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PROTECTION AND TREATMENT OF EXPERIMENTAL AUTOIMMUNE
ENCEPHALOMYELITIS BY THE ORAL ADMINISTRATION OF MYELIN BASIC
PROTEIN AND EXPANSION OF DENDRITIC CELLS USING FLT-3L

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Richard M. Wardrop, III, B.S.

*****

The Ohio State University
2000

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Approved by

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). The exact etiology is unknown but environmental, genetic, infectious, and immunological contributions to the disease have been described. Because MS shares striking clinical, histopathologic, and immunological similarities with the induced disease experimental autoimmune encephalomyelitis (EAE), EAE is widely used system to test putative therapies for MS. We have shown that the oral administration of the neuroantigen myelin basic protein (MBP) to rats and mice can both prevent and treat the progression of EAE. The induction of hypo-responsiveness via the oral administration of protein antigens is commonly referred to as oral tolerance. The induction of oral tolerance is under clinical evaluation for the treatment of several human inflammatory autoimmune diseases and allergies.

Little is known about the antigen presenting cells (APC) involved in the processing and presentation of orally introduced antigens. We hypothesized that dendritic cells (DC) participate in the presentation of orally administered antigens during oral tolerance induction. DC represent a unique population of APC in lymphoid and non-lymphoid tissues and are implicated in both tolerance and immunity in vivo. To test this
hypothesis, we expanded the normally trace population of DC using the DC growth factor Flt-3 ligand (FL). Administration of FL to EAE susceptible B10.PL mice expands both lymphoid derived and myeloid derived CD11c+/MHC Class II+ DC in the gut-associated lymphoid tissue (GALT) and periphery. To investigate the effects of DC expansion on oral tolerance, FL-treated or control mice were fed either a low or high dose of MBP prior to the induction of EAE. In FL-treated mice, the oral administration of both MBP doses caused a dramatic enhancement in disease protection versus oral antigen alone. Oral MBP in conjunction with DC expansion begun after resolution of the acute phase of disease profoundly decreased the severity of chronic disease when compared to controls. ELISPOT analyses showed that the frequency of IFN-γ, IL-2, IL-4, and TGF-β secreting cell numbers was dramatically decreased, suggesting an anergy/deletion mechanism for the inactivation of autoreactive T cells in vivo. Furthermore, DC isolated from the peripheral and mesenteric lymph nodes of FL-treated-MBP fed mice were capable of stimulating MBP specific CD4+ T cells ex vivo, indicating that DC became loaded with orally administered proteins in vivo.

Mechanistic studies in T cell receptor transgenic mice and in adoptive transfer experiments indicate that antigen specific T cells undergo rapid activation in vivo followed by multiple cell divisions following oral antigen administration in FL treated or control-treated mice. T cell activation was more extensive in FL-treated mice when compared to control mice indicating that activation and proliferation of MBP specific T cells may represent part of the tolerization process. MBP specific T cells from MBP-fed,
FL-treated mice are hyporesponsive to restimulation *in vitro* reinforcing the idea that antigen specific T cells are rendered anergic after oral antigen presentation.

These studies suggest that DC play a key role in presentation of MBP to self-reactive T cells, participate in the induction of oral tolerance, and that combining FL mediated DC expansion with oral antigen administration enhances oral tolerance in the face of an ongoing autoimmune disease process.
Dedicated to my wife Sarah and our daughter Mary Grace
ACKNOWLEDGMENTS

I wish to thank my adviser, Dr. Caroline C. Whitacre, for her support, both intellectual and financial over the past 3 1/2 years. I would also like to thank sincerely the members of the Whitacre lab both past and present without who, none of this work could have been done: Scott Stuckman, Ingrid Giennap, Dr. Fei Song, Dr. K.C. Dowdell, Dr. Connie Jo Rogers, Dr. Phillip Popovich, Dr. Jacqueline Benson, Dr. Najma Javed, Karen Cox, and Dr. Kim Campbell.

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PAPERS:


ABSTRACTS:


**FIELDS OF STUDY - Major Field: Molecular Virology, Immunology and Medical Genetics**
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xvii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xviii</td>
</tr>
</tbody>
</table>

## Chapters

1. Introduction

1.1 Multiple Sclerosis

1.2 Experimental Autoimmune Encephalomyelitis (EAE) as a Model for MS

1.3 Introduction to Oral Tolerance

1.3.1 General Mechanisms of Oral Tolerance Induction:
   - Active Suppression and Anergy/Deletion
   - Experimental Modulation of Oral Tolerance
   - Cytokines -administration and antagonism
   - Mucosal Adjuvants
   - Gamma Delta T Cells

1.4 Oral Tolerance for the Treatment of Inflammatory Autoimmune Diseases in Animal Models

1.4.1 Models of Autoimmune Arthritis
1.4.2 EAE..............................................................15
1.4.3 Models of Autoimmune Neuritis.................................18
1.4.4 Models of Autoimmune Diabetes.................................19
1.4.5 Models of Inflammatory Eye Disease.............................20
1.4.6 Other Models Where Oral Tolerance is effective in treatment... 21
1.5 Oral Tolerance for the Treatment of Disease in Humans ..........24
1.5.1 Multiple Sclerosis Clinical Trials..................................24
1.5.2 Rheumatoid Arthritis and Juvenile Rheumatoid Arthritis Trials...25
1.5.3 Uveitis Trials.......................................................26
1.5.4 Insulin Dependent Diabetes Mellitus Trials......................27
1.5.5 Oral Allergen Immunotherapy......................................27
1.6 Basic Considerations in the Establishment of Oral Tolerance ...28
1.6.1 The Fate of Ingested Protein Antigens............................29
1.6.2 Antigen Presenting Cells (APC) and Oral Tolerance............30
1.7 DC as immunomodulatory APC in vitro and in vivo................32
1.7.1 Flt3-L (FL) as a growth factor for DC in vivo.................33
1.8 Objectives............................................................34

2. Materials and Methods.....................................................35
2.1 Mice............................................................................35
2.2 FL Administration.......................................................36
2.3 Preparation of Neuroantigens and MBP..............................36
2.4 Induction of EAE........................................................36
2.5 Induction of Oral Tolerance and administration of oral antigens ...37
2.6 Cell Isolation Procedures................................................38
  2.6.1 Peripheral Lymphoid Organs....................................38
  2.6.2 Peyer's Patch (PP)..................................................38
  2.6.3 Intraepithelial Lymphocytes (IEL)...............................38
  2.6.4 Lamina Propria Lymphocytes (LPL)..............................39
2.7 Flow Cytometry..........................................................39
2.8 Purification of CD11c+ DC and CD4+ Transgenic T cells...........40
2.9 Ex vivo antigen presentation assays using Purified DC from MBP fed mice..................................................41
2.10 Proliferation Assays....................................................42
2.11 ELISPOT Analysis for Frequencies of Cytokine-Secreting Cells....43
2.12 CMFDA / CFSE Labeling of DC or CD4+ T cells and adoptive transfer..................................................44
2.13 Statistical analysis.....................................................45
3. Expansion of Functional DC in vivo using FL in B10.PL mice

3.1 FL administration to B10.PL mice expands CD11c⁺/MHC Class II⁺ DC of both myeloid and lymphoid lineage in multiple tissues

3.1.1 Administration of 10 μg/day FL for nine days expands CD11c⁺/MHC Class II⁺ DC in B10.PL mice

3.2 Expanded DC from FL mice are competent antigen presenting cells and can present both protein as well as peptide antigens to CD4⁺ TCR Tg cells

3.2.1 DC from FL treated mice are comparable to DC from non-FL treated mice

3.3 FL treatment does not exacerbate spontaneous EAE but does differentially alter the EAE disease course depending upon the time of administration

3.3.1 Expansion of DC with FL does not increase the rate of spontaneous EAE in two MBP specific TCR Tg mouse strains

3.4 Summary

4. Protection and Treatment of EAE by expansion of DC prior to feeding MBP in B10.PL mice

4.1 FL administration enhances oral tolerance to MBP resulting dramatic protection from EAE

4.1.1 Expanding Dendritic Cells prior to the oral administration of MBP enhances protection from EAE

4.1.2 Th1, Th2, and TGF-β cytokine responses are suppressed in protected mice despite no observable reduction in proliferative responses to MBP in vitro

4.1.3 CD11c⁺ DC from FL treated mice display low levels of accessory molecules but can upregulate expression in the presence of a pro-inflammatory stimulus

4.2 FL used in conjunction with oral MBP suppresses established EAE

xii
4.2.1 FL alone during REAE does not change the EAE but augments the therapeutic efficacy of oral MBP in REAE........  

4.3 Summary.............................................................................................................................................. 104

5. Fate of MBP specific T cells in vivo in FL treated MBP fed mice.......... 108

5.1 Rapid Activation of MBP specific T cells in vivo after feeding MBP... 108

5.1.1 Oral administration of MBP to FL treated TCR Tg mice leads to CD4+ T cell activation in multiple lymphoid organs in vivo.... 110

5.1.2 Development of an adoptive transfer model to study the fate of MBP specific T cells after their initial activation in vivo...... 114

5.1.3 MBP specific T cells are activated to a higher degree in FL treated mice than in control mice 18 hours following the oral administration of MBP.................................................... 121

5.2 Proliferation of MBP specific T cells in vivo after feeding.............. 124

5.2.1 MBP specific T cells proliferate in vivo after 20 mg oral administration of MBP but fail to accumulate as predicted.... 124

5.2.2 Tg T cells are hypo-responsive to in vitro restimulation 72 hours after the oral administration of MBP................................. 131

5.2.3 CD11c+ DC are pulsed with orally administered antigens soon after feeding antigen and can present it to T cells in vitro........ 136

5.3 Summary ............................................................................................................................................. 141

6. Discussion and Conclusions................................................................. 145

Bibliography................................................................................................................................. 172
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Administration of FL for nine days causes a marked expansion of total cell numbers in multiple tissues when compared to MSA controls</td>
<td>48</td>
</tr>
<tr>
<td>3.2</td>
<td>Administration of FL causes a marked expansion of CD11c$^+$ and MHC Class II$^+$ cells, but not CD3$^+$, CD4$^+$, or CD19$^+$ cells.</td>
<td>50</td>
</tr>
<tr>
<td>3.3</td>
<td>FL administration <em>in vivo</em> expands CD11c$^+$ / MHC Class II$^+$ DC</td>
<td>51</td>
</tr>
<tr>
<td>3.4</td>
<td>Analysis of CD11c$^+$ cell from FL treated mice demonstrates the CD11c is not expressed on CD3$^+$ T cells nor on CD19$^+$ B cells</td>
<td>52</td>
</tr>
<tr>
<td>3.5</td>
<td>FL expands both lymphoid and myeloid lineages of DC <em>in vivo</em></td>
<td>54</td>
</tr>
<tr>
<td>3.6</td>
<td>FL administration expands DEC-205$^+$ cells in the PP and myeloid DC in the liver</td>
<td>56</td>
</tr>
<tr>
<td>3.7</td>
<td>Positive selection of CD4$^+$ T cells from transgenic mice using anti-CD4-conjugated microbeads yields highly pure CD4$^+$/Tg TCR$^+$ cells</td>
<td>59</td>
</tr>
<tr>
<td>3.8</td>
<td>Purified naïve CD4$^+$ T cells from MBP specific TCR Tg mice proliferate in response to antigen or TCR stimulation but not as a result of purification</td>
<td>62</td>
</tr>
<tr>
<td>3.9</td>
<td>Antigen presenting capacity is dramatically enhanced in multiple cell populations by the administration of FL</td>
<td>64</td>
</tr>
<tr>
<td>3.10</td>
<td>Positive magnetic selection of CD11c$^+$ cells from FL animals yields highly pure DC from multiple lymphoid organs</td>
<td>67</td>
</tr>
<tr>
<td>3.11</td>
<td>Purification of DC using CD11c magnetic selection does not alter the surface expression on multiple costimulatory molecules</td>
<td>68</td>
</tr>
</tbody>
</table>
3.12 The DC based antigen presentation assay using CD4+ T cells is sensitive over a variety of antigen concentrations 71

3.13 DC purified from FL treated mice present both soluble protein and peptide antigens to CD4+ Tg cells 72

3.14 FL administration does not exacerbate the development of spontaneous EAE in either of two strains of MBP specific TCR transgenic mice 75

3.15 FL variably affects EAE in B10.PL mice depending on when it is administered 77

3.16 FL treated and control mice generate similar MBP proliferative responses in the LNC and spleen regardless of disease protection 80

4.1 FL enhances oral tolerance to MBP at both a low and high dose of oral MBP, but does not alter EAE 86

4.2 Th1, Th2, and TGF-β cytokine responses are suppressed in FL treated mice fed 2 or 20 mg oral MBP prior to sensitization 90

4.3 CD11c+ DC from FL mice express low levels of accessory molecules in the absence of pro-inflammatory stimuli 93

4.4 FL administration does not exacerbate EAE when administration is begun after acute disease has remitted 96

4.5 FL expands CD11c+ MHC Class II+ DC in mice with EAE similarly to naïve mice 97

4.6 Multiple oral administrations of MBP in conjunction with FL decrease the severity of EAE to a greater extent than feeding MBP alone 100

4.7 Cumulative clinical scores and mean clinical scores / day are improved in FL treated MBP fed mice 101

4.8 Proliferative responses to MBP are decreased in mice receiving 7 doses of oral MBP as therapy for REAE 103
5.1 Vβ8+ T cells are activated in the MLN of FL treated mice within 18 hours after oral MBP administration.

