10\(^6\) in 1B2-treated D2C mice vs. 2.84 \(\times\) 10\(^6\) in T3.70- treated D2C mice, 6 df, p,<0.001). Furthermore, treatment with 1B2 reduced the intensity of V\(\beta\)8.2 expression by an order of magnitude, as assayed by FACS\(^\circledR\) using F23.1 staining (Fig. 40E). Therefore the therapeutic effect of the 1B2 mAb likely involves the down-modulation of the 2C TCR, resulting from TCR cross-linking \textit{in vivo} and, furthermore, by blocking the interaction of this down-regulated receptor with cognate tissue Ag. These data highlight the necessary role of cognate Ag recognition by the 2C TCR in disease pathogenesis, which supports the hypothesis that 2C cells have a direct role in disease.

5.3.2 T\(_{\text{reg}}\) Preclude the Acute Activation of 2C Cells

Based on the ability of the blocking mAb 1B2 to abrogate 2C cell activation and disease development, it was hypothesized that previous interventions which mitigated the disease phenotype worked via controlling 2C cell activation. Serum transferred from S3 D2C animals to pre-diseased D2C mice had only a mild effect on the phenotype of disease (Fig. 28), suggesting that this treatment failed to prevent 2C cell activation. As expected, flow cytometric evaluation of peripheral lymphocytes from serum and vehicle control-treated mice revealed that this intervention only slightly reduced the percentage of 2C cells activated \textit{in vivo} (Fig. 41A). While these data suggest that high titers of opportunistic pathogen IgG can slightly modulate the
Figure 41. Effect of Previous Interventions on the Activation Status of 2C TCR DNTC in D2C Mice

(A) Pre-diseased D2C mice were treated with twice-weekly injections of 0.5-1.0 ml of diluted S3 D2C serum containing 2 mg/ml IgG as previously described. Control animals received twice-weekly injections of the irrelevant IgG1 mAb (T3.70, anti-idiotype mAb of the HY TCR). After four weeks of treatment, CD69 expression on 2C TCR DNTC was assayed by flow cytometry. Histograms are gated on live 2C cells. (B) Pre-diseased D2C recipients of unfractionated DBA/2 CD4 T cells or PBS were sacrificed four weeks post-cell transfer. LN were harvested, prepared for flow cytometry, and stained with the α-2C TCR mAb (1B2) and a mAb against CD69 (H1.2F3). Histograms are gated on live 1B2 cells. (C) 1-2x10^6 CD4^+CD25^+ T<sub>reg</sub> cells, 1-2x10^7 CD4^+CD25^- "helper" T cells or PBS was transferred to pre-diseased D2C mice. Four weeks post-transfer, animals were sacrificed and CD69 expression on 2C TCR DNTC was assayed by flow cytometry. Histograms are gated on live 1B2 cells. (D) Pre-diseased D2C mice were treated daily with 0.5 ml of a 20 µg/ml solution of dexamethasone in PBS or 0.5 ml of the PBS diluent alone. Four weeks post-treatment, animals were sacrificed and CD69 expression on 2C TCR DNTC was assayed by flow cytometry. Histograms are gated on live 1B2 cells.
disease phenotype, it is clear that even this supraphysiological level of antiserum cannot preclude 2C cell bystander activation, presumably due to the inability to sufficiently influence the inflammatory milieu of these animals.

The hypothesis that 2C cell activation occurs proximally to the development of severe skin disease predicted that both the unfractionated DBA/2 CD4 cell transfers (Fig. 26, 27) and purified DBA/2 T\textsubscript{reg} cell transfers (Fig. 30, 31), which completely abrogated disease, would be associated with CD69\textsuperscript{+} 2C cells. As shown in Figures 41B and 41C, both of these cell transfer experiments did in fact abrogate the acute activation of the 2C cells whereas the recipients of T\textsubscript{reg}-depleted DBA/2 CD4\textsuperscript{+}CD25\textsuperscript{-} cells, who developed a kinetically-enhanced course of disease (Fig. 30B, C), possessed an extensive population of CD69\textsuperscript{+} 2C cells (Fig. 41C). The finding that these latter recipients of DBA/2 CD4\textsuperscript{+}CD25\textsuperscript{-} T cells had fewer CD69\textsuperscript{+} 2C cells than littermate control D2C animals (Fig. 41C) is likely attributable to the faster disease kinetics in these animals rather than any protection afforded by the transfer of CD4\textsuperscript{+}CD25\textsuperscript{-} cells since, at the time of analysis, the recipients of CD4\textsuperscript{+}CD25\textsuperscript{-} cells were undergoing disease convalescence while control D2C mice were developing peak pathological changes (Fig. 30B). It is likely that immunophenotyping these animals at early time points would have demonstrated greater activation of the 2C cells relative to unmanipulated D2C mice.

D2C recipients of Dex were also protected against the development of disease which was associated with a massive reduction in LN cellularity relative to PBS-treated D2C animals (6.8 ± 3 x 10\textsuperscript{5} cells vs. 5.1 ± 2.1 x 10\textsuperscript{6} cells, respectively) which included a significantly reduced number of CD4 T cells (5.8 ± 2.1 x 10\textsuperscript{4} vs. 9.9 ± 2.5 x 10\textsuperscript{5} cells, respectively) and 2C cells (2.8 ± 1.4 x 10\textsuperscript{5} vs. 1.1 ± 0.4 x 10\textsuperscript{6} cells, respectively). Interestingly, while the majority of 2C cells were CD69\textsuperscript{-} in these animals, some 2C cells were found to be CD69\textsuperscript{+} (Fig. 41D). The failure of disease development in these mice, despite some 2C cell activation, could result from a number
of factors, including a reduction in the frequency of 2C cells below a critical threshold necessary for the induction of clinically significant bystander damage, or a Dex-induced biochemical block precluding the acquisition of full effector functioning of the 2C cells. More research on the effects of Dex upon 2C cells as well as $T_{reg}$ in D2C mice will be necessary to determine the mechanism by which Dex protects against the development of the disease phenotype.

### 5.3.3 2C Cells are not Sufficient for Disease Development

Since the protection from disease afforded by the administration of 1B2 demonstrated the necessity of 2C cells in D2C pathogenesis, acutely activated 2C cells were transferred to DBA/2 recipients to determine if these cells were sufficient for lesion development. Lightly irradiated DBA/2 mice received $5 \times 10^6$ purified CD69$^+$ 2C cells from S3 D2C donors (Fig. 42A). Three months post-cell transfer, these cells were still present in large numbers with an average of $2.43 \pm 0.5 \times 10^6$ 2C cells being recovered from each recipient. Despite the persistence of these cells in the disease susceptible DBA/2 background, the 2C cells lost their acutely activated immunophenotype (Fig. 42A) and recipient animals remained free of gross disease or psoriasiform histopathology (Fig. 42B, C). Given the number of 2C cells recovered from these recipients, it is unlikely that disease resistance could be explained solely by an inadequate transfer of 2C cells. Rather the disease resistance of these recipients is best explained by the failure to deplete critical cell populations which occurs in D2C mice as a result of TCR transgenesis. Although the administration of 550 rads facilitated the engraftment of the donor cells by clearing some space in the peripheral lymphoid compartment, a rapid replenishment of the lymphoid compartment due to the unperturbed thymic development in these animals would have quickly reversed the temporary lymphopenia induced by this measure. Furthermore, the recipient CD4$^+$ T cell repertoire was a diverse one, without skewing of V$\beta$ chain usage or $T_{reg}$
Figure 42. Adoptive Transfer of CD69⁺ 2C TCR DNTC to Recipient DBA/2 Mice

(A) CD69⁺ 2C DNTC were purified from S3 D2C mice as previously described and transferred to lightly irradiated (550 rads) DBA/2 recipient mice (5 × 10⁶ CD69⁺ 2C cells per mouse, n=5). Three months post-cell transfer, recipient mice were sacrificed and LN were collected and processed for flow cytometry. Representative histograms demonstrating CD69 expression on 2C DNTC before cell transfer and on the same cell population recovered from recipient mice are shown. Histograms are gated on live 2C cells. (B) Representative gross photograph of a recipient DBA/2 mouse. (C) Representative H&E histology of rostral skin from a recipient DBA/2 mouse.
lymphopenia as occurs in D2C mice. Other T cell populations, whose ontogeny is known to be disrupted by TCR transgenesis such as γδ T cells, would similarly be unaffected in these recipients. Therefore these immunocompetent recipients would not respond to cutaneous insults with the same prolonged inflammatory response and subsequent development of target tissue inflammation. This is consistent with the current dogma that prolonged inflammation is necessary to drive bystander stimulation-mediated autoimmune exacerbations of disease [156, 163]. Such cytokine enriched environments may promote the activation of anergized T cells as well as self-reactive T cell clones with low avidity for Ag by bypassing biochemical blocks or reducing the activation threshold of these cells, respectively. Therefore this data demonstrates that other effects of transgenesis, such as the induction of T cell lymphopenia and skewing of the T cell repertoire, likely have a necessary role in disease pathogenesis.