5.2 2 mg or 20 mg oral MBP activates a large proportion of Vβ8.2+/CD4+ TCR Tg T cells after feeding the peripheral lymph nodes.

5.3 CMFDA labeled cells are detectable *in vivo* as a discrete Vβ8.2+/CMFDA+ population.

5.4 CMFDA labeled cells remain naïve but can respond to antigen *in vivo* in B10.PL mice.

5.5 CFSE labeled cells persist *in vivo* for up to eight days as a distinct population.

5.6 Different degrees of MBP specific T cells activation in FL treated vs. MSA treated mice after a 20 mg feed of MBP.

5.7 MBP specific CD4+ T cells proliferate *in vivo* after 20 mg oral MBP.

5.8 CFSE+ cells do not accumulate in the PLN, MLN, or spleen of MBP fed mice after feeding.

5.9 MBP specific T cells are less responsive to restimulation *in vitro* 72 hours after oral administration of MBP.

5.10 MBP specific CD4+/CFSE+ cells display hypo-responsiveness when restimulated *in vitro* 72 hour after 20 mg oral MBP administration to FL treated mice.

5.11 APC from FL-treated-MBP-fed mice induce proliferation in MBP specific T cells *ex vivo*.

5.12 DC from FL-treated-MBP-fed mice induce proliferation in MBP specific T cells *ex vivo*.
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Animal models where oral tolerance is used to treat inflammatory autoimmune diseases</td>
<td>13</td>
</tr>
<tr>
<td>3.1</td>
<td>FL administration protects mice from EAE if administered before MBP/CFA immunization but not after</td>
<td>78</td>
</tr>
<tr>
<td>4.1</td>
<td>Enhanced protection from EAE when FL is administered prior to feeding 20 or 20 mg of oral MBP</td>
<td>87</td>
</tr>
<tr>
<td>5.1</td>
<td>Different degrees of activation of transferred Tg T cells after oral MBP administration to FL or MSA treated mice</td>
<td>123</td>
</tr>
<tr>
<td>5.2</td>
<td><em>In vivo</em> proliferative responses to MBP in FL and MSA treated mice are variable but appear to fall into 1 of 2 categories</td>
<td>130</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Adjuvant induced arthritis</td>
</tr>
<tr>
<td>ACAID</td>
<td>Anterior chamber-associated immune deviation</td>
</tr>
<tr>
<td>AChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AIA</td>
<td>Antigen induced arthritis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell(s)</td>
</tr>
<tr>
<td>CCII</td>
<td>Chicken type II collagen</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster designation</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund's Adjuvant</td>
</tr>
<tr>
<td>CH</td>
<td>Contact hypersensitivity</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen induced arthritis</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
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<tr>
<td>CTB</td>
<td>Cholera toxin b-subunit</td>
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<td>Cyc</td>
<td>Cychrome</td>
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<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo nucleic acid</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco's phosphate buffered saline</td>
</tr>
<tr>
<td>DPT-1</td>
<td>Diabetes Prevention Trial - Type I</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
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<td>EAMG</td>
<td>Experimental autoimmune Myesthenia Gravis</td>
</tr>
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<td>EAN</td>
<td>Experimental autoimmune neuritis</td>
</tr>
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<td>Experimental autoimmune thyroiditis</td>
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<td>EAU</td>
<td>Experimental autoimmune uveoretinitis</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FL</td>
<td>Human recominant Flt-3L</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
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<td>GBS</td>
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</tr>
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<td>HA</td>
<td>Influenza Hemaglutnin</td>
</tr>
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</tr>
<tr>
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<td>Human gama globulin</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
</tbody>
</table>

xviii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<tr>
<td>IDDM</td>
<td>Insulin-dependant diabetes mellitus</td>
</tr>
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<td>IEC</td>
<td>Intestinal epithelial cell</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial lymphocytes</td>
</tr>
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<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
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<td>Interleukin</td>
</tr>
<tr>
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</tr>
<tr>
<td>IRBP</td>
<td>Inter-photoreceptor retinoid binding protein</td>
</tr>
<tr>
<td>IV</td>
<td>Intra-vascular</td>
</tr>
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<td>JRA</td>
<td>Juvenile Rheumatoid Arthritis</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>LNC</td>
<td>Lymph node cells</td>
</tr>
<tr>
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<td>Lamina propria</td>
</tr>
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<td>LPL</td>
<td>Lamina propria lymphocytes</td>
</tr>
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</tr>
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<td>Monoclonal antibody</td>
</tr>
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<td>Myelin basic protein</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MDC</td>
<td>Myeloid dendritic cell</td>
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<tr>
<td>Mφ</td>
<td>Macrophage</td>
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<tr>
<td>MG</td>
<td>Myesthenia Gravis</td>
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<td>Major histocompatibility complex</td>
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<td>Macrophage inflammatory protein -1 alpha</td>
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<tr>
<td>MLN</td>
<td>Mesenteric lymph node</td>
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<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
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</tr>
<tr>
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<td>Mycobacterial heat shock protein</td>
</tr>
<tr>
<td>NAc 1-11</td>
<td>N-acetylated 1-11 peptide of MBP</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Ovalbumin</td>
</tr>
<tr>
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<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth-factor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PIA</td>
<td>Pristane induced arthritis</td>
</tr>
<tr>
<td>PLN</td>
<td>Peripheral lymph node</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid protein</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer's patch</td>
</tr>
<tr>
<td>PT</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>REAE</td>
<td>Relapsing experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>RIP</td>
<td>Rat insulin promoter</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>ThG</td>
<td>Thyroglobulin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Ts</td>
<td>T suppressor cell</td>
</tr>
</tbody>
</table>
1.1 Multiple Sclerosis

MS is a chronic inflammatory demyelinating disease of the CNS with an unknown etiology. Although environmental, genetic, infectious, and autoimmune factors have been implicated in the disease process (1-3), no single factor seems to be operating alone. The MS disease course can be variable and is currently classified as relapsing remitting, secondary progressive, primary progressive, or progressive relapsing (4). Recently, evidence for axonal transection in MS plaques has been reported, which could contribute to the permanent and progressive neurological impairment of the disease (6).

There is significant evidence for an autoimmune component in MS, since various immunologic abnormalities are detectable in the brain, cerebrospinal fluid, and peripheral blood of MS patients (1). Further evidence for an autoimmune component in MS consists of currently approved immunomodulatory agents as therapy including copolymer-1, corticosteroids, beta interferon, methotrexate, and cyclophosphamide (5). Additional evidence for autoimmunity is that the clinical, immunological, and histopathologic
features of MS can be reproduced by immunization with myelin antigens, resulting in experimental autoimmune encephalomyelitis (EAE). EAE is an animal model which has been used successfully to explore the pathogenesis and test therapeutic interventions into the MS disease process (1).

1.2 EAE as a Model for MS

EAE is induced by immunization of susceptible rodents with myelin antigens including MBP, proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), or peptides derived from these proteins. EAE is mediated by activated CD4\(^+\) Th1 cells that recognize myelin antigens in the context of MHC Class II. The immune response results in infiltration of mononuclear cells into the CNS, CNS inflammation, demyelination, edema, and an ascending paralysis. EAE in susceptible mouse strains, (B10.PL, SJ/L, and PL/J) is characterized by a relapsing remitting (R-EAE) disease course wherein animals exhibit an initial acute phase, followed by remission, and progression to multiple relapses. R-EAE is established in mice by either immunization with myelin antigens and complete Freund's adjuvant with pertussis toxin (PT) or by adoptive transfer of activated myelin antigen specific CD4\(^+\) T cells (7, 8). The immunodominant epitope of MBP recognized by encephalitogenic CD4\(^+\) T cells in B10.PL mice is the N acylated 1-11 (NAc 1-11) peptide.

CD4\(^+\) T cells of the Th1 subtype are required for EAE induction in immunocompetent animals (9, 10). Once activated by an APC, naïve CD4\(^+\) cells (Th0)
can develop distinct effector phenotypes depending on the local cytokine milieu and co-stimulatory molecules expressed by the APC. IL-12 released by APC biases naïve T cells towards the Th1 phenotype, characterized by production of high amounts of IL-2 and the pro-inflammatory cytokines IFN-γ and TNF-α. Th1 cells antagonize the development of the Th2 subtype of CD4⁺ T cells which typically produce IL-4, IL-5, IL-10, and IL-13 (11). Th2 cytokines, such as IL-10 and TGF-β likewise antagonize Th1 development directly and indirectly by inhibiting APC IL-12 production (11). The presentation of myelin antigens by APC to Th0 cells in the presence of CFA results in expansion of predominantly Th1 cells (9). Studies in vitro and in transgenic mice have implicated TNF-α and IFN-γ in the process of demyelination (12-14), although studies in TNF-α and lymphotoxin (LT) knock-out mice have shown that neither TNF nor LT are an absolute requirement for EAE induction (15). Factors required for generating an encephalitogenic Th1 response include co-stimulatory molecule signaling through the B7.1/CD28 pathway, as well as endogenous IL-12 production. (16, 17). A dependence on expression of Fas and/or FasL has also been demonstrated for EAE as lpr and gld mice are not susceptible to EAE despite the ability of immunized mice to mount a Th1 response (19, 20). Thus, by modulating the expression of critical surface molecules and cytokine mediators on T cells or APC, it is possible to modify or abrogate the disease process.
1.3 Introduction to Oral Tolerance

Treating autoimmune diseases is a difficult and complicated task, since the etiology is often unknown and the immune responses directed against a variety of self antigens. In seeking therapeutic options, it would be advantageous to select immunological intervention strategies that are organ or antigen specific. Administering antigens via a mucosal route has been recognized as a means to induce tolerance, as measured by subsequent challenge with the same antigen in an immunogenic form. The phenomenon of orally induced tolerance was first described by Wells in 1911 (23). In these early studies, guinea pigs were protected from systemic anaphylaxis by first administering egg proteins orally. In 1946, Chase (24) demonstrated inhibition of contact hypersensitivity (CH) responses to haptons after first feeding the hapten. These experiments along with others using oral administration of ovalbumin and red blood cells to animals represent the seminal reports in the mucosal tolerance field (25, 26).

A milestone in the area of mucosal tolerance was the recognition that self antigens could be administered to prevent and treat autoimmune diseases. Early studies showed that animals receiving self antigens orally prior to immunologic challenge were protected from subsequent disease induction (27-30). These early studies have been expanded to include a variety of model systems representing a spectrum of human diseases with autoimmune components (31).
1.3.1 General Mechanisms of Oral Tolerance: Active Suppression and Anergy/Deletion

In many autoimmune diseases, the specific autoantigen is either unknown or there are multiple antigens against which the immune response is directed. This poses a potential problem for initiating antigen-specific interventions, especially when multiple antigens are recognized. Therefore, treatment modalities that provide site-specific immunomodulation are desirable in these settings. Early studies of oral tolerance in EAE in the Lewis rat indicated that feeding low doses of MBP induced CD8$^+$ T suppressor (Ts) cells with site-specific suppression of EAE in fed animals (32). However, subsequent studies showed that oral tolerance could be induced in CD8$^+$ depleted animals (33, 34, 35). Subsequently, a population of immunomodulatory T cells within the CD4$^+$ T cell fraction was reported in mice (35, 36). Since most inflammatory autoimmune diseases are mediated by CD4$^+$ Th1 cells, any population of CD4$^+$ cells that would antagonize Th1 mediated responses could be viewed as "suppressor cells". Deviating an immune response from a predominantly Th1 response to a Th2 response may be one way of protecting animals from EAE.

Studies aimed at unraveling the mechanism(s) of oral tolerance induction have focused on changes in T cell phenotype, dynamics of T cell trafficking, or changes in the cytokine milieu of the gut that take place following oral antigen administration. As a result, two major mechanisms have been put forward to explain oral tolerance: active suppression by antigen specific induction of regulatory cells and T cell clonal anergy / deletion. Several factors have been identified as playing a role in determining the
mechanism of tolerance induction with the most critical factor being the dose of fed antigen (31, 37).

The active suppression mechanism has typically been associated with 1) lower doses of fed antigen and 2) the induction of TGF-β secreting regulatory cells that suppress the development of Th1 immune responses in lymphoid tissue and in the target organ (38). There is evidence that after repeated low doses of oral antigen, populations of TGF-β, IL-4, and IL-10 secreting cells are generated in the GALT, and these cells are capable of modulating the development of Th1 immunity (31, 37 ). The importance of TGF-β for the induction of oral tolerance has recently been questioned. Barone et. al (39) reported that oral tolerance was achieved in TGF-β null mice by the oral administration of both high and low doses of OVA. Interestingly, the TGF-β deletion did not affect high dose oral tolerance in these mice at all, and low dose oral tolerance was only minimally affected. Interpretation of these results is complicated, however, by the dependence of this mouse strain on injections of soluble LFA-1 for survival. Nevertheless, these observations provide insight into the role of TGF-β in oral tolerance induction.

The clonal anergy / deletion mechanism has been associated with 1) higher doses of fed antigen, 2) reversal of unresponsiveness with IL-2 treatment, and / or 3) deletion of antigen-reactive cells by apoptosis. In cases where relatively high doses of antigen are administered (40-43), antigen-specific cells display impaired functional properties such as diminished Th1 cytokine production and decreased antigen-specific proliferation (44). Evidence for deletion of antigen-specific T cells by apoptosis has been best seen in TCR Tg animals fed extremely high doses of antigen (45).
Since Th1-mediated autoimmune diseases such as EAE are suppressed by the oral administration of autoantigens, it has also been proposed that oral tolerance may involve a shift from Th1 to Th2 type responses. This idea is particularly appealing given the predominantly Th2 environment of the gastrointestinal tract. The immune deviation hypothesis is supported by work of Caspi et al. (46), who showed that administration of IL-4 or IL-10 at the time of feeding enhanced oral tolerance to retinal antigens. However, other studies indicate that neither IL-4 nor IL-10 are required for the establishment of high dose oral tolerance, or that oral antigen administration inhibits both Th1 and Th2 responses specific for the fed antigen (43, 47, 48). In any case, in diseases such as EAE and EAU, it appears clear that the inhibition of Th1 responses to the autoantigen is vital to the establishment of oral tolerance. Administration of anti-IL-12 antibody at the time of oral antigen administration inhibits Th1 responses and causes a dramatic enhancement in TGF-β and IL-4 secretion in the GALT (49). MCP-1 has also been shown to play a role in oral tolerance induction, since inhibition of this chemokine with monoclonal antibody treatment abolished oral tolerance (50). Moreover, a correlation between oral tolerance induction and increased MCP-1 levels with feeding has been suggested, which results in decreased mucosal IL-12 (51). Thus, it appears from a number of studies that inhibition of IL-12 release at the time of antigen presentation in the GALT is a key determinant in establishing oral tolerance.
1.3.2 Experimental Modulation of Oral Tolerance

Since most experimental autoimmune diseases are mediated by CD4\(^+\) Th1 cells, antagonism of these cells would be expected to enhance oral tolerance. In contrast, augmentation of Th1 responses or inhibition of Th2 responses would be expected to inhibit tolerance induction. The administration of specific cytokines or cytokine antagonists has been utilized to elucidate the role of specific cytokine environments in oral tolerance.

Cytokines -administration and antagonism

Zhang et al. (52) examined the role of IFN-\(\gamma\) in oral tolerance and showed that tolerance could be abrogated by the systemic administration of IFN-\(\gamma\). In this study, mice were fed 50 mg BSA five times to induce tolerance. Treatment of animals with IFN-\(\gamma\) prior to feeding abolished oral tolerance while feeding first had no effect.

Administration of IL-2 in oral tolerance studies has shown mixed results. In EAU, B10.A mice fed 3 times with IRBP in combination with hrIL-2 (administered via i.p. injection) showed enhanced protection from disease relative to animals not receiving IL-2. This protection was comparable to animals that received 5 feedings of IRBP with no IL-2. Further, IL-2 did not cause an exacerbation of EAU when given with oral antigen and in fact was associated with increases in Th2 cytokine production (53). In our hands, IL-2 has been shown to abrogate low dose oral tolerance in EAE in the Lewis rat. Multiple low dose feedings (5 x 1 mg) of MBP protected rats from EAE, but in vivo administration of recombinant IL-2 resulted in re-appearance of disease, arguing for a
reversal of anergy. Interestingly, this reversal was not apparent at higher doses of fed antigen (4 x 5 mg), suggesting deletion of autoreactive cells at the higher antigen dose (41).

Administration of IL-12 has been shown to redirect mucosal immune responses toward a Th1 phenotype. In a recent study examining the response to a cholera toxin (CT) / tetanus toxoid oral vaccine, administration of IL-12 either orally or by i.p. injection changed the response to this vaccine from a Th2 to a Th1 profile. This change was determined by measuring IgG isotypes and cytokine production following in vitro challenge (54). Using an alternative approach, Marth et al. (49) showed that administration of anti-IL-12 mAb enhanced the development of oral tolerance. In the OVA TCR transgenic mouse, systemic anti-IL-12 mAb enhanced the suppression of OVA specific proliferative responses observed with oral OVA treatment. This diminution was also seen after OVA challenge in OVA-fed mice. The administration of anti-IL-12 with oral OVA was associated with both TGF-β production and an increase in apoptotic cells in the Peyer’s patches of fed animals.

The effects of IL-4 and IL-10 administration on oral tolerance development have been examined. It has been reported that parenterally administered IL-4, oral IL-4, and oral IL-10 can enhance oral tolerance (55). However, IL-4 deficient animals were shown to be capable of oral tolerization using high oral doses of OVA (48). This suggests that IL-4 is not required for the development “high dose” oral tolerance, which has been associated with anergy / deletion mechanisms (48, 56).
The chemokine MCP-1 has recently been suggested to play a role in tolerance development following oral administration of antigen. Antagonism of MCP-1 abrogated oral tolerance to human gamma globulin (HGG), while antagonism of MIP-1α had no effect (50). The role of MCP-1 in oral tolerance is currently unclear but two possibilities deserve consideration. MCP-1 has a well-defined role as a chemotactic factor for a variety of cell types. The capacity of MCP-1 to effect lymphocyte and macrophage trafficking into and out of the lamina propria may explain why antagonism of this chemokine disrupts tolerance induction (51). Inhibition of the influx of either tolerogenic APC or naïve antigen-specific T cells into the GALT could explain the abrogation of peripheral tolerance development. We have observed an influx of antigen-specific T cells into the gut following oral administration of MBP in MBP TCR transgenic mice (57). Another possibility is the observation that MCP-1 down-regulates mucosal IL-12 production. Since IL-12 is the major cytokine involved in Th1 development, MCP-1 may act primarily on local APC to inhibit Th1 responses to fed antigens (51). Therefore MCP-1 may play a prominent role in oral tolerance through its ability to attract cells to the gut and/or through down-regulating mucosally derived IL-12.

**Mucosal Adjuvants**

Various adjuvants have been shown to either abrogate or enhance oral tolerance when co-administered with antigen orally. For example, cholera toxin (CT) is a potent mucosal adjuvant, that has been shown to augment mucosal IgA as well as Th1 and Th2 responses, even though Th2 responses predominate following mucosal immunization without CT (58-60). Co-administration of CT orally together with KLH, results in
abrogation of oral tolerance to KLH (61). However, CT has also been shown to enhance oral tolerance to bovine peripheral myelin (BPM) and to enhance protection from EAN in animals receiving BPM and CT (62).

Studies using the β subunit of CT (CTB) combined with oral antigen delivery have yielded more consistent results. CTB given simultaneously with a variety of antigens orally has been shown to enhance oral tolerance, to inhibit ongoing disease, and to lower the dose of antigen required to achieve tolerance (63-66). These studies indicate that CTB could potentially improve oral tolerance in humans by requiring a lower dose of oral antigen as well as by improving the efficacy of tolerance initiated during chronic disease.

Bacterial lipopolysaccharide (LPS) has also been shown to enhance oral tolerance under certain conditions. In the Lewis rat, LPS enhanced protection from EAE when MBP and LPS were both given orally, while orally administered MBP paired with subcutaneous LPS exacerbated EAE (67). In EAN, enhancement of tolerance was time dependent; if EAN was induced soon after oral antigen / LPS treatment, exacerbation of disease was observed (62).

**Gamma Delta T Cells**

Gamma delta (γδ) T cells have recently been shown to be important in oral tolerance induction following feeding of OVA. Depletion of these cells with mAb or by genetic disruption results in diminished “high dose” oral tolerance (68, 69). These cells are also implicated in nasal tolerance studies, since human recombinant insulin delivered
intranasally induced regulatory γδ T cells, which were shown to inhibit the development of diabetes in the NOD mouse (70).
1.4 Oral Tolerance for the Treatment of Inflammatory Autoimmune Diseases in Animal Models

Several animal models are currently used to study human inflammatory autoimmune diseases. Many of these systems also serve as models for studying oral tolerance. Table 1.1 illustrates the relevant animal models and the antigens reported to induce oral tolerance.

<table>
<thead>
<tr>
<th>Human Disease</th>
<th>Animal Model</th>
<th>Fed Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple Sclerosis</td>
<td>EAE</td>
<td>MBP, MBP peptides, myelin PLP, PLP peptides</td>
</tr>
<tr>
<td>Rheumatoid Arthritis</td>
<td>Collagen-Induced Arthritis</td>
<td>Type II collagen, human collagen Mycobacterial heat shock proteins collagen peptides</td>
</tr>
<tr>
<td></td>
<td>Adjuvant-Induced Arthritis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pristane-Induced Arthritis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antigen-Induced arthritis</td>
<td></td>
</tr>
<tr>
<td>Insulin Dependent Diabetes Mellitus</td>
<td>NOD Mouse</td>
<td>GAD; human, porcine insulin</td>
</tr>
<tr>
<td>Inflammatory Autoimmune Uveitis</td>
<td>EAU</td>
<td>S-antigen, S-antigen peptides IRBP, human HLA peptides</td>
</tr>
<tr>
<td>Myasthenia Gravis</td>
<td>EAMG</td>
<td>Acetylcholine Receptor</td>
</tr>
<tr>
<td>Thyroiditis</td>
<td>EAT</td>
<td>Thyroglobulin</td>
</tr>
<tr>
<td>Peripheral Demyelinating Neuronal Disease</td>
<td>EAN</td>
<td>peripheral myelin</td>
</tr>
<tr>
<td>Transplant Rejection</td>
<td>Cardiac transplants</td>
<td>allogeneic cells, MHC peptides</td>
</tr>
</tbody>
</table>

Table 1.1 Animal models where oral tolerance is used to treat inflammatory autoimmune diseases
1.4.1 Models of Autoimmune Arthritis

Models of Rheumatoid Arthritis (RA) include collagen-induced arthritis (CIA), adjuvant arthritis (AA), pristane-induced arthritis (PIA), and antigen-induced arthritis (AIA) are different models used to study the immunopathology of human rheumatoid arthritis (71-77). These diseases are induced in rats and susceptible strains of mice, which exhibit clinical signs and histopathologic changes similar to those seen in RA.