To address the contribution of these other factors in disease pathogenesis, D2C mice homozygous for the Rag-1 KO mutation were created by interbreeding D2C mice with DBA/2 Rag-1"^/-" animals. DBA/2 Rag-1"^/-" animals are deficient in all B and T cells since the Rag-1"^/-" mutation precludes V(D)J rearrangement and thereby impairs lymphocyte development [234]. DBA/2 Rag-1"^/-" mice were completely resistant to the development of gross or microscopic D2C pathology (Fig. 43A), despite being severely immunocompromised as evidenced by the absence of circulating serum Ig (Fig. 43C). The disease resistance of these animals is not unexpected as both self-reactive 2C cells and dysregulated CD4 T cells, which these animals lack, were previously shown to be critical for disease pathogenesis. Furthermore, other immunologically-devastating mutations on the "susceptible" BALB/c background, such as the severe combined immunodeficiency (SCID) and Nu/Nu (nude) mutations, do not have an observable cutaneous phenotype [329]. Therefore, these data confirm that overwhelming susceptibility to infection is insufficient for disease. D2C Rag-1"^/-" mice were similarly resistant to disease while age-matched
Figure 43. Characterization of the DBA/2 Rag-1$^-$/ Mice

(A) The Rag-1$^-$/ mutation was backcrossed from the B6 to the DBA/2 background for 6 generations. Representative gross and microscopic images of DBA/2 Rag-1$^-$/ and littermate DBA/2 Rag-1$^+$/ mice are shown. (B) Tail vein blood from DBA/2 Rag-1$^-$/ and littermate DBA/2 Rag-1$^+$/ mice was stained with a goat $\alpha$-mouse Ig FITC Ab and analyzed by flow cytometry to assay for circulating B lymphocytes to confirm PCR-based genotyping results. The dot plots are gated on live cells. (C) Serum was collected from DBA/2 Rag-1$^-$/ and littermate DBA/2 Rag-1$^+$/ mice and assayed by ELISA for serum IgG.
D2C Rag-1\textsuperscript{+/−} littermate controls developed the typical D2C disease phenotype (Fig. 44A). D2C Rag-1\textsuperscript{+/−} mice are deficient in all but 2C TCR-expressing lymphocytes (Fig. 44B, C), and thus have a similar level of immunocompromise as DBA/2 Rag-1\textsuperscript{+/−} animals. Given the absence of a dysregulated population of CD4\textsuperscript{+} T cells, the disease resistance of D2C Rag-1\textsuperscript{+/−} mice was predicted by the results of previous experimentation. Unexpectedly, while the majority of 2C cells derived from D2C Rag-1\textsuperscript{+/−} mice were CD69\textsuperscript{+}, a variable but significant population of 2C cells in these animals was CD69\textsuperscript{−} (Fig. 44C). This result is consistent with the ability of mediators other than CD4 T cell-derived IL-2 to cause bystander activation of the 2C cells, such as IL-15 [61, 62]. While this data demonstrates that it is likely that IL-15 derived from infected epithelium and other sources of inflammatory cytokines may also contribute to the bystander activation of 2C cells (Fig. 44C), these sources do not predispose animals to the development of clinically-relevant autoimmune disease, and perhaps may reflect additional roles of dysregulated CD4 T cells in disease pathogenesis.

The conclusion from these findings is that 2C transgenesis has a multitude of effects which are critical for disease pathogenesis. Although the production of self-reactive cells is important for disease, the generation of an environment lymphopenic of both conventional CD4 "helper" T cells and CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{reg} is equally critical. The resultant dysregulated expansion of the “helper” T cell population in some way synergizes with the self-reactive 2C cells to cause organ-specific autoimmune disease.

**5.4 Conclusion**

While a number of different TCR transgenic model system of spontaneous skin inflammation have been recently generated [330-332], D2C mice differ from these other systems in that the cutaneous pathological changes in these other models are seen extensively, often
Figure 44. Characterization of D2C Rag-1\(^{-/-}\) Mice

(A) D2C Rag-1\(^{-/-}\) mice were created by mating D2C mice with DBA/2 Rag-1\(^{-/-}\) mice. Representative gross and cutaneous microscopic images of D2C Rag-1\(^{-/-}\) and littermate D2C Rag-1\(^{+/+}\) mice are shown. (B) Tail vein blood from D2C Rag-1\(^{-/-}\) and littermate D2C Rag-1\(^{+/+}\) mice was stained with a goat α-mouse Ig Ab and analyzed by flow cytometry to assay for circulating B lymphocytes to confirm PCR-based genotyping results. The dot plots are gated on live cells. (C) LN from D2C Rag-1\(^{-/-}\) and littermate D2C Rag-1\(^{+/+}\) mice were harvested, prepared for flow cytometry, and stained with the α-2C TCR mAb 1B2 and a mAb against CD69 (H1.2F3). Histograms are gated on live 1B2\(^{+}\) cells.
leading to the development of a lethal wasting disease with pathology more closely resembling graft versus host disease [330-332], whereas the waxing/waning, milder, and uniquely distributed cutaneous pathology of the D2C mice is limited to the seborrheic regions of the skin and has a psoriasiform pattern [123]. The different morphological changes and differing pathophysiologies of these model systems clearly demonstrate the uniqueness of the D2C model, studies on which will generate novel insights into the poorly understood psoriasiform conditions.

The data described herein demonstrate that the bystander activation of 2C cells occurs distal to the dysregulated expansion of CD4 T cells, as precluding this CD4 expansion by the provision of Treg abrogates the acute activation of the 2C cells (Fig. 41C) without correcting the immunocompromised state of these recipients (Fig. 31D). This suggests that the activation of 2C cells likely represents a terminal effector mechanism in the generation of disease. These data have also demonstrated the source of the cytokine support fueling the bystander activation and the subsequent autoimmune exacerbation of disease. Both IL-2 and IL-15 have been shown to be capable of causing bystander stimulation in vitro [61, 62]; however, these results suggest that CD4-derived cytokines are the dominant source of this bystander cytokine support (Fig. 44C). Although the skin from immunodeficient D2C Rag-1\(^{-/}\) animals would be expected to produce abundant amounts of IL-15 in response to infection [333], only a minority of 2C cells in these disease resistant animals expressed high levels of CD69 (Fig. 44C). This suggests that while other sources of cytokine can cause limited 2C cell bystander activation, only T cell-derived IL-2 or other CD4\(^{+}\) effects are capable of predisposing to the D2C disease phenotype. To further investigate the effect of CD4\(^{+}\)CD25\(^{-}\) T cell expansion and the subsequent liberation of T cell-derived IL-2 on the disease phenotype, it is planned to transfer CD4\(^{-}\)CD25\(^{-}\) cells to non-transgenic DBA/2 Rag-1\(^{-/}\) mice as well as D2C Rag-1\(^{-/}\) mice. The development of disease in the D2C Rag-1\(^{-/}\) but not the DBA/2 Rag-1\(^{-/}\) recipients would provide further evidence for a role of
self-reactive 2C cells in disease pathogenesis, while demonstrating that CD4 T cell
dysregulation is not sufficient for lesion development.

Although it is likely that the cutaneous pathology in D2C mice is largely the result of
inflammatory cytokines causing the bystander stimulation of memory phenotype self-reactive
cells, these data also indicate that the recognition of Ag by the 2C TCR is critically important for
this process. While the Ag recognition by such terminal effector cells may be envisioned to
focus the resultant immune response upon specified targets, the cognate Ag of 2C cells in D2C
mice is expressed systemically [110]. Therefore the peculiar distribution of disease caused by
2C cells in D2C mice seems inconsistent with a TCR-directed mechanism. However, the
possibility that the target inflammation induced by the overgrowth of opportunistic pathogen
was associated with an upregulation of epithelial stress ligands may reconcile this discrepancy as
2C cells in diseased D2C mice were found to express high levels of the stress ligand receptor
NKG2D (Fig. 38B). As the ligation of NKG2D has been shown to trigger cytotoxic T cell
responses [322], it is possible that the expression of stress ligands by lesional tissue may thereby
direct the subsequent immunopathological onslaught. While the recognition of Ag by TCR may
simply be a permissive step in this effector response, the interruption of TCR signaling may
abrogate this effector functioning and thereby inhibit the development of autoimmune disease.
As "normal" mice and humans possess populations of circulating memory phenotype cells that
express NKG2D [334], this mechanism may represent a real autoimmune threat in the presence
of sufficient target tissue inflammation.

The mechanism by which 2C cells induce psoriasiform lesions is likely related to the
propensity of these cells to liberate IFN-γ [61], since this cytokine has been shown to have an
important role in the generation of psoriasiform disease [159, 335]. Interestingly, the
administration of a blocking mAb against IFN-γ in a pilot experiment was found to have a
mitigating effect upon disease, (data not shown), and the development of D2C IFN-γ KO mice is in progress to definitively address the role of this cytokine in D2C pathophysiology. It is also planned to collect 2C cells from dexamethasone-treated mice, as well as D2C-Rag-1−/− mice to determine if the self-reactive 2C cells in these animals, some of which were found to upregulate the acute activation marker CD69, were functionally impaired with respect to the liberation of IFN-γ.