The application of oral tolerance to autoimmune disease was first reported in CIA. In these initial studies, rats and mice were protected from the development of CIA by the oral administration of type II collagen (27, 28). Subsequent studies have shown that CIA induced with chicken type II collagen (CCII) can be prevented by feeding human type II collagen peptides prior to challenge (78).

AA, induced by the intradermal injection of Mycobacterial antigens, was shown to be prevented by the oral administration of CCII (79). Experiments in AIA have also shown the protective effects of CCII. Arthritis in rats induced with bovine serum albumin (BSA) was abrogated by both the oral administration of CCII as well as BSA (80). These studies argue for a mechanism of active suppression occurring at the level of the joint (31). Alternatively, Haque et. al (81) showed that the oral administration of a 65 kilodalton Mycobacterial heat-shock protein (MT HSP) suppressed AA. MT HSP has been shown to have some cross reactivity with CCII, and HSP has been suggested to play a role in human RA (82), although this idea is controversial (83, 84).

Clonal anergy has been pinpointed as an important mechanism mediating tolerance in AIA (85). Lewis rats fed either low dose or high dose ovalbumin (OVA) and
later challenged with OVA, showed evidence of clonal anergy in OVA-reactive cells. Suppressed antigen specific proliferative responses following feeding could be reversed with IL-2. Further, lymphocytes from OVA-fed animals could not transfer tolerance, nor could they inhibit proliferation of primed lymphocytes in vitro.

1.4.2 EAE

As mentioned previously, EAE is an induced autoimmune disease affecting the central nervous system (CNS) and resulting from the immunization of susceptible animals with myelin antigens including myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte protein (MOG), or purified peptides derived from these proteins, in complete Freund’s adjuvant (CFA). EAE is mediated by activated CD4+ T helper type 1 (Th1) cells that recognize the immunizing antigen in the context of major histocompatibility complex (MHC) Class II glycoproteins. The resulting immune response generates an infiltrate of mononuclear cells into the CNS, inflammation and CNS demyelination, with associated edema leading to an ascending paralysis. EAE, induced in susceptible mouse strains, including B10.PL, SJ/L, and PL/J, is characterized by a relapsing-remitting (R-EAE) disease course, wherein animals undergo an initial acute disease episode, followed by a brief remission, with progression to multiple relapses. R-EAE can be established in mice by either immunization with myelin antigens in CFA with pertussis toxin (PT), or by adoptive transfer of activated myelin antigen-specific CD4+ T cells (7). In the B10.PL mouse, the immunodominant peptide recognized by encephalitogenic CD4+ T cells is the N acetylated 1-11 (NAc 1-11) peptide.
of MBP. Because of its resemblance to MS in clinical, immunological, and histopathologic features, R-EAE is used widely to test interventions in MS.

Early oral tolerance studies performed in the Lewis rat showed that feeding guinea pig MBP or its fragments prevented animals from developing EAE following challenge with MBP in CFA (29, 30). This protection from disease was manifested by suppression of clinical signs and CNS histopathologic changes as well as decreased antigen-specific proliferation to MBP. Subsequent studies indicated the importance of antigen dose for the establishment of oral tolerance. MBP given at low doses (μg to low mg range) produces a transferable suppression of EAE, attributed to CD8+ suppressor T cells in the rat and CD4+ suppressor T cells in the mouse (32, 35, 86). Higher antigen doses (mg range) produce a reversible anergy, specific for the fed antigen, with evidence for ultimate clonal deletion of MBP-reactive cells (40). The majority of evidence supports these two mechanisms for tolerance induction, dependant upon antigen dosage. However, other evidence has emerged which challenges the model. For example, in our hands we have found that treatment of tolerant rats with IL-2 reverses low-dose but not high-dose tolerance. While this is consistent with the involvement of deletion in high-dose tolerance, it suggests that anergy may also be involved in low-dose oral tolerance (41). Further studies in the Lewis rat revealed that the induction of oral tolerance is antigen specific, with the best protection afforded when the same antigen is used for feeding and challenge (87). In our hands, the induction of oral tolerance to MBP in the Lewis rat is exquisitely specific, extending to the individual amino acid level (88).
An important consideration in the use of oral tolerance as a treatment modality for autoimmune diseases is its effect on established disease. Early studies in the Lewis rat and guinea pig chronic relapsing EAE models indicated that the oral administration of myelin antigens could protect diseased animals from further relapse (89). Recently, murine models of EAE have received greater attention for study of established disease because of their repeated remissions and relapses. Studies in the mouse looking at R-EAE have shown that mice can be protected from developing EAE following oral administration of MBP or proteolipid protein (PLP) 139-151 peptide (90, 91). Our own work has demonstrated that repeated oral administrations of MBP, but not whole myelin following the appearance of clinical disease or at the point of recovery from acute EAE can significantly protect these mice from further relapses (90, 92). Mice fed MBP twice per week following recovery from the acute disease episode had an overall decrease in mean score/day, in the number of relapses, and in cumulative clinical score. Additionally, when compared to feeding whole myelin to mice, feeding purified MBP proved superior to treating established REAE in mice, highlighting the importance of antigen homogeneity in oral tolerance (92). These studies demonstrate the safety of oral administration of self-antigen during an ongoing disease process as well as indicate the potential for treating chronic autoimmune diseases in humans by mucosal antigen delivery.

Recent studies in oral tolerance have utilized MBP-specific T cell receptor (TCR) transgenic (Tg) mice, developed by Goverman et. al (Vα2.3/Vβ8.2) and Lafaille et. al (Vα4/Vβ8.2) (93, 94). Both mouse strains have >95% of their CD4⁺ T cell repertoire
These transgenic mouse lines have enabled investigators to determine the fate and function of the encephalitogenic T cells following oral antigen administration. Using the Vα4/Vβ8.2 TCR Tg mouse, we found that a single (100 mg) oral dose of MBP protects these mice from EAE when challenged soon after feeding (95). Thus, tolerance is demonstrable in the Tg mice, in spite of the large number of encephalitogenic T cells.

1.4.3 Models of Autoimmune Neuritis

Experimental Autoimmune Neuritis (EAN) is an induced autoimmune disease that mimics the clinical and histopathologic features of the human disease Guillain-Barre syndrome (GBS) as well as other relapsing chronic inflammatory demyelinating neuropathies. EAN is induced in animals by immunization with peripheral nerve myelin (bovine peripheral myelin - BPM), P2 protein, or its neuritogenic peptide plus CFA (96). Gaupp et. al (62) examined the feeding of myelin and various myelin proteins together with the mucosal adjuvants lipopolysacceride (LPS) and cholera toxin (CT) to effect protection from EAN. Protection was observed only under conditions where the fed antigen was the same as the immunizing antigen, and both active suppression and anergy were implicated as causative mechanisms. Interestingly, there was an augmentation of oral tolerance with both CT and LPS administration together with neuroantigen.
1.4.4 Models of Autoimmune Diabetes

Models of Insulin Dependent Diabetes Mellitus (IDDM) include the nonobese diabetic mouse (NOD), a frequently used model for the human disease insulin dependent diabetes mellitus. This mouse strain develops diabetes spontaneously and the disease is polygenic. Mice of both sexes spontaneously develop insulitis at about 3-4 weeks of age with virtually all mice exhibiting insulitis by 3 months. Overt diabetes is present in 80-90% of females and 40-50% of males depending upon the particular mouse colony.

Protection from insulitis and overt diabetes can be conferred by feeding porcine insulin, and this protection could be transferred using spleen cells from porcine or human insulin fed donors. This protection was attributed to the CD4\(^+\) component of transferred cells. Other models of diabetes have also been explored for their applicability to oral tolerance. Von Herrath et al. utilized mice transgenic for the nucleoprotein gene of lymphocytic choriomeningitis virus (LCMV) under the control of the insulin promoter. These mice express the viral transgene on pancreatic islet cells and develop diabetes when immunized with LCMV. The oral administration of insulin prior to challenge with LCMV provided protection from disease and suggested a bystander suppression mechanism. The oral administration of the immunodominant portion of the insulin β chain also protected mice from overt diabetes in a co-transfer system. When cells from fed animals were transferred together with diabetogenic cells, development of overt diabetes was slowed. Further, subsequent feeding of the recipients largely prevented diabetes. This protection was associated with a Th1 → Th2 cytokine shift as well as an increase in antigen-specific TGF-β production.
been shown that feeding transgenic plants expressing glutamic acid decarboxylase (GAD) to NOD mice protected them from developing diabetes (101).

1.4.5 Models of Inflammatory Eye Disease

Experimental Autoimmune Uveoretinitis (EAU) is an induced organ-specific autoimmune disease mediated by T cells. Immunization of Lewis rats or B10.A mice with retinal antigens together with appropriate adjuvant or passive transfer of activated CD4⁺ T cells produces the histopathologic features of EAU, which are similar to uveoretinitis in humans (102, 103). The histopathology is characterized by posterior retinal and choroid lesions, damage to micro-vasculature, photoreceptor damage, as well as granuloma formation (104). Like EAE, the disease course in rats is acute and monophasic, while the mouse presents with a more chronic-relapsing disease course.

Studies in both acute and chronic-relapsing EAU have indicated that the oral administration of S-Ag, S-Ag derived peptides, inter-photoreceptor retinoid binding protein (IRBP), and HLA derived peptides can protect animals from EAU (53, 56, 105-107). Oral tolerance was shown to be enhanced by the co-administration of IRBP and IL-2 (53). Gregerson et. al (56) showed that the oral administration of microgram doses of S-Ag peptide to rats resulted in tolerance mediated by active suppression. However, feeding milligram doses of S-Ag resulted in tolerance mediated by an anergy / deletion mechanism, since peptide feeding protected animals from peptide-induced but not S-Ag induced EAU. In chronic-relapsing EAU, mice were protected from relapses when IRBP was orally administered as late as during the recovery period following acute uveitis.
Recently, it was shown that oral feeding of recombinant *E. coli* engineered to express retinal S-antigen could also mediate oral tolerance (109).

### 1.4.6 Other Models Where Oral Tolerance is effective in treatment

Experimental Autoimmune Thyroiditis (EAT) is an inflammatory autoimmune disease induced in susceptible strains of mice by immunization with human or mouse thyroglobulin (ThG) plus adjuvant or by passive transfer of activated CD4\(^+\) T cells. The disease is characterized by mononuclear cell infiltration of the thyroid and subsequent thyroid follicle damage. Measures of disease activity include histopathologic changes in the thyroid, anti-ThG antibody production, and T cell proliferative responses to thyroid antigens (110-114).

Studies of oral tolerance in EAT have shown that passive transfer of disease can be inhibited if donor mice are given oral porcine ThG before challenge with murine-ThG (115). Also, feeding human ThG protects mice from EAT when immunized with human ThG, and this protection was associated with lower antigen-specific proliferation, lower anti-ThG antibody responses, a reduction of Th1 cytokines, and an augmentation of regulatory cells that secrete IL-4 and TGF-\(\beta\) (116, 117).

Experimental Autoimmune Myasthenia Gravis (EAMG) is a model for the human disease myasthenia gravis (MG). MG is a T cell-dependent antibody-mediated autoimmune disease of humans in which auto-antibodies are directed against the nicotinic acetylcholine receptor (AChR) found at the motor end plate of neuromuscular junctions.
EAMG is an antibody-mediated disease that can be induced by immunizing animals with purified AChR emulsified in CFA (118). These antibodies interfere with motor end-plate function and contribute to the turnover and degradation of AChR at this site, leading to the clinical picture of muscle weakness and fatigue observed in affected animals (119). The oral administration of AChR is effective in preventing and / or delaying EAMG in Lewis rats (120). Feeding purified AChR but not cruder preparations resulted in lower anti-AChR antibody levels, decreased IFN-γ secreting cells, and an up-regulation of TGF-β secreting cells relative to non-fed controls (121-123). Recently, a state of double tolerance has been reported using a dual feeding strategy of MBP and AChR with protection from both EAE and EAMG (124).

The oral administration of alloantigen is a potentially simple strategy for inducing allograft tolerance and prolonging graft acceptance in humans and experimental animals. Sayegh et. al (125) and Hancock et. al (126) utilized a model of accelerated graft rejection of cardiac grafts in which prior sensitization of recipients was effected by skin grafting. Accelerated rejection was largely prevented by prior oral administration of donor splenocytes (125, 126). Lewis rats fed allopeptides for 5 days before sensitization with alloantigen exhibited decreased antigen-specific DTH responses compared to controls (127). Further, in a model of corneal transplantation, mice fed for 10 days with a mixture of cultured donor epithelial and endothelial cells exhibited prolonged survival of corneal grafts and a decreased incidence of rejection (128).