While the results of these studies provided a solid basis for the understanding of psoriasiform pathophysiology in D2C mice, the resultant model was unable to explain certain observations, namely the confusing results of bone marrow chimera experiments. For example, while the finding that B2Cbone marrow could transfer disease to DBA/2 recipients was explained by the necessity of DBA/2 non-hematopoic factors for disease pathogenesis, the failure of D2C bone marrow to transfer disease to DBA/2 recipients was perplexing. A promising avenue of research which may reconcile these unexplained observations will be the investigation of the cutaneous immune network in these animals. The suspicion that derangements in cutaneous immunity may be involved in D2C disease pathogenesis was based upon the finding of significant differences in the composition of cutaneous lymphocytes between disease resistant and disease susceptible bone marrow chimeras. This fortuitous data suggested that the loss of sentinel cutaneous γδ lymphocytes might contribute to the susceptibility to disease. Given that the γδ KO mutation on certain “weak” backgrounds is known to result in spontaneous cutaneous inflammation [126, 139], the likelihood of this hypothesis was considered sufficiently possible to warrant the investigation of γδ T cells in D2C pathophysiology.
Chapter 6: Cutaneous $\gamma \delta$ T Lymphocytes and Their Role in Disease

Pathophysiology

6.1 Introduction

One of the skin's first lines of defense is a population of $\gamma \delta$ TCR-expressing intraepithelial lymphocytes (IEL), which have been shown to play a major role in the maintenance of the epidermal barrier as well as the regulation of cutaneous inflammation [126]. Cutaneous $\gamma \delta$ IELs can respond quickly [126] to kill epithelial cells that are stressed by infection [336-338] or by transformation [322, 336, 339], without the delay necessary for the expansion of rare cognate antigen-specific clones that occurs in conventional adaptive immune responses [1]. $\gamma \delta$ IELs perform "innate" surveillance [126] by virtue of their constitutively-expressed stress ligand receptor, NKG2D [340], and their canonical $V\gamma3V\delta 1$ TCR [341] which together coordinately bind to separate epitopes on stress ligands [15, 342]. In multiple murine TCR$\delta^+$/ models, mice develop spontaneous cutaneous inflammation [139] and/or exaggerated immunopathology in response to epithelial insults [142, 143]. The similarities between these other model systems and D2C mice suggested that a natural deficiency of such sentinel cutaneous lymphocytes might underlie disease pathophysiology in the D2C model system.

To first investigate the hypothesis that an impairment in cutaneous $\gamma \delta$ cells played a role in disease, the immunophenotype of IELs was ascertained in various 2C transgenic and non-transgenic animals. Interestingly, no cutaneous $\gamma \delta$ T cells were found in any 2C-expressing animals, all of which possessed a cutaneous compartment occupied almost exclusively by 2C TCR-expressing lymphocytes. These intraepidermal 2C cells had a dendritic morphology analogous to cutaneous $\gamma \delta$ cells and were found in roughly the same concentration as $\gamma \delta$ cells in normal murine epidermis. Interestingly, the frequency of intraepidermal lymphocytes on the DBA/2 background, both 2C cells in D2C mice and $\gamma \delta$ cells in DBA/2 mice, was much reduced
relative to intraepidermal lymphocytes in B2C and B6 mice, respectively. On a mixed
(B6xDBA/2)N1 background, the frequency of IELs for both 2C and non-2C animals was equal to
that of B2C and B6 mice, respectively, indicating that the low frequency of cutaneous IEL in the
DBA/2 background did not follow an autosomal dominant inheritance pattern. Rather, transgenic
and non-transgenic N2 backcrosses to the DBA/2 background had approximately equal numbers
of animals possessing the B6-like and DBA/2-like frequency of intraepidermal lymphocytes,
indicating a possible autosomal recessive mode of inheritance. Intriguingly, the pattern of
inheritance of the low IEL phenotype was somewhat similar to the pattern of disease penetrance
N22C DBA/2 backcrosses. To see if this low frequency of intraepidermal lymphocytes correlated
with disease susceptibility in N22C backcrosses to the DBA/2 background, this parameter was
assayed in N22C backcrosses being followed for the development of cutaneous pathology.
Interestingly, N22C backcrosses possessing lower levels of 2C IELs were found to be less
susceptible than littermates possessing higher number of 2C IELs, suggesting that the lower
number of 2C IELs did not predispose these animals to disease. Rather, this data lent further
support to the important role of the self-reactive 2C cell in disease pathogenesis. The conclusion
that the 2C IEL were not protective was supported by the finding that 2C mice, from all
backgrounds, had an impairment in their cutaneous barrier function equal to that of TCR8⁻/⁻ mice,
as assayed by the application of the cutaneous irritant croton oil. These data suggested that TCR
transgenesis rendered these animals deficient in cutaneous immunosurveillance functioning.
Given this new understanding, the seemingly discordant bone marrow transplant results were re-
investigated with respect to this new parameter. The finding that B2C⁺ bone marrow but not D2C
bone marrow could transfer disease to lethally irradiated DBA/2 recipients was correlated with a
preserved population of cutaneous γδ sentinel cells in recipients of D2C marrow, whereas, in
DBA/2 recipients of B2C⁺ marrow, the epidermis was inhabited almost exclusively by 2C TCR-
bearing T cells. To further address the role of \( \gamma\delta \) T cell deficiency in disease pathogenesis, the TCR\( \delta^{--} \) mutation was backcrossed from the B6 to the DBA/2 background. DBA/2 TCR\( \delta^{--} \) mice were completely devoid of any features of the D2C phenotype; however, when these mice were used as recipients of D2C bone marrow, the resulting chimeric animals developed typical features of D2C disease. Analysis of the epidermis from these animals revealed that, in contrast to \( \gamma\delta^{++} \) recipients, these animals were deficient in cutaneous \( \gamma\delta \) T cells and replete with cutaneous 2C cells. The analysis of peripheral 2C cell activation in these aforementioned experiments also revealed that disruption of the cutaneous barrier is a proximal event in disease pathogenesis which is necessary for the subsequent development of bystander stimulation of the 2C DNTC.

6.2 Intraepithelial Lymphocytes in 2C Mice

6.2.1 Characterization of IELs in 2C TCR Transgenic and Non-Transgenic Mice on the B6, DBA/2, and Mixed (B6xDBA/2)\( N_1 \) Backgrounds

To address the role of cutaneous \( \gamma\delta \) T cells in the model, flow cytometry was performed on cell suspensions prepared from the epidermal sheets of 2C-expressing and non-transgenic B6, DBA/2 and mixed (B6xDBA/2)\( N_1 \) mice to assay for the presence of \( \gamma\delta \) T cells and 2C cells. As expected, the epidermis from B6, DBA/2 and mixed (B6xDBA/2)\( N_1 \) mice lacked 2C cells and the vast majority of cutaneous IEL in these animals were \( \gamma\delta \) T cells, expressing the canonical \( V\gamma 3V\delta 1 \) TCR (Fig. 45). Surprisingly, a sizable reduction of \( \gamma\delta \) T cells was observed in DBA/2 mice relative to B6 and (B6xDBA/2)\( N_1 \) animals (Fig. 45). Such an observation is not entirely unexpected as a reduction in the frequency of cutaneous \( \gamma\delta \) IEL is known to occur in several inbred strains including BALB/c mice [343]. Although the observation that different inbred strains can harbour different frequencies of cutaneous IEL is intriguing, its relevance to disease
Figure 45. Characterization of Cutaneous Intraepithelial Lymphocytes in B6, DBA/2 and Mixed (B6xDBA/2)N1 Mice With or Without the 2C TCR Transgenes

Epidermal cell suspensions from the specified animals were prepared using dispase digestion, dissection with jeweler's forceps under a dissecting microscope, and disaggregation of the epidermis by grinding through a steel sieve. Cells were stained with the α-2C TCR mAb 1B2 and a pan-γδ TCR mAb (GL3). Representative dot plots are gated on live lymphocytes. Note: numbers indicate percentage of total cutaneous cells (non-enriched sample).
pathogenesis is unclear.

Next, the frequency and immunophenotype of cutaneous IEL in B6, DBA/2, and mixed (B6xDBA/2)N1 mice expressing the 2C TCR transgenes was assayed by flow cytometry. In 2C-expressing animals, no cutaneous γδ T cells were appreciated in any of the backgrounds while all of the αβ-expressing cells within the epidermis of these animals expressed the 2C receptor (Fig. 45). Furthermore, D2C mice were observed to have a reduced number of cutaneous 2C IELs relative to B2C and (B6xDBA/2)N2C animals, analogous to that seen with non-transgenic animals (Fig. 45). The replacement of cutaneous γδ cells by Tg TCR cells has been described previously, with the completeness of this process determined by the timing of the Tg TCR expression in the thymus [113]. The complete absence of γδ cells suggested that the 2C TCR was expressed before day 14 of development, after which Vγ3Vδ1 ontogeny begins [93].

Immunohistochemical analysis of the skin from B2C, (B6xDBA/2)Nγ2C, and D2C mice using the 2C TCR-specific mAb 1B2 revealed that 2C cells assume a dendritic arrangement with fine branching processes reaching between adjacent keratinocytes, a configuration seen with cutaneous γδ T cells (Fig. 46A). 1B2 immunohistochemical staining of the gut from these same animals also revealed large numbers of 2C IELs and 2C lamina propria cells (Fig. 46B). These data suggested that all IEL populations in 2C mice express the 2C TCR while the development of dendritic morphology in cutaneous IELs may signify that these cells are behaving like their cutaneous γδ counterparts. However, previous studies have demonstrated that TCR transgenic mice with a complete replacement of cutaneous γδ cells with Tg T cells are akin to TCRδ−/− mice [344]. These studies demonstrated that the failure to express the canonical Vγ3Vδ1 TCR, which coordinately binds to stress ligands together with the stress ligand receptor NKG2D [15, 342], impairs the ability of these cells to perform their normal immunosurveillance duties [344].
Figure 46. 2C Cells Located within Epithelial Sites

(A) Acetone-fixed frozen tissue sections of (B6xDBA/2)N12C mouse skin were stained with the biotinylated α-2C TCR mAb 1B2. HRP-labeled Streptavidin and the Vector® Nova Red HRP immunohistochemistry kit were used to develop the staining. Representative photomicrographs are shown. (B) Acetone-fixed frozen tissue sections from the small intestine of (B6xDBA/2)N12C mice were stained with 1B2 as described above. Representative photomicrographs are shown.
Nevertheless, other studies have demonstrated that cutaneous IELs expressing a TCR other than the canonical Vy3Vδ1 receptor may also behave like typical DETC [345]. Therefore, although these data indicate that expression of the 2C TCR transgenes abrogated the normal development of cutaneous γδ IEL, this neither supports nor refutes the possibility that 2C TCR-expressing IELs are capable of protecting against cutaneous insults.