Oral tolerance has also been used experimentally to improve the efficacy of gene therapy. Adenoviruses represent excellent vectors for transducing foreign genetic
material into non-dividing cells. The widespread use of these systems has been shown to be hampered by the immune response generated against adenoviral proteins. Such a response by the host limits the duration of transgene expression, the readministration of the vector, and thus the efficacy of treatment by the therapeutic gene (129, 130). Recently, a potential solution to this problem has been reported, i.e., by feeding animals adenoviral proteins prior to vector administration. Animals receiving orally administered viral antigens prior to a first parenteral exposure to adenovirus showed evidence of effective gene transfer after a second adenovirus injection, while control animals did not. This suggests a diminution in cell-mediated and humoral immunity to the adenoviral vector in the fed recipients (131). These studies have been extended to rats with pre-existing immunity to adenoviruses and show that oral tolerization will permit repeated administration of adenoviral vectors (132).

Within the arena of molecular biology, investigators have taken advantage of the immunoregulatory properties of the intestinal immune system by transfecting intestinal epithelial cells with a gene encoding a protein involved anaphylactic peanut allergy in mice. Oral administration of DNA nanoparticles synthesized by complexing plasmid DNA with chitosan, resulted in peanut protein gene expression in the intestinal epithelium(133). This novel technique circumvents the danger of feeding an otherwise allergic protein to individuals sensitized to that protein in order to "retolerize" to that particular antigen. For food allergies where the allergic epitpeote(s) is known, this novel DNA tolerizing vaccine may be useful to treat food allergies, a proposition impossible via the oral route of administration.
1.5 Oral Tolerance for the Treatment of Disease in Humans

Oral tolerance was demonstrated in humans using keyhole limpet hemocyanin (KLH) administered to volunteers. Subjects consumed a total of 500 mg KLH, with 50 mg / day for 10 days. Subjects were then immunized and subsequently challenged with KLH. Fed subjects showed decreased T cell proliferative responses and DTH responses specific to KLH. However, some B cell priming was observed as fed subjects showed higher KLH-specific antibody levels than controls (134).

These results along with the animal studies outlined earlier have led to the careful design of clinical trials testing oral antigen administration in human autoimmune diseases. Trials have been conducted in multiple sclerosis, rheumatoid arthritis, juvenile rheumatoid arthritis, inflammatory uveitis, autoimmune thyroiditis, and insulin dependent diabetes mellitus. No toxicity or exacerbation of disease has been observed following oral autoantigen administration (31). Although instances of improvement have been noted in some patients treated with oral antigen, broad ranging clinical improvement has not been observed. It can be argued that the choice of antigen (requirement for purified form), dose of antigen (low-dose versus high-dose tolerance), or timing of antigen administration (during disease relapse or remission) could have a marked effect on the usefulness of oral tolerance for the treatment of human autoimmune disease.

1.5.1 Multiple Sclerosis Clinical Trials

A small double-blind Phase I/II trial was conducted in MS in which early remitting-relapsing patients received orally 300 mg of bovine myelin per day. Results
showed that bovine myelin could be safely administered to humans with MS without adverse effects or exacerbations of disease (135). Males fed myelin were observed to have fewer exacerbations than placebo treated controls. This trend was not observed in female patients. A recently completed Phase III multi-center trial with 515 patients showed no differences in exacerbation rate between groups that received bovine myelin and those that received placebo (31). Magnetic resonance imaging carried out on improved patients was suggestive of significant changes favoring oral myelin in some patient groups (31). In some patients receiving oral myelin, TGF-β secreting cells specific for PLP and MBP could be demonstrated. The relative frequencies of these cells was significantly higher in patients that received oral myelin compared to placebo-treated patients (136).

1.5.2 Rheumatoid Arthritis and Juvenile Rheumatoid Arthritis Trials

In an open label study testing the oral administration of CCII to children with juvenile rheumatoid arthritis, 8 of 10 children showed no toxicity or treatment-related exacerbation (137). A randomized, double-blind trial of 60 patients with severe active rheumatoid arthritis was conducted in which patients received oral CCII or placebo daily for 3 months. There was improvement in the group receiving oral collagen as measured by a decrease in the number of swollen and tender joints. There were no observed side effects resulting from the treatment (138). The importance of the dose of orally administered collagen was addressed in a dose-ranging study testing 20 - 2500 μg collagen daily. The group receiving the 20 μg oral dose showed significant benefit (139).
In contrast, in a separate trial, oral administration of larger doses of bovine collagen (1-10 mg/day) showed no differences between collagen-fed and control groups (140).

1.5.3 Uveitis Trials

Orally administered S-Ag has been shown to be a potentially promising treatment option for uveitis. In an initial pilot study, two patients received oral S-Ag for the treatment of pars planitis and uveoretinitis. These patients reported improvement and their dosage of anti-inflammatory drugs was either decreased substantially or stopped (141). A larger, 45 patient, randomized, masked phase I/II study compared treatment groups which received either soluble S-Ag alone, a mixture of S-Ag and retinal peptides, retinal peptides alone, or placebo. Those patients receiving S-Ag alone were more easily tapered off of anti-inflammatory treatment as compared to controls (142).

Recently, a synthetic peptide mimic of disease-associated HLA-B haplotype sequences was administered orally to patients with autoimmune uveitis. This peptide was shown to cross react with S-Ag and to induce EAU in rats. This peptide protected rats from EAU if fed prior to challenge with S-Ag or IRBP. Five patients received oral peptide therapy three times weekly for 12 weeks. There was no observed toxicity and several patients were able to discontinue their steroid treatment due to improvements in ocular inflammation (107).
1.5.4 Insulin Dependent Diabetes Mellitus Trials

Trials are currently underway to study prevention strategies in those individuals at risk for developing diabetes (143, 144). In the Diabetes Prevention Trial - Type I (DPT-1), patients judged by their family history to be at an intermediate risk for developing diabetes were randomized to receive either oral insulin or placebo (143). At the completion of this trial, it will be clearer whether oral tolerization strategies can be used early on in the course of autoimmunity.

A potential danger of oral antigen administration is exacerbation of autoimmune disease. In fact, the induction of diabetes was recently reported following oral administration of OVA (145). A mouse transgenic for OVA under the control of the rat insulin promoter (RIP) was injected with cytotoxic T lymphocytes directed against OVA. Diabetes resulted from the oral administration of OVA and was mediated by activated CD8⁺ T cells. Although this system was artificial in nature, these findings indicate that care must be taken when designing therapeutic interventions into the treatment of autoimmune conditions using the oral administration of antigen (146).

1.5.5 Oral Allergen Immunotherapy

The oral administration of allergens, when compared to the more traditional subcutaneous route, is a more convenient and safer route of antigen administration (147). Recent experiments in mice have suggested a new approach to oral administration of allergens. OVA was encapsulated with an acid-resistant polymer, and encapsulated OVA was compared with soluble OVA in feeding studies. Mice receiving encapsulated OVA exhibited strong Th2 responses with increases in OVA-specific IgA, IgG1, and IgE

27
antibody responses (147-149). This approach was applied to allergy by feeding encapsulated ragweed antigens in both Phase I and Phase II double-blind studies. In a Phase I open label study, 9 treated patients displayed no significant gastrointestinal side effects and reported clinical improvement during the ensuing ragweed season (147). Treated patients had increased anti-ragweed IgG and displayed regulated IgE increases during ragweed season (147). Subsequent double-blind studies demonstrated both the safety and clinical benefit in some patients, as manifested by decreased nasal reactivity and regulated IgE levels during ragweed season (150, 151).

1.6 Basic Considerations in the Establishment of Oral Tolerance

The general assumption when studying oral tolerance induction is that the establishment of oral tolerance is measurable by assessing oral antigen induced changes to CD4+ T cells. This assumption is justified by a large body of evidence that demonstrate a requirement for CD4+ T cells in the establishment of oral tolerance (26, 35, 43). Since CD4+ T cells are restricted in their responses to antigens presented in the context of MHC class II, we make another assumption that oral tolerance involves antigen presentation by bone marrow derived antigen presenting cells. However, the lack of evidence for a non-conventional (i.e. IEC) cell acting as an APC to CD4+ T cells does not constitute proof that these cells do not participate in oral tolerance induction. Therefore, it is impossible to rule out the contributions of these types of cells in the establishment of a tolerogenic T cell response.
1.6.1 The Fate of Ingested Protein Antigens

When antigen is administered orally, it first interacts with the immune system in a network of mucosal tissues collectively known as the gut-associated lymphoid tissue (GALT). Cells that comprise the GALT include epithelial cells that line the mucosa of the small and large intestine, lymphocytes located between epithelial cells (intraepithelial lymphocytes or IEL), lymphocytes found within the lamina propria of the gut wall (lamina propria lymphocytes or LPL), and T and B lymphocytes found within specialized lymphoid follicles known as Peyer’s patches (PP) and in the mesenteric lymph nodes (MLN). Dendritic cells (DC) as well as macrophages (Mφ) are also present in the GALT and can act as antigen presenting cells (APC).

Once antigen is ingested, much of it is degraded by proteolytic digestion. However, it is clear that some of the ingested antigen is absorbed intact through the gut epithelium and enters the mesenteric lymph, systemic, or hepatic portal circulation (152, 153). Early studies in humans revealed the importance of an intact hepatic portal system for maintaining tolerance to gut microflora and dietary antigens (154, 155). Further, injection of allogeneic spleen cells directly into the portal vein of the rat, but not systemically was shown to result in antigen-specific tolerance to hepatic allografts (156). Surgical approaches using portal-caval shunts have also shown that the development of oral tolerance is dependent on the hepatic portal circulation (157, 158). It has been hypothesized that peptides fed at high doses could permeate the gut and enter the portal circulation for uptake by liver resident immune cells (152). It also becoming clear that a measurable and significant proportion of ingested protein or peptide antigens appear in
the circulation quickly after the oral administration of antigen. In studies where pigeon
cytochrome-C (PCC) peptide was fed to mice transgenic for PCC, PCC specific T cell
activation was apparent in multiple peripheral lymphoid sites, including the spleen in as
little as 6 hours after antigen administration suggesting a rapid appearance of antigen in
this site (159). Further reinforcing this view, splenic derived APC from mice fed 5 mg
PCC peptide were able to stimulate PCC specific T cells \textit{in vitro} while APC from non-fed
mice were not (159).

1.6.2 \textit{APC and Oral Tolerance}

There are several cell types that have emerged as possible candidate APC for oral
tolerance induction based on their location in the gut and close proximity to intestinal
lymphocytes. These include intestinal epithelial cells (IEC), B cells, macrophages, and
DC (160). IEC are the most numerous of the cell types mentioned and they have been
shown to acquire oral antigen, to express MHC class II in the absence of co-stimulatory
molecules, and to present antigen to T cell hybridomas (160). The role of these cells in
stimulating immune responses has been investigated more than their role in tolerance
induction (161, 162).

B cells, Mφ, and DC are present primarily in the LP, PP, and MLN of the GALT
(160). All three cell types have been shown to promote immunologic tolerance in
experimental animal models (163-169). However, the individual contribution of each cell
type to oral tolerance is just now beginning to be explored. Studies in B cell deficient
mice have suggested that B cells are not required for the establishment of peripheral
tolerance (170). Similar studies have not been reported to date examining the role of
GALT B cells in initiating oral tolerance. B cells are present in the GALT and can
acquire and present soluble antigen on MHC class II (171). Mφ could play a role in oral
tolerance induction as well, since they are known to promote anterior chamber-associated
immune deviation (ACAID) (172).