Interestingly, BALB/c mice, which are also known to develop spontaneous inflammatory skin disease when expressing the 2C TCR, share with DBA/2 mice a lymphopenia of cutaneous IEL [343]. This observation suggested that this cutaneous IEL "defect" might represent an important factor in disease pathogenesis. To determine if this lymphopenia of cutaneous IEL on the DBA/2 background was involved in controlling disease penetrance, the inheritance pattern of this phenotype was first ascertained by quantifying the number of lymphocytes in anti-CD3-stained epidermal sheets by immunohistochemistry. As previously seen by FACS® analysis, the frequency of CD3 positive cells in the skin of DBA/2 and D2C mice was considerably lower than in B6 and B2C mice respectively while the equivalent number of cutaneous IELs in B6 and (B6xDBA/2)N1 mice as well as in B2C and (B6xDBA/2)N12C mice was once again appreciated (Fig. 47A, B). In the second backcross generation, equal numbers of N22C mice had high levels (B6-like) and low levels (DBA/2-like) of cutaneous 2C cells (Fig. 47C). These data were consistent with an autosomal recessive pattern of inheritance of this phenotype and, interestingly, this was similar to the pattern of disease penetrance in N22C backcrosses to the DBA/2 background.

Given this similar inheritance pattern and the important immunosurveillance role of cutaneous IELs, it was hypothesized that a reduced frequency of cutaneous IEL might confer an increased susceptibility to disease. To determine if a correlation existed between the frequency of 2C IEL and the susceptibility to disease, these parameters were compared in (B6xDBA/2)N22C
Figure 47. Microscopic Examination of Cutaneous Intraepithelial Lymphocyte Density

(A) Shaved skin from the indicated animals was floated dermal side down in a solution of ammonium thiocynate, followed by the manual separation of the intact epidermis. Epidermal sheets were acetone-fixed and stained with a biotinylated mAb against CD3 (145.2C11). HRP-labeled Streptavidin and the Vector® Nova Red HRP immunohistochemistry kit were used to develop the staining. Representative photomicrographs are shown. (B) Epidermal sheets were collected and stained as described above. The number of CD3$^+$ intraepidermal T cells in epidermal sheets was quantified per high power field (HPF) for both 2C transgenic and non-transgenic animals on the B6, DBA/2 and mixed (B6xDBA/2)N$_i$ background (n=4 animals per group). Data is demonstrated in bar graph format. (C) Epidermal sheets were collected and stained as described above. The number of CD3$^+$ intraepidermal T cells in epidermal sheets was quantified per HPF for both 2C transgenic and non-transgenic (B6xDBA/2)N$_2$ backcrosses to the DBA/2 background (n>10 animals per group). The number of intraepidermal CD3$^+$ T cells per HPF was arbitrarily divided into a "low" group (0-6) and a "high" group (7-14). Data is presented as the percentage of animals within each group in bar graph format.
backcrosses to the DBA/2 background (Fig. 48). Interestingly, (B6xDBA/2)N$_2$2C mice having the highest concentration of cutaneous 2C cells were found to have the most severe disease, while animals with the fewest 2C cells were more resistant to the development of disease and possessed a milder disease phenotype (Fig. 48). Contrary to what would be expected for a population of sentinel lymphocytes, these data suggest that an increased frequency of 2C cells in the skin is not more protective, further supporting the notion that transgenic lymphocytes cannot perform the surveillance functions of normal $\gamma\delta$ cells. Rather, these data seems to suggest that having a higher percentage of self-reactive cells in the skin is a risk factor for the development of increased cutaneous pathology, which is not unexpected especially given the known role of the self-reactive 2C cells in disease pathogenesis (Fig. 40).

6.2.2 Cutaneous Immunoregulatory Function of 2C IELs in D2C Mice

Since the specialized immunosurveillance properties of $\gamma\delta$ T cells such as wound healing and keratinocyte growth factor (KGF) liberation are believed to be dependent upon the presence of the V$\gamma$3V$\delta$1 $\gamma\delta$ TCR [344], it is suspected that transgenic mice devoid of $\gamma\delta$ cells may be akin to $\gamma\delta$ KO mice [344]. The finding that a high frequency of 2C IELs was not more protective than a lower frequency of these cells in (B6xDBA/2)N$_2$2C animals seems to assert this belief. However, given the possibility that cutaneous 2C IEL may represent $\gamma\delta$ cells incognito [97, 113], it is conceivable that these cells may still afford some protection to these animals. While the 2C receptor would not be expected to recognize MHC class 1B stress ligands, the constitutive expression of the stress ligand receptor NKG2D by cutaneous IEL [340] may impart some immunosurveillance function to the 2C IELs.

To address whether the 2C cells in the skin of 2C mice behave as sentinel lymphocytes, TCR$\delta^{+/}$, 2C, and wildtype control mice were challenged with an application of a 2% croton oil
Figure 48. Correlation of Intraepithelial CD3\(^+\) Cell Density with Susceptibility to the Development of Cutaneous Pathology

(A) Epidermal sheets were collected from dorsal skin and stained as described previously. The number of CD3\(^+\) intraepidermal T cells in epidermal sheets was quantified per HPF for (B6xDBA/2)N\(_2\)2C backcrosses to the DBA/2 background (n=20 animals). The number of intraepidermal CD3\(^+\) T cells per HPF was arbitrarily divided into a "low" group (0-6) and a "high" group (7-14). Prior to skin harvest for this analysis, animals had been observed daily for the development of disease, which was graded according to the previously described scale. Data is presented as a scatterplot demonstrating the relationship between the density of CD3\(^+\) IEL and susceptibility to disease. (B) This same data is presented in bar graph format.
solution [346]. In normal animals, a 2% solution of this cutaneous irritant causes local erythema, which peaks within 2–3 days after application and thereafter resolves [347]. However, in TCRδ−/− mice, the application of croton oil results in a more profound and prolonged cutaneous inflammatory response [139] due to an impaired cutaneous barrier and an absence of cutaneous immunoregulatory function in these mice [139, 344]. Consistent with the literature, both B6 and DBA/2 wildtype mice were resistant to lesion development (Fig. 49), while TCRδ−/− mice developed ulcerated cutaneous defects. Interestingly, croton oil painting of both B2C d and D2C mice resulted in the development of lesions similar to those observed on TCRδ−/− animals (Fig. 49). These results indicate that, at least in this irritant assay, neither a high (B2C d) nor a low (D2C) frequency of cutaneous 2C IELs are capable of maintaining a normal barrier function or repelling circulating inflammatory cells, functions previously ascribed to cutaneous γδ IEL [139, 344]. These data are therefore consistent with those previous studies proclaiming that TCR transgenic animals lacking Vy3Vδ1 T cells in the skin are akin to TCRδ−/− mice [344]. Furthermore, the replacement of these immunoregulatory cells with self-reactive 2C lymphocytes, capable of undergoing bystander stimulation, imparts to these mice a strong susceptibility to the development of spontaneous, localized autoimmune disease.

6.2.3 Adoptive Transfer of Disease is Associated with the Replacement of Cutaneous γδ T Cells with 2C Lymphocytes

With the knowledge that 2C mice were akin to TCRδ−/− animals and that 2C cells in the skin exacerbated rather than protected from disease, an attempt to understand the results of previous adoptive transfer experiment was made. The finding that (B6xDBA/2)NI2C and B2C d, but not D2C, bone marrow could adoptively transfer disease to DBA/2 recipient mice was particularly perplexing (Fig. 50A, B), especially since all of these resulting chimeric animals had
A 2% croton oil in acetone solution was applied with a cotton swab to the trimmed dorsal trunk of indicated mice (n=5 animals per group). Five days post-application, mice were euthanized and the extent of gross disease was documented. Representative H&E-stained histological sections taken from the croton oil application area are shown.
Figure 50. Susceptibility of DBA/2 Recipient Mice to Disease Following Transfer of D2C and B2Cd Bone Marrow

(A) Lethally irradiated (1150 rads) DBA/2 mice were transplanted with T cell-depleted bone marrow from either D2C or B2Cd donor animals (n=4 animals in each of three individual experiments). Representative gross and microscopic photographs are shown. (B) The effectiveness of the bone marrow transplant in transferring disease was quantified by observing and grading resultant disease in the indicated bone marrow chimeras (BMC). The data is summarized in bar graph format. (C) Three months post-transplant, LN were harvested from the indicated BMC, prepared for flow cytometry, and stained with mAbs against the 2C TCR (1B2) and CD69 (H1.2F3). Representative histograms are gated on live 1B2+ cells.
nearly indistinguishable hematopoietic systems upon reconstitution (data not shown). Given the similar thymic and peripheral lymphoid compartments in these chimeric animals (data not shown), an evaluation of the cutaneous immune network was undertaken to determine whether differences in the skin could account for the differing susceptibility of these chimeric animals to disease (Fig. 51A). This possibility was plausible given the incomplete reconstitution of skin immune cells following bone marrow transplant. For example, skin dendritic cells may not be cleared by the γ-irradiation used to prepare recipients for a bone marrow transfer, as this generally requires UV irradiation [348], and cutaneous IEL may be replenished by a radio-resistant precursor within the thymus [349], thwarting the colonization of the skin by donor-derived cells.

Examination of the skin of disease-susceptible DBA/2 recipients of B2C or (B6xDBA/2)N12C bone marrow by flow cytometry revealed that more than two-thirds of the cutaneous IEL were 2C cells with the remainder expressing the canonical Vγ3Vδ1 TCR (Fig. 51A, B; Table 6), while the epidermis of disease-resistant DBA/2 recipients of D2C bone marrow was populated almost exclusively by Vγ3Vδ1 γδ T cells (Fig. 51A, B; Table 6). As the 2C hematopoietic system does not support the development of γδ T cells (Fig. 45), these cutaneous γδ sentinels had to have been derived from the DBA/2 recipients. This fortuitous discovery argued in favour of the possibility that replacement of the immunoregulatory γδ cutaneous IEL with self-reactive 2C lymphocytes was a critical factor in disease pathogenesis.
**Figure 51. Characterization of Cutaneous Intraepithelial Lymphocytes in DBA/2 Recipients of D2C and B2C<sup>d</sup> Bone Marrow**

(A) Acetone-fixed frozen tissue sections from the dorsal trunk skin of indicated bone marrow chimeras (BMC) were incubated with the biotinylated α-2C TCR mAb (1B2). HRP-labeled Streptavidin and the Vector<sup>®</sup> Nova Red HRP immunohistochemistry (IHC) kit were used to develop the staining. Representative photomicrographs are shown. (B) Epidermis from indicated BMC animals was separated from underlying dermis using jeweler's forceps and a dissecting microscope after incubation in a solution of dispase type II. Epidermal cells were disaggregated by vigorous pipetting and straining through a steel sieve. The cells were stained with 1B2 and a pan-γδ TCR mAb (GL3). Representative dot plots are gated on live cells. Note: numbers indicate percentage of total cutaneous lymphocytes.
Table 6. Cutaneous Lymphocyte Subsets in the Various Mouse Strains Studied

<table>
<thead>
<tr>
<th>Subjects</th>
<th>$\gamma\delta^+\text{ DETC}$ (Relative %)</th>
<th>$2C^+\text{ DETC}$ (Relative %)</th>
<th>Developing Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2</td>
<td>100% ± 0%</td>
<td>0% ± 0%</td>
<td>0%</td>
</tr>
<tr>
<td>D2C</td>
<td>0% ± 0%</td>
<td>100% ± 0%</td>
<td>100%</td>
</tr>
<tr>
<td>$B2C^d$ bone marrow $\rightarrow$ B6$^d$ recipient</td>
<td>91.5% ± 6%</td>
<td>8.5% ± 6%</td>
<td>0%</td>
</tr>
<tr>
<td>$B2C^d$ bone marrow $\rightarrow$ DBA/2 recipient</td>
<td>27.9% ± 3%</td>
<td>72.1% ± 3%</td>
<td>100%</td>
</tr>
<tr>
<td>D2C bone marrow $\rightarrow$ B6$^d$ recipient</td>
<td>99.3% ± 0%</td>
<td>0.7% ± 0%</td>
<td>0%</td>
</tr>
<tr>
<td>D2C bone marrow $\rightarrow$ DBA/2 recipient</td>
<td>92.1% ± 4%</td>
<td>7.9% ± 4%</td>
<td>0%</td>
</tr>
<tr>
<td>D2C DNTC $\rightarrow$ DBA/2 recipient</td>
<td>100% ± 0%</td>
<td>0% ± 0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

6.2.4 Adoptive Transfer of Disease to DBA/2 TCR$\delta^+$ Mice

Based on the suspected importance of $\gamma\delta$ lymphopenia in the D2C model system and the development of spontaneous skin inflammation in TCR$\delta^+$ mutant mice on some inbred strains, it was of interest to see whether the expression of the TCR$\delta^+$ mutation on the DBA/2 background would be associated with the development of spontaneous skin inflammation. In order to test the sufficiency of $\gamma\delta$ lymphopenia for development of the D2C disease phenotype, the TCR$\delta^+$ mutation was partially backcrossed from the B6 to the DBA/2 background (5 backcross generations). The resultant DBA/2 TCR$\delta^+$ mice remained disease-free and were indistinguishable from DBA/2 TCR$\delta^{+/c}$ littermates (Fig. 52A, B). Furthermore there was no microscopic evidence of subclinical disease (Fig. 52A), or other features of D2C pathology such as hypergammaglobulinemia (Fig. 52C). These findings were not unexpected since DBA/2 TCR$\delta^+$ mice are not lymphopenic of conventional lymphocytes nor do these animals have a deficiency of CD4$^+$CD25$^+$ $T_{reg}$, and thus these animals do not experience the massive dysregulated expansion of T lymphocytes typical of unmanipulated D2C animals. Furthermore,
Figure 52. Characterization of DBA/2 TCRδ⁻/⁻ Mice

(A) B6b TCRδ⁻/⁻ mice were backcrossed 5 generations to the DBA/2 background. Representative gross photographs of a DBA/2 γδ⁻/⁻ mouse and a DBA/2 γδ⁺/+ littermate control mouse are shown. Representative photomicrographs of rostral skin from a DBA/2 γδ⁻/⁻ mouse and a DBA/2 γδ⁺/+ littermate control mouse are shown. (B) The extent of clinical disease in DBA/2 TCRδ⁻/⁻ and DBA/2 TCRδ⁺/+ littermate controls was monitored and depicted in the shown bar graph (n=25 animals per group). (C) Serum was collected from DBA/2 TCRδ⁻/⁻ and DBA/2 TCRδ⁺/+ littermate controls and assayed by ELISA for serum IgG as previously described.
these animals do not have large populations of circulating and intraepithelial self-reactive 2C lymphocytes. Therefore, despite the importance of γδ dysfunction in the model system, a lymphopenia of cutaneous γδ IELs is not sufficient for the induction of spontaneous psoriasiform disease.

Given the purported role of γδ T cells in the disease resistance of DBA/2 recipients of D2C bone marrow (Fig. 50, 51; Table 6), it was hypothesized that D2C bone marrow could adoptively transfer the disease phenotype to DBA/2 TCRδ+/− mice. In these chimeric mice, no radio-resistant thymic precursor cells would be able to successfully compete with donor-derived 2C lymphocytes for epithelial colonization. Therefore D2C bone marrow was adoptively transferred to littermate TCRδ+/− and TCRδ−/− mice. DBA/2 TCRδ−/− recipients developed a moderate disease phenotype characterized by S1-S2 cutaneous pathology and typical psoriasiform histopathology while TCRδ+/− recipients remained free from disease (Fig. 53A, B). Furthermore, the majority of 2C cells in DBA/2 TCRδ−/− recipients of D2C marrow were acutely activated, expressing high levels of CD69 while only a small minority of 2C cells were acutely activated in TCRδ+/− recipients (Fig. 53C). The lesser phenotype of these DBA/2 TCRδ+/− recipients (Fig. 53B), relative to "wildtype" D2C mice (Fig. 17) or DBA/2 recipients of B2Cld bone marrow (Fig. 18B, 50B), could be attributable in part to the incomplete backcrossing of the TCRδ mutation to the DBA/2 background, as only five backcrosses were performed. Therefore, it is plausible that residual B6 genes may have precluded the generation of the full disease phenotype. Other considerations such as the age of the recipient mice as well as the prevailing housing conditions may have also impacted upon the ultimate disease phenotype of these chimeras. Nevertheless the spontaneous disease development in DBA/2 TCRδ+/− but not DBA/2 TCRδ+/− bone marrow chimeras demonstrated the necessity of cutaneous γδ lymphopenia in D2C
Figure 53. Characterization of DBA/2 TCRδ−/− Recipients of D2C Bone Marrow

(A) Lethally irradiated (1150 rads) DBA/2 TCRδ−/− and littermate control DBA/2 TCRδ+/- mice were transplanted with T cell-depleted D2C bone marrow (n=4 animals per group). Ten weeks post-transfer, animals were sacrificed for necroscopic examination. Representative gross and microscopic photographs of the rostral skin are shown. (B) The effectiveness of disease transfer with D2C bone marrow to DBA/2 TCRδ+/- and DBA/2 TCRδ−/− recipients was quantified by observing and grading resultant disease in the indicated bone marrow chimeras (BMC). The data is summarized in bar graph format. (C) Ten weeks post-transplant, LN were harvested from the indicated BMC, processed for flow cytometry, and stained with mAbs against the 2C TCR (1B2) and CD69 (H1.2F3). Representative histograms are gated on live 1B2+ cells. (D) Epidermis from indicated BMC animals was separated from underlying dermis using jeweler’s forceps and a dissecting microscope after incubation in a solution of dispase type II. Epidermal cells were disaggregated by vigorous pipetting and straining through a steel sieve. The cells were stained with 1B2 and a pan-γδ TCR mAb (GL3). Representative dot plots are gated on live cells. Note: numbers indicate percentage of total cutaneous lymphocytes.
psoriasiform pathology (Fig. 53). Future studies on these chimeric mice will focus upon whether an intact cutaneous barrier in DBA/2 recipients of D2C bone marrow, maintained by a resident population of γδ T cells, prevented the microbial Ag-specific expansion of CD4 T cells, relative to the DBA/2 TCRδ−/− recipients of D2C bone marrow in which this expansion would be predicted to occur. Such a finding would demonstrate that the disruption of the cutaneous barrier occurs proximal to the dysregulated CD4 T cell expansion and thus represents the proximal-most defect in disease pathogenesis.