Antigen-laden cells can be detected in the LP, PP, and mesenteric lymph drainage
following oral antigen administration, suggesting that cells at these sites acquire orally
administered antigen in or traffic to these sites. In these studies, the antigen-laden cells
were identified as DC. Functionally, these DC were demonstrated to lead to immune
activation and T cell priming in vitro and in vivo (173-175). It has recently been
suggested that the priming events observed could have been due to DC activation as a
result of the isolation procedures used (160). The importance of DC in oral tolerance
induction has recently been reinforced by published studies using the protein growth
factor Flt-3L (FL) (176). In these experiments, animals received multiple injections in
vivo of FL and exhibited massive increases in numbers of DC in all lymphoid organs. The
expanded DC populations in the GALT of FL-treated mice exhibited a resting phenotype.
FL-treated mice had enhanced oral tolerance to OVA compared with animals fed OVA
alone (176). We have obtained similar results in B10.PL mice studying oral tolerance to
MBP. Mice receiving FL and oral MBP displayed enhanced protection from EAE relative
to feeding MBP alone. Moreover, protection from EAE was achieved with a much lower
dose of oral MBP in the presence of FL.
1.7 DC as immunomodulatory APC in vitro and in vivo

The role of DC as immunostimulatory APC is well established in a variety of experimental models including acquired immunity to infectious agents, immunizing protocols, as well as in vitro experiments. In fact the immunostimulatory powers of DC are being used in a variety of immuno-therapeutic strategies aimed at eradicating certain kinds of tumors by stimulating the immune system using either DC in situ (177), or through some sort of cell based transfer system (178-182). However, along with the well established potential for DC to be immunostimulatory in vitro and in vivo, other more regulatory activities have been ascribed to this complex cell type. Studies in transplantation have shown that immature DC retain the capacity for inducing graft non-responsiveness in vitro and in vivo and that donor DC can be recovered from the bone marrow of recipients (164, 166, 183). The aforementioned studies' basic conclusions about the immunomodulatory properties of these tolerance inducing DC focussed on the relative lack of costimulatory molecule expression on these APC. This conclusion is justified by subsequent studies that reversed this spontaneous acceptance of hepatic and cardiac grafts in mice with an expanded population of high costimulator expressing DC (184, 185). Other studies have shown in vitro that DC posses the ability to either kill newly activated CD4\(^+\) T Cells via a Fas/FasL mechanism or to limit the expansion of antigen specific CD8\(^+\) T cells by interfering with normal cytokine production.(186, 187). The relative importance of these two pathways is still under question but there is broad based speculation that DC under the right conditions induce antigen specific tolerance in
vivo (188-191). It was with these studies in mind that the following studies were performed using the DC growth factor FL.

1.7.1 Flt3-L (FL) as a growth factor for DC in vivo

FL is a type I trans-membrane protein that is the ligand for the Flt-3 (for fms-like tyrosine kinase-3) receptor (192, 193). Flt-3 (CD135) is a type III protein tyrosine kinase of the platelet derived growth-factor (PDGF) receptor sub-family and the human homologue of the murine receptor Flk-2 (for Fetal liver kinase-2)(194, 195). Flt-3 / Flk-2 expression is restricted in adults to cells of the bone marrow (194). FL expression, on the other hand, is not as restricted and is detected in a variety of cell types as either a type I trans-membrane protein or as a secreted form (192). Human FL has been cloned and can be synthesized in and harvested from Chinese hamster ovary (CHO) cells (192, 196). The potential of this growth factor for studies of hematopoiesis and lymphopoiesis is enormous. Administering human recombinant FL to mice and non-human primates produces a variety of effects on lymphoid and myeloid progenitor cells in vivo (192, 196, 197). The cellular effects of this growth factor reflect a transient mobilization and maturation of multi-lineage progenitor cells from the bone marrow. The effect of FL administration to mice is the dramatic increase in numbers of functional CD11c+ DC in lymphoid and non-lymphoid organs including the GALT (176, 184, 198-200). The expanded DC acquire and present antigen, and initiate immune responses to exogenous antigens (198). Interestingly, no observed functional or numerical changes in T cells result from the FL mediated expansion of DC (176, 198). The expansion of DC populations peaks after 9 days of FL administration and declines to baseline levels after
FL is stopped. However, the selectivity of this growth factor with respect to the exclusive expansion of DC relative to other cell types has yet to be described entirely, as FL has been shown to be a common cofactor *in vivo* for the development of other cell types including thymic B cells and NK cells but the majority of expanded mature cells fall into the classical murine DC phenotype of CD11c* / MHC Class II* cells (201, 202, 203-205).

### 1.8 Objectives

We wished to explore the roles of DC in oral tolerance to MBP. Initial experiments were designed to adapt the use of FL in B10.PL mice where the requirements for oral tolerance were known. Firstly, the DC expansion was characterized in this mouse strain with particular attention being paid to the phenotypes of DC expanded, and whether other possibly confounding cells types were being changed as well. Secondly, the effects of this expansion on EAE disease course was examined since this was not described anywhere in the literature. Thirdly, we examined the effects of DC expansion on the development of oral tolerance to MBP prior to and during EAE, with particular attention being paid to T cell responses in tolerant mice. Fourthly, the actual role of DC as APC during oral tolerance induction was studied using rapid purification of DC from fed animals. Lastly, we considered the mechanisms of oral tolerance in FL treated mice as we examined the in vivo fate of MBP specific T cells in both TCR transgenic and adoptive transfer experiments.
CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

Male B10.PL mice, aged 6-8 weeks, were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed at The Ohio State University (OSU, Columbus, OH). Vα4/β8.2 TCR transgenic (Tg) mice were bred at OSU using founder stock obtained from Dr. Charles Janeway. Tg mice for experimentation were obtained by breeding transgenic male or female mice with B10.PL mice obtained from The Jackson Laboratory. Progeny were screened for expression of the transgenic TCR by flow cytometry using peripheral blood lymphocytes labeled with monoclonal antibodies directed against the Tg TCR Vβ8.2 (Pharminen, San Diego, CA). Transgene positive animals were used at 6-10 weeks of age.
2.2 FL Administration

hrFlt-3L (FL) was a kind gift from Immunex Corporation, (Seattle, Washington). Lots used in these studies were either diluted from frozen concentrated stock solutions (LOTS: 5479-071, 6870-007, 6870-004) or reconstituted from lyophilized powder (LOTS: 002626) and stored in stock solutions of 1 mg/ml at -80°C. Dilutions of concentrated frozen stocks were made in sterile PBS pH 7.4 containing 0.1% mouse serum albumin (Sigma, St. Louis, MO). Lyophilized powders were reconstituted using sterile water. Mice were injected subcutaneously in the nape of the neck with either 200 μl of vehicle PBS containing 0.1% MSA or 10 μg FL in 200 μl PBS containing 0.1% MSA daily for nine days as previously reported (198, 206).

2.3 Preparation of Neuroantigens and MBP

MBP was extracted from guinea pig spinal cords (Harlan Sprague-Dawley, Indianapolis, IN) according to the method of Swanborg et al. (207). MBP used for the induction of EAE was further purified on a Sephadex G50 column and eluted with 0.01 N HCL. Individual fractions containing a single band were pooled, dialyzed against water and lyophilized. The N-acetylated immunodominant peptide of MBP (NAl-11), MBP 43-61, and PLP 139-151 were synthesized by The Ohio State University peptide facility.

2.4 Induction of EAE

EAE was induced in B10.PL mice by subcutaneous immunization over four sites on the flank with 0.1 ml of an emulsion containing 200 μg guinea pig MBP in complete
Freund's adjuvant (CFA) (containing 200 µg heat killed *Mycobacterium tuberculosis*, Jamaica strain). Pertussis toxin (PT) (List Biochemical, Campbell, CA) was injected i.p. (200 ng/injection) at the time of MBP immunization and 48 hours later. Clinical signs were assessed daily and scored as follows: limp tail, 1; ataxia with no limp tail, 1.5; ataxia with limp tail, 2; partial hind limb paralysis, 3; full hind-limb paralysis, 4; moribund, 4.5; or death, 5.

2.5 Induction of Oral Tolerance and administration of oral antigens

Male B10.PL or TCR transgenic mice were deprived of food but not water for 4-5 hours preceding oral antigen administration. Mice were given either PBS, MBP, or OVA in 500 µl PBS by gastric intubation under mild ether anesthesia. In EAE protection studies, mice were immunized with MBP/CFA/PT 7 days after antigen feeding. For treatment of established EAE, mice were fed a loading dose of 20 mg MBP or PBS at the point of greatest recovery from acute EAE followed by daily injections of FL (10µg) or MSA for 9 days. Maintenance treatment consisted of twice/week oral doses of 10 mg MBP or PBS and injections of FL or MSA 3 times / week through 50 days post-immunization. For studies in transgenic mice and adoptive transfer recipients, mice were sacrificed for analysis from 6 hrs. - 7 days following oral antigen administration.
2.6 Cell Isolation Procedures

2.6.1 Peripheral Lymphoid Organs: Single cell suspensions from spleen, peripheral lymph nodes (PLN) (inguinal, axillary, brachial, cervical, and peri-aortic nodes), and mesenteric lymph node (MLN) were prepared by mechanical dissociation through sterile wire mesh, centrifuged, washed, and resuspended in complete medium containing RPMI 1640 supplemented with 25 mM HEPES buffer, 2-mercaptoethanol, 2mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5% - 10% by volume heat inactivated fetal bovine serum (FBS).

2.6.2 Peyer's Patch: PP were excised from the wall of the small intestines, slit using a surgical blade and teased gently in medium. The cell suspension was passed through a stainless steel screen to remove cell debris and washed.

2.6.3 Intraepithelial Lymphocytes (IEL): IEL were obtained from gut tissues as originally described by Cerf-Benussan et al. (208), with minor modifications (209, 210). Briefly, the small intestine was flushed with PBS followed by excision of PP. The intestine was opened longitudinally, and cut into 5-10 mm pieces. The intestine fragments were placed into Medium 199 (GIBCO, Grand Island, NY) supplemented with 1 mM dithiothreitol with shaking at 37 °C for 30 min. These suspensions contained epithelial cells and IEL. The IEL were purified on a 44-67.5% Percoll (Pharmacia Biotech, Santa Ana, CA) gradient, and resuspended in culture medium.
2.6.4 Lamina Propria Lymphocytes (LPL): The remaining gut pieces after removal of IEL were used to isolate LPL. The tissue was digested with collagenase type VIII (Sigma) with constant shaking for 60 min at 37 °C. Cells in the supernatant were harvested, washed, filtered through a 70 μm cell strainer (Becton Dickinson, Franklin Lake, NJ) and purified using a discontinuous 40-100% Percoll gradient. Lymphoid cells were collected, washed, and resuspended in medium.

2.7 Flow Cytometry

Freshly isolated single cell suspensions or purified cell preparations were incubated with approximately 1 μg of directly labeled monoclonal antibodies following a 15 minute blocking step using 30% mouse serum / PBS / 0.1% sodium azide at 4°C. Antibody labeling was performed in the dark at 4°C for 30 minutes, followed by washing in PBS pH containing 0.1% NaN₃ and fixation using 1% paraformaldehyde. All antibodies were obtained from Pharmingen, and included FITC, PE, or CyChrome conjugated anti-Vβ8.2 TCR, anti-CD3ε, anti-CD19, anti-CD11c, anti-CD8α, anti-CD11b, anti-MHC Class II, anti-CD4, anti-DX5, anti-B80, anti-B86, anti-CD40, anti-CD40L, anti-CD54, anti-CD122, anti-CD62L, anti-CD69, anti-Fas, anti-FasL, anti-CD49d, anti-LFA-1, anti-CD25, anti-CD45, or isotype matched controls. Analyses were performed on an Epics XL flow cytometer (Coulter Corp.) Lymphocyte gates were set using forward scatter / side scatter parameters and selected cell populations analyzed using double parameter fluorescence histograms. Most data were collected in listmode for
further analysis at a later time. The majority of data are presented as plots derived from exported listmode data processed using the public domain program WINMDI version 2.8.

2.8 Purification of CD11c⁺ DC and CD4⁺ Transgenic T cells

CD11c⁺ DC were isolated and purified for flow-cytometric analysis or assays of antigen presentation by positive selection using magnetic selection columns (Miltenyi Biotec Corporation, Auburn, CA) following the manufacturer's instructions. Briefly, single cell suspensions were obtained from spleen, MLN, PLN, or PP were subjected to gradient centrifugation using Lympholyte M (Cedar Lane Laboratories, Hornby, Ontario) to deplete dead cells and tissue fragments. Approximately 50 x 10⁶ cells were overlaid on 5ml of Lympholyte M in a 15 ml conical tube. Gradients were centrifuged at 2000 g for 20 minutes at room temperature. Cells localizing at the interface were harvested and were washed in 4°C buffer containing PBS pH 7.2, 0.5% enzyme grade BSA (Sigma, St. Louis MO.), 0.01% NaN₃, and 2 mM EDTA (MACS Buffer). Nonspecific binding was blocked by addition of a solution containing 30% heat inactivated mouse serum and 100 µg/ml mouse IgG (Sigma Chemical Corp., St. Louis, MO) in MACS buffer at 4°C for 15 minutes. Cells were washed, counted, and incubated with magnetic bead conjugated hamster anti-mouse CD11c for 15 minutes at 4°C according to the manufacturer's recommendations. Magnetically labeled cells were washed followed by positive selection using magnetic selection columns (Miltenyi Biotec Corporation, Auburn, CA). Positive fractions were washed, counted, and either assayed directly in antigen presenting assays or analyzed for purity by flow cytometry. Purity of CD11c⁺ cells was routinely ~95%. 

40
Purification by positive selection did not increase the expression of co-stimulatory molecules when compared to nonseparated cells after 2 hours in culture at 37°C.

CD4⁺ T cells were purified from pooled PLN of 6-8 week old male TCR Tg mice by positive selection. Single cell suspensions were prepared by gradient centrifugation as described above, washed, counted and incubated with magnetic bead conjugated anti-mouse CD4 (L3T4) (Miltenyi Biotec Corporation) followed by positive selection using magnetic selection columns. Purity of CD4⁺ cells was routinely >95%. During the selection process and in culture overnight in the absence of antigenic stimulation, T cells maintain a naïve phenotype with no evidence for T cell activation as measured by CD69 expression, CD62L downregulation, or proliferation in culture. Purified CD4⁺ cells were co-cultured with APC from various sources for 72 hours at 37°C for antigen presentation assays. For adoptive transfer studies, CD4 cells were isolated from pooled PLN, MLN and spleen prior to labeling with fluorescent dyes.

2.9 Ex vivo antigen presentation assays using Purified DC from MBP fed mice

FL-treated mice were given a single dose of PBS 25, 50, or 100 mg MBP orally. Six hours after PBS or oral antigen administration, MLN and PLN were obtained from MBP fed and PBS-fed mice. APC populations consisted of either single cell preparations from lymph nodes (3 x 10⁵/ well) of FL treated mice or CD11c⁺ DC (1-2 x 10⁵/ well) purified from fed mice as described above. T cell activation by in vivo antigen-pulsed APC was detected by proliferation during 72
hour cultures compared with control fed animals. In separate experiments, we
determined that this assay could detect as little as 0.05 μg/ml MBP peptide (NAc 1-
11) on the surface of DC. To detect the presence of orally administered antigens on
APC from fed mice, APC were exposed to MBP NAc1-11 specific CD4+ T cells
purified from Vα4/Vβ8.2 TCR transgenic mice.

2.10 Proliferation Assays

Single cell suspensions from spleens, PLN, MLN, were cultured in complete
medium and plated in 96 well round bottom plates at 4 x 10^5 cells / well. In some assays,
purified DC were cultured with purified T cells. In those experiments varying (2.0 x 10^4
- 1 x 10^5 cells / well) numbers of purified DC were plated with 5 x 10^4 purified CD4+ Tg
T cells. In other experiments, non-purified cell populations were used as APC by plating
varying numbers cells from spleen, PLN, MNL, PP, and lamina propria (3 x 10^3 - 3 x 10^5
cells / well) with 5 x 10^4 purified CD4+ Tg T cells. 5 x 10^4 Tg T cells / well did not
proliferate significantly above baseline without exogenous APC added, regardless of the
presence of antigen. In all proliferation experiments, cells were stimulated with MBP (40
μg/ml or titrated), MBP NAc 1-11 peptide (10 μg /ml or titrated), MBP 43-67 peptide (10
μg /ml), PLP 139-151 (10 μg /ml), or anti-CD3ε (4 μg/ml) (Pharmin gen, San Diego,
CA). Cells were plated in triplicate or quadruplicate for 72 hours including a final 18
hour pulse with 3H-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ). Cultures
were harvested onto filter mats using a Skatron harvester (Skatron Corporation, Norway)
and proliferation was quantified by liquid scintillation on an LKB Beta plate Analyser
Data were expressed as the average counts per minute (CPM) of triplicate or quadruplicate wells +/- the standard error of the mean. In some experiments, delta CPM (ΔCPM) are expressed representing the antigen specific proliferation that is calculated by subtracting the mean of media stimulation from the mean of a specific antigen stimulation. In proliferation experiments after adoptive transfer of CMFDA or CFSE labeled cells, some results are expressed as CPM / transgenic cell which is calculated as [(mean CPM of antigen stimulated cells) – (mean CPM of unstimulated cells)] / # of transgenic cells / well. The number of transgenic cells / well is determined by flow cytometric analysis of single cell suspensions gating of CFSE⁺ / CD4⁺ cells.

2.11 ELISPOT Analysis for Frequencies of Cytokine-Secreting Cells:

ELISPOT analysis was performed as described previously (92). Briefly, ELISPOT plates (Polyfiltronics, Rockland, MA or Millipore Corporation) were coated with specific capture antibodies (Ab) 24h before culture. Capture and detection Ab were all obtained from Pharmingen (Pharmingen, San Diego, CA) with the exception of the TGF-β reagents. Ab for capture were plated at 2 µg/ml anti-IL-2 (JES6-1A12), 4 µg/ml anti-IL-4 (BVD4-1D11), 5 µg/ml anti-IL-5 (TRFK5), 4 µg/ml anti-IFN-γ (R4-6A2), and 4 µg/ml chicken anti-TGF-β (R&D Systems, Minneapolis, MN). After coating, plates were blocked with DMEM (Life Technologies, Grand Island, NY) with 1% BSA (Sigma) for 1h. PLN were harvested, washed and resuspended in HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with 1% L-glutamine and 50 µg/ml gentamicin (Life
Technologies, Grand Island, NY). PLN cells (5 x 10^5/well) were added to the plates in triplicate with 40 μg/ml MBP or with medium alone. Positive control wells were incubated with 1 μg/ml anti-CD3ε (PharMingen). Cultures were incubated at 37°C for 24 h (for IL-2, IFN-γ) or 48 h (for IL-4, IL-5, and TGF-β). Plates were then washed and cytokine-specific secondary antibodies were added: 2 μg/ml anti-IL-2-biotin (JES6-5H4), 2 μg/ml anti-IL-4 -biotin (BVD6-24G2), 4 μg/ml anti-IL-5-biotin (TRFK4), 2 μg/ml anti-IFN-γ-biotin (XMG1.2), and 2 μg/ml mouse anti-TGF-β (Genzyme, Cambridge, MA). After overnight incubation at 4°C, plates were washed and incubated with either alkaline phosphatase (AP)-conjugated goat anti-biotin IgG or AP-conjugated horse anti-mouse IgG (Vector, Burlingame, CA) for 2 h. Plates were developed with BCIP/NBT phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and analyzed by computer-assisted image analysis using an Immunospot Analyzer (The Ohio State University Campus Microscopy Facility, Columbus, OH) or a KS ELISPOT Image Analysis System (Carl Zeiss Microscopes, Thornwood, NY). Frequencies are expressed as the number of MBP-responsive cells per million +/- SEM.

2.12 CMFDA / CFSE Labeling of DC or CD4^+ T cells and adoptive transfer:

Purified DC from FL treated mice or purified / pooled LN (PLN + MLN) and spleen cells from naïve Vα4/Vβ8.2 mice were labeled with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) or 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Eugene, OR)
according to the manufacturers instructions and modified as described in other reports (211) (212). Briefly, cells were washed in warmed 37°C Dulbecco's PBS (D-PBS) and incubated with 5 μM CFSE or 5 μM CMFDA in D-PBS containing 0.1% enzyme grade BSA for 15 minutes. Cells were washed and incubated at 37°C for 30 min in RPMI + 10% FCS. Cells were washed again with D-PBS, resuspended in warmed D-PBS, and transferred to sex matched mice by IV tail injection (DC and CD4+ cells) or via subcutaneous injection into the footpad and flank (DC). Labeling efficiency was determined to be greater than 98% by flow cytometry.

2.13 Statistical analysis

Minitab statistical software or Microsoft Excel 97 was used for all analyses. For EAE protection and ELISPOT analyses, a non-parametric ANOVA with Tukey's post-hoc test was performed to determine differences between groups. Groups were considered significantly different at p<0.05. For analyses of differences between two group means, Student's T-test was used with p values < or = 0.05 considered significant.
3.1 FL administration to B10.PL mice produces a dramatic expansion of CD11c^+/MHC Class II^+ DC of both myeloid and lymphoid lineage in multiple tissues.

3.1.1 Administration of 10 μg/day FL for nine days expands CD11c^+ / MHC Class II^+ DC in B10.PL mice.

Studies on the role of DC in the establishment of oral tolerance were initiated in the EAE susceptible B10.PL mouse treated with FL. Published reports have shown increases in murine DC in multiple lymphoid and non-lymphoid tissues following FL administration (176, 198). Mice receiving daily injections of FL for 9 days demonstrated a consistent and dramatic increase in total cellularity in the spleen, peripheral lymph nodes (PLN), mesenteric lymph nodes
(MLN), Peyer's patches (PP), and lamina propria when compared to animals receiving injections of mouse serum albumin (MSA) alone. As shown in Figure 3.1 general increases in total cell number were seen in all lymphoid organs examined as well as the lamina propria.
Figure 3.1 Administration of FL causes an increase in cell number in multiple tissues compared to MSA controls.

Mice received 10 μg FL every day for nine days as described in Materials and Methods. Animals were sacrificed and single cell suspensions isolated as described in Materials and Methods from spleen, PLN, MLN, PP, and lamina propria. Total cell numbers are expressed as the mean +/- SEM 2 mice / group. Data are representative of 4 independent experiments.
Flow cytometric analysis of leukocytes isolated from these tissues indicated that the largest increase in cells, represented as a percent of the total, is seen in the CD11c^+ fraction (Figure 3.2). Murine CD11c is generally considered a DC restricted marker. CD3ε T cells and CD4^+ T cells actually decrease as a percent of total cells numbers in FL treated mice, while the percent of CD19^+ B cells percent remains similar with FL treatment. Class II^+ cells generally increase with FL treatment as shown in the MLN, but only slightly in other organs. Large increases are seen in MHC Class II^+ / CD19^-cells in all tissues examined with the exception of the lamina propria. MHC Class II^+ / CD19^- cells most likely represent DC. Further examination these tissues, as shown in Figure 3.3, determined that a large majority of the MHC Class II^+ cells from FL treated mice are CD11c^+ as well, in contrast to non-FL treated mice, where the majority of Class II^+ cells are also CD19^+. Phenotypic analysis of CD3ε^+ T cells and CD19^+ B cells from either MSA or FL treated mice, shown in Figure 3.4, demonstrates that neither of these populations of lymphocytes co-expresses CD11c^+ in the MLN of FL treated mice. This expression pattern was seen in multiple tissues including the PP, PLN, and spleen as well.
Figure 3.2 Administration of FL for nine days causes a marked expansion of CD11c⁺ and MHC Class II⁺ cells, but not CD3ε⁺, CD4⁺, or CD19⁺ cells.

Mice received MSA or 10 μg hrFlt-3L for 9 days. Animals were sacrificed and single cell suspensions isolated and stained with fluorochrome conjugated monoclonal antibodies as described in Materials and Methods. Percents of total cells are expressed as the mean +/- SEM from 2 mice / group. Data are representative of 4 independent experiments.
Figure 3.3  FL administration in vivo expands CD11c⁺ / MHC Class II⁺ DC

Mice received MSA or 10 μg hrFlt-3L for 9 days. Single cell suspensions were isolated and stained with fluorochrome conjugated monoclonal antibodies as described in Materials and Methods. Histogram dotplots were generated by gating on all live cells by forward and side scatter and are representative of approximately 10 experiments.
Figure 3.4 Analysis of CD11c⁺ cell from FL treated mice demonstrates the CD11c is not expressed on CD3ε⁺ T cells nor on CD19⁺ B cells.