6.3 Conclusion

Despite the implication of cutaneous opportunistic pathogens, a population of self-reactive 2C cells, and a dysregulated expansion of CD4⁺CD25⁻ T cells in D2C disease pathogenesis, these elements alone were insufficient for disease as demonstrated by the failed adoptive transfer of disease to DBA/2 recipients by D2C bone marrow. The data presented in this chapter demonstrates an important collaborative role for cutaneous γδ T cell lymphopenia in disease pathogenesis, which is necessary (Fig. 53) but not sufficient (Fig. 52) for disease development. It is not surprising that cutaneous γδ T cells, or rather a lack there of, was found to have a role in D2C disease pathogenesis as these cells are known to have a critical role in the immunosurveillance against cutaneous infection, the immunoregulation of inflammatory cells, and the general homeostasis of the skin. These critical functions of cutaneous γδ T cells, made a possible defect in these important sentinel lymphocytes a potentially unifying factor in disease pathogenesis worthy of further investigation.

As opportunistic pathogen overgrowth represents a component of SD disease pathogenesis [203, 219], the finding that stress ligands are upregulated in the lesional skin of SD
patients is not unexpected [227]. Normally, the recognition of such stress ligands by the coordinate binding by the Vγ3Vδ1 TCR and the NKG2D receptor on sentinel cutaneous γδ T cells [15, 342] would activate these cells to perform essential functions aimed at restoring cutaneous homeostasis [344]. For example, the γδ T cell recognition of stressed cells [322, 323] would result in the apoptotic death of infected keratinocytes [336], thereby abrogating the profound release of preformed and newly synthesized inflammatory mediators from keratinocytes [350]. Moreover, the release of the anti-chemotactic factor pTβ4 and the upregulation of more potent splice variants of this factor by activated γδ T cells [139, 140] would repel circulating inflammatory cells, preventing their exocytosis into the skin [139], while the secretion of KGF by γδ T cells would result in the proliferation and maturation of the epidermis [344], restoring the cutaneous barrier against pathogen invasion. Given the results of these D2C studies and the findings in human psoriasiform studies of high cytokine concentrations [167, 226, 351], prominent inflammatory cell infiltration [167, 228], and an impaired cutaneous barrier in lesional skin [167, 352], it is tempting to speculate that an impairment in the stress ligand/sentinel γδ-T cell axis may exist in psoriasiform disease. This hypothesis is made even more intriguing by the known association between psoriasis susceptibility and the 5.1 allelic variant of the MICA molecule [199, 200], which is characterized by a naked transmembrane protein deficient in its extracellular “signalling” domain that results from a premature stop codon. Therefore it is possible that an impaired recognition of stressed keratinocytes in psoriasiform disease and the subsequent failure to activate sentinel γδ T cells may result in the persistence of stressed cells and a chronic outpouring of inflammatory cytokines which orchestrate a massive exocytosis of inflammatory cells into the skin, including those capable of undergoing bystander stimulation and upregulating NKG2D receptor.
Given the possible impairment in immunosurveillance by cutaneous γδ-T cells in patients with psoriasiform disease, it is likely that these individuals have a lower resistance to microbial challenge, which is consistent with the known role of opportunistic infection in SD pathophysiology. It is therefore not surprising that disease is aggravated by conditions favoring greater microbial colonization, such as the presence of damp, oily skin [165, 204]. These observations are not unlike what is observed in D2C mice where disease was found to be aggravated by unhygienic conditions [123]. A similar phenomenon was observed in a related model of cutaneous inflammation which involves the expression of the TCR δΔ mutation on the FVB background [139], where it was found that mice can be made resistant to disease by being housed individually in ventilated cages. These housing conditions in which continuous airflow lowers the ambient humidity, reduces the levels of irritants and diminishes the potential for enhanced microbial colonization of the skin [126] would likely have a similar effect upon D2C disease since this condition is known to involve microbial overgrowth (Fig. 10). Although the current unavailability of reagents procludes this investigation, it is predicted that lesional skin in D2C mice will be shown to have an increased expression of stress ligands. This finding would suggest that opportunistic infection of rostral skin with lipophilic organisms induces susceptibility to a localized autoimmune attack from NKG2D-expressing effector cells by causing a prolonged expression of epithelial stress ligands.

The disruption of the cutaneous barrier and the increased colonization by opportunistic organisms in D2C mice likely results in a greater load of microbial Ag presented in the draining lymph nodes, which in part drives the massive CD4 expansion occurring in these mice (Fig. 23B, D). Although D2C Rag-1Δ mice are deficient in γδ cells and have a slightly higher burden of opportunistic pathogens on the skin as assessed by histology (data not shown), these animals were resistant to disease (Fig. 44A) due to their deficiency of CD4ΔCD25Δ T cells. Future
experiments are planned to see whether the adoptive transfer of CD4⁺CD25⁻ T cells will restore the disease susceptibility of D2C Rag-1⁻⁻ mice. To further implicate the lymphopenia of γδ T cells in the model system, it is also planned to transfer DBA/2 Vγ3Vδ1 T cells to D2C Rag-1⁻⁻ recipient mice prior to the administration of CD4⁺CD25⁻ T cells. It is expected that the transferred γδ T cells will abrogate disease development in these recipient animals by restoring the epidermal barrier, thereby precluding the delivery of microbial Ag and the subsequent dysregulated expansion of CD4⁺ T cells.

The conclusion that cutaneous γδ T cell deficiency was critical for disease pathogenesis was first suggested by the results of bone marrow chimera studies (Fig. 53). This fortuitous observation is strongly indebted to a peculiarity of IEL homing which exists between inbred stains. Why B2C⁺ but not D2C bone marrow was able to seed the recipient epidermis with 2C cells may somehow be related to the different frequencies of IEL in these inbred mouse strains (Fig. 45), as has been seen in other inbred strains [343]. The data suggest that given the same background genes, an IEL with the canonical Vγ3Vδ1 can outcompete cells with an “incorrect” receptor for epidermal colonization (Table 6). However, the data also suggests that the homing potential of DBA/2 cells to the epidermis is so “impaired” that a cell expressing an incorrect TCR (ie., the 2C TCR) can outcompete a DBA/2 Vγ3Vδ1 cell for epidermal residence if derived from a background with more favourable homing properties, for example the B6 background (Table 6). A cursory investigation of this DBA/2 skin homing impairment included an assay for the expression of the αE integrin CD103, which has been shown to be critical for lymphocyte residence in epithelial compartments [353]. While previous studies demonstrated that deficiencies in this receptor predisposed to inflammatory skin disease in certain mouse models [354], DBA/2 Vγ3Vδ1 T cells were shown to express high levels of CD103 (data not shown). Nevertheless, the ill-defined DBA/2 “impairment” in cutaneous γδ homing or survival was not
identified nor was this found to be of any physiological significance, as the lower concentration of cutaneous γδ cells in the DBA/2 recipients of D2C marrow was fully capable of suppressing the entire disease phenotype.

The greater understanding of the cutaneous immune network afforded by the aforementioned bone marrow chimera experiments was also instrumental in interpreting the results of previous experimentation. For example, the transfer of peripheral lymphoid CD69+ 2C cells to irradiated DBA/2 mice was unsuccessful in the adoptive transfer of the disease phenotype, demonstrating that 2C cells were not sufficient for disease (Fig. 42). The disease resistance in these recipient animals was attributed to a lack of T cell lymphopenia and CD4 repertoire skewing, and a bountiful T_{reg} population. Although these factors are known to be important for disease development, examination of the skin of these recipients demonstrated a full complement of Vγ3Vδ1 cells and a complete absence of 2C IEL (Table 6). Therefore, even if these animals were rendered immunodeficient and lymphopenic of T_{reg} by concomitant experimental manipulations, these recipients would be resistant to disease as a result of a full complement of cutaneous sentinel γδ T cells. The failure of the donor 2C cells to colonize the skin (Table 6) likely reflects the fact that skin homing requires the requisite developmental programming of IEL precursors in the thymus [349], an education not received by the peripheral 2C T cells used as donor lymphocytes in this experiment.

Although these data are exciting and may potentially contribute to our understanding of human psoriasiform disease, several discrepancies between murine and human cutaneous anatomy may limit the application of this data. Specifically, the epidermis of murine skin is only a few cell layers thick while human epidermis is significantly thicker (8-10 cell layers thick) providing a more formidable barrier to opportunistic pathogens [202]. However the lesional skin of D2C mice appears to conform to the typical psoriasiform reaction pattern observed in human
psoriasiform diseases (Fig. 4, 6, 9), indicating that the morphological differences in the skin between these species may be less applicable to this model system. Another obvious difference between murine and human skin is the lymphocyte subsets residing within the epidermis. While $\gamma\delta$ T cells comprise the principal cutaneous lymphocyte population in murine skin (>95% of all T cells) [341, 355], human skin has a more heterogeneous population of cells with only a minority of cells expressing a V81 TCR [356]. Given the unique monoclonal population of $\gamma\delta$-T cells in murine skin, the generalization of cutaneous findings in murine model systems to other organisms is a valid concern. However, the $\gamma\delta$ T cell population in human epithelia is known to respond to the stress-induced expression of MICA [15] and therefore these cells likely behave similarly to their murine counterparts. Therefore, even after due consideration of these differences in cutaneous architecture and immunity, this reliable disease model will still undoubtedly contribute to our understanding of human psoriasiform disease.

The innumerable parallels between human SD and D2C inflammatory skin disease suggest that an impairment in cutaneous $\gamma\delta$ function may be a central defect in SD pathophysiology. Emerging studies on human psoriasiform disease support this possibility [199, 200, 227] which will need to be confirmed by additional research. The ability of the D2C model to direct relevant experimentation on human psoriasiform disease, and to serve as a reliable tool for in-depth study of disease pathophysiology, exemplifies the utility of this unique model system.
Chapter 7: Implications of the D2C Model on Contemporary Hypotheses of Psoriasiform Disease Pathogenesis

7.1 Introduction

The psoriasiform diseases are amongst the earliest diseases documented, as Hippocrates wrote about psoriasis [191] and ancient Egyptians used sap from the sycamore tree and extracts from the fig to treat such lesions [357, 358]. Despite the long history of these diseases, and over twenty thousand research publications to date, no clear consensus about disease pathophysiology has emerged with a divergent group of hypotheses including infection [193, 195], autoimmunity [178, 359], and a primary hyperproliferative disorder of the skin [189, 190] all considered to be facets of disease pathogenesis. The initial work with D2C mice has demonstrated that this model is an accurate representation of the human psoriasiform disease SD, and this work has already contributed to the understanding of how the various aspects of SD disease pathogenesis fit together in this complex skin disorder. The impact of the D2C model on the various theories of disease pathogenesis in psoriasiform disorders will be discussed in turn.

7.2 Role of Intrinsic Keratinocyte Defects in Psoriasiform Disease Pathogenesis

It was first theorized that the hyperproliferation of keratinocytes was the primary etiological factor in psoriasiform disease pathophysiology [178], leading to the introduction of anti-proliferative treatments such as selenium sulfide and zinc pyrithione [218] as well as anthralin, which inactivates enzymes associated with cell proliferation [176]. Psoralens became a mainstay of treatment in the 1970's when they were found, in association with UV irradiation, to interfere with DNA synthesis [175, 358]. The success of these treatments reinforced the belief that psoriasiform diseases were hyperproliferative disorders, and subsequent treatments popularized in the 1970's included methotrexate [182] and hydroxyurea [360], both of which are
known to inhibit keratinocyte proliferation [359, 360]. More recently, systemic and topical retinoids were added to the armamentarium of psoriasiform treatments [361], which have been shown to reduce keratinocyte proliferation by inhibiting DNA synthesis [362]. All of these treatments remain in use today [181, 361]; however, recognition of alternate mechanisms of action for these therapies has led to new insights into the mechanism of psoriasiform diseases. For example, both selenium sulfide and zinc pyrithione have been shown to possess anti-fungal properties [203, 363], implicating a role for opportunistic pathogens in psoriasiform disease. Similarly, anthralins have subsequently been shown to possess anti-inflammatory activity, such as the upregulation of the IL-10 receptor on cultured keratinocytes [364], which has shifted the focus of psoriasiform research primarily towards the role of immunity in disease pathogenesis. While methotrexate and hydroxyurea reduce keratinocyte proliferation [359, 360], these systemic therapies have also been shown to have a profound effect on inflammatory cells [359, 365]. For example, the effect of hydroxyurea on inhibiting ribonucleoside diphosphate reductase [366] is particularly toxic to activated T cells [365], which is consistent with the possibility that keratinocyte proliferation in psoriasiform disease may be a secondary response to the presence of activated T cells [178, 185]. Similarly the effect of systemic retinoids on treating disease may be more directly related to the effect of these drugs on inducing apoptosis in T cells [367], with the reduction of keratinocyte proliferation being a secondary effect. These observations have caused a major change in the thinking about psoriasiform disease with a recent emphasis on the role of autoimmune disease and/or an aberrant immune responses against benign epidermal commensal organisms taking central stage. However, this changing paradigm is not unanimously accepted as new evidence supporting the idea that psoriasis is a primary disease of the keratinocyte is still emerging [189-191]. For example, in psoriatic lesions, epidermal keratinocytes have reduced expression of JunB, a gene localized in the psoriasis susceptibility region PSORS6, and the
inducible epidermal deletion of JunB and its functional partner c-Jun in mice leads to a psoriasiform disease phenotype [189].

While studies in the D2C mouse support the new paradigm of psoriasiform pathogenesis, with strong evidence in favor of a dysregulated interaction between several T cell populations culminating in disease, these data do not definitively exclude the possibility that a primary keratinocyte disorder is also a feature of the disease model. Although the ability to adoptively transfer the disease phenotype to DBA/2 recipients by B2C\textsuperscript{d} or (B6xDBA/2)N\textsubscript{1}2C bone marrow (Fig. 18, 50) was originally interpreted as reflecting a requirement for DBA/2 non-hematopoietic factors, this data was later complicated by the realization that the variability of disease transfer was in part attributable to the ability to transfer cutaneous \(\gamma\delta\) lymphocytes between various strains (Fig. 51, Table 6). While disease was never transferred to a “disease-resistant” background such as non-transgenic B6\textsuperscript{d} or (B6xDBA/2)N\textsubscript{1} mice, this failure likely reflected an inability to eliminate recipient derived cutaneous \(\gamma\delta\) lymphocytes. Future experiments which address this issue will be pursued to determine whether DBA/2 skin is in fact uniquely susceptible to psoriasiform pathology, perhaps reflecting a primary defect of the keratinocyte.

7.3 Role of Infection in Psoriasiform Disease

Despite the initial demonstration of the presence of opportunistic fungi in lesional SD skin, early investigators could find no relationship between these organisms and disease pathogenesis [194, 203, 205]. However, the effective treatment of SD withazole antifungal agents [203, 363], and the resultant reduction in the number of Malassezia on the skin [368], clearly established a relationship between opportunistic pathogens and SD. Furthermore, a greater understanding of fungal biology has illustrated why these lipophilic organisms are trophic for “seborrheic areas” of the body [204, 369, 370], and how the direct fixation of complement
and the liberation of both free fatty acids and arachidonic acid [194, 370, 371] by these organisms results in considerable cutaneous inflammation. Despite this evidence, it is unclear as to why "normal" numbers of these pathogens can initiate an inflammatory reaction in susceptible individuals whereas those with an inherent resistance to disease can tolerate large burdens of these organisms on the skin [203, 205, 372]. These findings suggest that SD patients may be predisposed towards infection with these organisms or an aberrant immune response against them. The finding that the development of SD is associated with both complement deficiencies [210, 211] as well as AIDS [206, 208] supports this hypothesis; however, the susceptibility to SD in seemingly immunocompetent individuals may also be the result of subtle, unrecognized immunodeficiency. The finding that the skin of SD patients is characterized by the expression of high levels of MICA stress ligands [227] suggests that an impairment in the recognition and elimination of infected keratinocytes from the skin by sentinel γδ T cells may be one form of this unrecognized immunodeficiency. Evidence from the D2C model demonstrates that, while complement deficiency (Fig. 19) and AIDS-like CD4 lymphopenia (Fig. 26, 27) have a role in disease pathogenesis, a defect in cutaneous γδ T cell function is a critically important facet of psoriasiform pathophysiology (Fig. 53). Interestingly, in the related psoriasiform disease psoriasis, a mutation in MICA that results in a truncated stress ligand devoid of an extracellular component has been linked to psoriasis susceptibility [199, 200]. Such defects in the γδ-stress ligand pathway would not only predispose to overgrowth by opportunistic pathogens but would also result in the failure to activate sentinel cutaneous γδ lymphocytes, and thereby the failure to maintain a protective cutaneous barrier by KGF production [344]. Furthermore, a reduced output of pTβ4 from activated γδ cells in stressed skin [140] would fail to repel circulating memory phenotype cells [139] which, upon bystander stimulation, upregulate NKG2D [373] and thus have the ability to unleash an immunological assault upon stress ligand-bearing keratinocytes.
Since these circulating cells are not equipped to fight disease in the delicate epithelial environment, the resulting immune responses tend to be prolonged and inefficient [139, 163]. Interestingly, the formation of typical psoriasiform lesions in response to mild trauma in psoriasis (koebnerization) as well as the considerable exocytosis of neutrophils into the epidermis in SD and psoriasis [165, 166, 221] may be a manifestation of this impaired pTβ4 production. Therefore, the failure of γδ IEL to recognize cutaneous infection would result in inflammation attributable to: the microbial colonization itself; the prolonged release of preformed, inflammatory mediators from stressed keratinocytes; and the failure to exclude circulating inflammatory cells which exacerbate the cutaneous inflammation.

Therefore, infection arising in a background of a variety of immunodeficient states likely has a role in most cases of SD. Given the recent recognition of epithelial γδ T cell subsets and their specialized functions, as well as the unfolding understanding of stress ligand biology, it is not surprising that defective γδ T cell function and/or stress ligand immunobiology have not yet been widely recognized in SD.

### 7.4 Role of the Immune System in Psoriasiform Disease:

Much like many of the so-called "anti-proliferative" drugs used to treat psoriasiform disease were found to have antimicrobial effects, many drugs in the armamentarium of psoriasiform disease treatment have been shown to have anti-inflammatory properties. For example, recent studies have shown that anthralins [364], fumaric acid esters (FAE) [374], as well as vitamin D3 and its analogs [375] stimulate expression of IL-10 and/or its receptor, while phototherapy with UVB irradiation has been shown to result in a significant depletion of T cells from psoriatic lesions [359, 376]. Although retinoid-based therapies such as targretin have been
shown to suppress keratinocyte proliferation [377], these agents also work by inducing T cell apoptosis [367] and facilitating the upregulation of retinoic acid inducible stress ligands [378] that are recognized by sentinel Vδ1 IEL [15, 342]. Glucocorticoids, which have strong efficacy in the treatment of psoriasiform disease [171, 205], have been shown to possess both anti-inflammatory and immunosuppressive activities including their ability to abrogate the proliferation of T cells and inhibit inflammatory cytokine gene transcription [181]. Moreover, some azole antifungal treatments, some of which were instrumental in supporting the fungal theory of SD pathogenesis [203], have been shown to have potent anti-inflammatory activity [363, 379]. These findings have contributed to the belief that the immune system may be the central orchestrator of disease pathogenesis in psoriasiform disease, with keratinocyte hyperproliferation occurring secondary to T cell activation [178]. Such an interpretation is consistent with the findings that activated T cells are a major component of psoriasiform inflammation [228, 380] and the effective treatment of psoriasiform diseases with the calcineurin-inhibiting immunosuppressants [213, 359] involves the inhibition of T-lymphocyte signal transduction, IL-2 transcription, and the expression of IL-2R on T cells [381]. These findings in human psoriasiform disease are consistent with data from the D2C mouse where the administration of dexamethasone to pre-diseased D2C mice protected against the development of the disease phenotype (Fig. 34) and was associated with a massive reduction in the total number of T cells (Fig. 35).

The implication of immune dysfunction in psoriasiform disease pathogenesis has led to the production of numerous targeted "biologic" therapies, mostly in the form of monoclonal Ab which block critical immunological pathways or deplete critical lymphocyte populations [359, 382, 383]. For example, etanercept (soluble TNF-α receptor) as well as infliximab (humanized mAb against TNF-α) block the effect of TNF-α [359, 382, 383]. Daclizumab and Basiliximab,
mAbs against CD25, block IL-2 from interacting with IL-2R on T cells [359, 382, 383] while Efaluzimab, a mAb against the CD11a subunit of LFA-1, has been shown to block the interaction of LFA-1 and ICAM-1 and subsequently mitigate disease by decreasing T-cell activation and migration [359, 382]. Numerous other mAbs have been developed to block T cell activation including: Siplizumab, an mAb against CD2 that blocks CD2/LFA-3 interaction [359, 383]; OKTcdr4a, a humanized anti-CD4 IgG4 mAb that inhibits APC/T cell interaction [359, 382, 383]; and Galiximab a mAb against CD80 that blocks the provision of co-stimulatory molecules [359, 382-384]. A number of biologic agents such as the CD2-binding, LFA-3-Ig fusion protein Alefacept and the diphtheria toxin-IL-2 fusion protein ONTAK have been shown to deplete activated/memory phenotype cells in psoriasis [185, 359, 382]. Although none of these agents are specific enough to implicate one particular subset of lymphocytes in lesion development, data from the D2C model suggests that NKG2D-expressing, IFN-γ-secreting, memory phenotype T cells can contribute non-specifically to an ongoing inflammatory response though the process of bystander T cell activation (Fig. 37, 38, 40). Interestingly, histochemical studies on SD have demonstrated that an irritant-like, target tissue inflammation that occurs in this condition, can in fact result in an increased number of non-specific, memory phenotype lymphocytes (NK1⁺CD16⁺ NK cells) in SD lesional tissue [226].

The results from the D2C model suggest that disease resolution in these animals occurs after the established of sufficient T cell regulatory control. Since convalescent D2C mice have a persistent defect in cutaneous γδ-Tcells, these data also suggest that CD4⁺CD25⁺ Treg can compensate for this IEL deficiency. Interestingly, tolerization to ubiquitous commensal organisms has been shown to be dependent upon the function of IL-10-secreting Treg [385, 386]. While IFN-γ is consistently upregulated in psoriasiform disease [167, 387] and shown experimentally to be sufficient for lesion development in genetically susceptible individuals
[159], IL-10 which is a known downregulator of IFN-γ expression [387], together with its receptor (IL-10R) are consistently deficient in psoriasiform lesions [167, 387, 388]. Furthermore, a polymorphism in the IL-10 promoter is associated with the familial form of psoriasis [389]. This deficiency of IL-10 in human psoriasiform diseases [387] would be expected to result in a more stimulating milieu for T lymphocytes since IL-10’s anti-inflammatory functions include the induction of Ag-specific regulatory T cells [390, 391], reducing the expression of MHC class II [392, 393] and co-stimulator molecules [394], suppressing IL-2 transcription [387, 395] as well as contributing to T cell anergy [387, 396]. The invasion of opportunistic pathogens into such an environment would likely be associated with a vigorous, Ag-specific CD4 T cell response against microbial Ag; and not surprisingly a massive influx of CD4 T cells is present in the lesional skin of D2C mice (Fig. 9I) as well as in lesional skin from SD patients [228]. Results from the D2C mouse suggest that these CD4 cells play a pivotal role in psoriasiform disease pathogenesis as D2C mice devoid of CD4+CD25- "helper" T cells are resistant to disease (Fig. 44). The products of this T cell expansion, while initially harmful due to massive cytokine liberation, i.e., copious cytokine liberation, may eventually afford mild protection against disease as a result of partially-protective Ab production (Fig. 28); however, convalescence in these animals only occurs after T cell regulatory control has been established. Therefore it is tempting to speculate that a defect in IL-10 secreting T_{reg} is a critical feature of human psoriasiform disease pathogenesis. Such a prediction is consistent with the known ability of corticosteroids, which are highly effective in the treatment of psoriasiform disease [171, 205], to increase the relative number of T_{reg} [289], and the results of recent studies, which have demonstrated T_{reg} dysfunction to be a feature of psoriasiform disease [137].
7.5 Conclusion

After more than two millennia, an understanding of psoriasiform disease remains elusive and, with each insight gained, new questions arise. This illustrates the critical importance of a reliable animal model to test new hypotheses as well as potential treatments. The D2C model of psoriasiform disease clearly fits the current understanding of human psoriasiform diseases and demonstrates how a susceptibility to infection, development of target tissue inflammation, dysregulated T cell expansion due to defects in T_{reg} function, and cytokine-mediated bystander stimulation are intertwined in these complex disease processes (Fig. 54).
Figure 54. Summary of Factors Contributing to Disease Development

A schematic demonstrating the various components of disease pathophysiology is shown.
Bibliography


216


Appendices

Appendix 1: Contributions of Others

Scientific research often requires teamwork and collaborations to be successful. In this appendix, these contributions are outlined. In all of the collaborations detailed below, I played a significant role in some or all of the following: experimental design, data collection, data analysis, and data presentation. Furthermore, under the supervision of Dr. Hung-Sia Teh, I was responsible for overseeing and managing the many facets of the project.

Overall

The following Teh lab technicians provided superb technical assistance throughout the course of these studies: Edward Kim, Becky Dineson, and especially Soo-Jeet Teh.

Chapter 3

All experiments were done by myself at UBC, with the exception of the following:

- The identification of fungal isolates by initial ITS amplification was performed by Dr. Shelley Rankin (Assistant Professor, University of Pennsylvania, USA).
- The Vitek Yeast Biochemical card identification and ITS sequence analysis were performed by Dr. Koichi Makimura (Associate Professor, Teikyo University Institute of Medical Mycology, Japan).
- An attempt at ITS sequencing of DNA extracted from paraffin-embedded tissue was performed by Dr. Jacques Guillot (Professor, École Nationale Vétérinaire d'Alfort, France).
Chapter 4

A UBC project student, Mindy Hsieh, assisted with the fractionated CD4$^+$ T cell transfer experiments as well as the dexamethasone treatment studies. A UBC undergraduate project student, Lina Tang, played a major role in the acquisition and analysis of TCR Vβ repertoire studies as well as Ig ELISA experiments. A volunteer, Elisabeth Collett, assisted with many of the experiments and had an integral role in genotyping mice and the analysis of FACS® data.

Chapter 5

A volunteer, Elisabeth Collett, played a major role in genotyping and assaying both the DBA/2 Rag-1$^{-/-}$ and D2C Rag-1$^{-/-}$ mice.

Chapter 6

UBC undergraduate project students, Malene Ambjørn and Jenny Law, assisted with the cutaneous IEL analysis, DETC density experiments, and bone marrow chimeric studies.
Appendix 2: List of Publications

Journal Articles - Published

Oble DA and Teh H-S. 2001. Tight skin mouse subcutaneous hypertrophy can occur in the absence of $\alpha\beta$ TCR Bearing Lymphocytes. J Rheumatol. 28(8):1852-5.


Abstracts

Does the immune system have a role in the development of the TSK phenotype? 2000. American Association of Immunologists & the Clinical Immunology Society Joint Annual Meeting (Seattle, USA). Poster Presentation. FASEB abstract number 150.35.


228