Mice received MSA or FL for nine days. Single cell suspensions were labeled with fluorochrome conjugated monoclonal antibodies. Data shown are from the MLN of MSA or FL treated mice. Histogram dotplots were generated by gating on all live cells by forward and side scatter and are representative of results from in the spleen, PLN, MLN, and PP from 3 independent experiments.
Taken together these results indicate that FL treatment resulted in a profound increase in the proportion of CD11c+ / MHC class II+ cells in all lymphoid organs examined and the lamina propria, representing a 3-20 fold increase depending upon the organ assayed. Further, there was no significant increase in the percentage of either CD19+ B cells or CD3+ T cells similarly to what has been described for the C57Bl/6 and Balb/C strains (176, 198).

DC arise in vivo from distinct lineages, viz., from myeloid or lymphoid progenitors, which may represent functionally distinct populations in vivo based on their dynamics and location in lymphoid organs during the steady state and during acute inflammatory responses (190, 213, 214) (186, 187, 215). Myeloid-derived DC (MDC) and lymphoid-derived DC (LDC) can be distinguished based on the expression of CD8α and CD11b. LDC are MHC ClassII+/CD11c+/CD8α+/CD11blo while MDC are MHC ClassII+/ CD11c+/CD8α+/CD11bhi (213). Analysis by flow cytometry indicated that within the expanded CD11c+ / MHC Class II+ population in FL treated B10.PL mice, both CD8α+ and CD8α- cells could be identified (Figure 3.5). This was most apparent in the spleen, PLN, MLN, and PP where both types of cells were detectable, while in the lamina propria, such cells were mostly CD8α- (data not shown). It is notable that within the CD11c+ population, very few cells labeled with both CD8α and CD11b, consistent with the distinction between the two lineages.

53
Figure 3.5 FL expands both lymphoid and myeloid lineages of DC in vivo.

Single cell suspensions from FL treated mice were labeled with fluorochrome conjugated anti-CD11c, anti-CD8α, and anti-MHC Class II or anti-CD11b. Dotplot histograms were obtained gating on live CD11c⁺ cells. Data are representative of 2 independent experiments of 2 mice/group.
The expansion of cells bearing DC lineage markers in other tissues was examined using immunohistochemical techniques. As shown in Figure 3.6, FL treatment expands DEC-205\(^+\) DC in the PP of FL recipients compared with MSA treated controls. These DC are located mostly in the parafollicular regions of the PP and are thought to represent functionally mature DC. Similarly, CD11b\(^+\) cells are expanded in the liver of FL treated mice compared to MSA controls. These cells presumably are myeloid DC that have been described elsewhere (185). Thus, in the B10.PL mouse, both lymphoid and myeloid lineages of DC are expanded with FL treatment in multiple tissues, including the spleen, liver, PLN, MLN, PP, and lamina propria.
Figure 3.6 FL administration expands DEC-205⁺ cells in the PP and myeloid DC in the liver.

Mice received MSA or FL for nine days as described in Materials and Methods. Tissues were harvested, frozen, cut, fixed, and labeled on glass slides as described in Materials and Methods. Primary monoclonal antibodies used in these experiments were derived from either culture supernatants (DEC-205) or in purified form (CD11b). Sections shown are 7 μm thick shown at 200x from a digital image.
3.2 Expanded DC from FL mice are competent antigen presenting cells and can present both protein as well as peptide antigens to CD4\(^+\) TCR Tg cells

3.2.1 DC from FL treated mice are comparable to DC from non-FL treated mice

We next examined the functional capabilities of the CD11c\(^+\) DC that were expanded by FL. We hypothesized that despite the similar percentages of Class II\(^+\) cells in the tissues of FL and control mice the overall antigen presenting capability of cell populations from FL treated mice would be higher. This hypothesis was based on the superior antigen presenting capacity of DC. Therefore we expected the Class II\(^+\)/CD11c\(^+\) cells to exert a positive effect on the overall antigen presenting activity of cells isolated from FL treated mice.

To test this hypothesis, we devised an MBP and NAc1-11 peptide specific antigen presentation assay that could analyze a variety of antigen presenting cell populations for their ability to stimulate proliferation of naïve CD4\(^+\) T cells. CD4\(^+\) T cells were purified from pooled PLN and MLN of MBP TCR Tg mice. We found that magnetic selection using anti-CD4 monoclonal antibody conjugated microbeads yielded consistently pure populations of CD4\(^+\)/TCR Tg\(^+\) T cells when cells were isolated from either pooled lymph nodes or spleen. This rapid purification method is described in the literature for a variety of applications with no evidence for T cell activation as a result of the purification procedure (216-220). Purity using this method is regularly >95% from pooled lymph nodes and >90% from spleen. As Figure 3.7 demonstrates, purified cells are largely
devoid of CD19+ B cells and contain primarily Vβ8.2+/CD4+ T cells with the purity of LNC preparations being consistently higher than those of the spleen. Interestingly, the yield from this procedure is quite high, with recovery of CD4+ cells approaching 80% based on initial starting percentages and number of cells isolated. Negative fractions are devoid of CD4+ T cells and enriched mostly for B cells (data not shown). Because of the consistently higher purity achieved using the PLN as a source of cells, splenic T cells were not used as a responding cell population in further experiments, except where otherwise noted.
Figure 3.7 Positive selection of CD4+ T cells from transgenic mice yields highly pure CD4+.

CD4+ T cells were purified using positive magnetic selection as described in Materials and Methods. Unfractionated and positively selected cells were labeled with fluorochrome conjugated monoclonal antibodies to CD4, Tg TCR VB8.2, and CD19. Graphs were generated from either single or double parameter flow cytometry histograms by gating on all live events. Results are representative of multiple experiments.
Initial antigen presentation assays utilized purified CD4+ TCR Tg T cells stimulated with MBP or TCR stimulation via anti-CD3ε monoclonal antibody. APC used in these experiments consisted of splenocytes from naïve sex-matched B10.PL mice, where T cell reactivity to MBP would be minimal. APC received either gamma irradiation at 3500 rads or no irradiation to determine the effect of irradiation on APC function. As shown in Figure 3.8A, T cells cultured with media alone produced minimal proliferation above baseline, even when APC were not irradiated prior to culture with T cells. Importantly, T cells alone at all concentrations of cells / well did not incorporate 3H-thymidine, indicating that the selection procedure is not activating the T cells. It is also noteworthy that purified CD4+ T cells maintain a naïve phenotype when cultured for 24 hours with no antigenic stimulation. This indicated that neither the purification procedure itself nor the lack of APC irradiation was activating the T cells to proliferate or modulating the expression of cell surface activation molecules, namely CD69, CD25 (IL-2Rα), or CD62L (L-selectin) (Data shown later, in Figure 3.12). In Figure 3.8B, addition of exogenous MBP to these cell mixtures produced significant proliferation at multiple Tg T cell concentrations. Interestingly, non-irradiated APC stimulated greater proliferation over background at multiple T cell densities, suggesting that irradiation was decreasing the antigen processing or presentation capability of the splenocytes. Reinforcing this point as illustrated in Figure 3.8C, CD3ε stimulation showed similar results with significant proliferation of T cells in the presence of non-irradiated APC. It is worth mentioning that T cells alone did not proliferate in response to CD3ε stimulation,
presumably since TCR stimulation was occurring in the absence of co-stimulatory molecules from an APC population. It should be noted that the significant proliferation seen in non-irradiated APC only was probably due to the proliferation of live T cells within the splenic APC population. Future experiments utilized these same parameters using non-irradiated APC at densities less than or equal to $3.5 \times 10^5$ well and $5 \times 10^4$ purified CD4$^+$ T cells.
Figure 3.8 Purified naïve CD4+ T cells from MBP specific TCR Tg mice proliferate in response to antigen or TCR stimulation but not as a result of purification.

CD4+ T cells were purified from pooled lymph nodes as described in Materials and Methods and cultured with 3.5 x 10^5 splenocytes from naïve sex-matched non-transgenic B10.PL mice and stimulated with medium, MBP, or anti-CD3e monoclonal antibody. APC received gamma irradiation (3500 rads) prior to the assay or no irradiation. For comparison of irradiated and non irradiated APC, 3 T cell concentrations were compared (1 x 10^3 – 2.5 x 10^4 T cells / well). Cells were incubated at 37°C for 72 hours including an 18 hour 3H-thymidine pulse. Data are expressed as the mean CPM +/- SEM for quadruplicate wells.

62
With a sensitive proliferation assay in hand, we began testing the hypothesis that cells isolated from FL-treated mice, when used as APC, would induce more vigorous proliferative responses in naïve T cells as a result of the massive expansion of professional APC, i.e. DC. Mice received either FL or MSA for nine days. Cells were harvested from spleen, PLN, MLN, PP, and lamina propria from both groups and cultured with purified naïve CD4+ T cells as described above. Figure 3.9 shows that in all cell populations examined, FL treated cells induced more vigorous proliferative responses than did cells from non-FL treated mice. This is despite the fact that Class II+ cells are present in these mice at similar levels. What is different between the cell populations is that FL-treated mice have a higher percentage of DC than their non-FL-treated counterparts, suggesting that it is the DC that is increasing the APC activity in these cell populations.
Figure 3.9 Antigen presenting capacity is dramatically enhanced in multiple cell populations by the administration of FL.

Single cell suspensions were prepared without further purification as described in Materials and Methods from GALT and peripheral tissues of FL and control treated mice. Cells were used as APC to stimulate MBP specific CD4$^+$ Tg T cells in a 72 hour proliferation assay. $5 \times 10^5$ T cells were cultured with three differing concentrations of APC. DC percentages in these experiments were comparable to what was seen in Figure 3.3. Data are presented as mean CPM +/- SEM for quadruplicate wells of cells pooled from 3 mice / group. *=P<0.05 and **=p<0.005 for differences between cells from FL and MSA treated mice by Student’s T-Test.
We next compared the relative antigen presenting capability of DC from FL-treated mice with DC from control-treated mice. It important that we compare the APC activity of expanded DC for interpretation of later results. Literature reports comparing DC from FL and control mice utilize allogeneic stimuli or peptide presentation in other strains of mice. We hypothesized, based on these reports, that DC from FL treated mice would be similar to those from control mice in the B10.PL mouse as well (198). We chose to isolate CD11c+ DC using magnetic positive selection as opposed to other methods like antibody antibody depletion of non-DC populations (198) for multiple reasons. First, we had experience using this selection protocol for purification of CD4+ cells. Second, other reports using this technology suggested that this gentle procedure produced a high level of purity in isolated cells, sufficient for a variety of assays (221, 222). Thirdly, this method is rapid and can be accomplished within 3 hours with no plastic adherance that could change the activation state of the DC.

Initial DC purification efforts proved that DC could be rapidly purified from the MLN, PLN, PP, and spleen of FL treated mice. Figure 3.10 illustrates the high purity achieved in a typical isolation of CD11c+ DC from PLN and spleen. Similar results were achieved in the MLN as well, but not the PP. In our hands, this protocol was only able to enrich cells from PP to ~65% pure CD11c+ cells with a considerable loss of cells as well (data not shown). As a result, we did not routinely purify cells from the PP for any further comparisons. Purification of DC of from other lymphoid tissues using this procedure normally approaches 95% with no detectable contamination of CD3+ T cells or CD19+ B cells (data not shown). It is worth noting that selecting for CD11c+ cells does not change
the relative proportions of LDC (CD8α+/CD11c+/MHC Class II⁺) to MDC (CD8α−/CD11c+/MHC Class II⁺) in these isolates, as shown in Figure 3.10.

We also considered changes in co-stimulatory molecule expression on DC as a result of the purification process. Any manipulation of these cells \textit{ex vivo}, whether it is by positive selection, gradient centrifugation, or plastic adherence, has the potential for changing their activation state. We compared the expression of multiple APC accessory molecules that have been shown to be modulated upon activation, namely CD40, ICAM-1 (CD54), CD80 (B7.1), and CD86 (B7.2) on purified and non-purified spleen DC from FL treated mice. Relevant comparisons of the expression of these molecules on purified and non-purified DC are shown in Figure 3.11 following similar treatment of both cell populations. Both cell populations appeared to exhibit similar expression profiles following the five-six hour treatment protocol.
Figure 3.10 Positive magnetic selection of CD11c<sup>+</sup> cells from FL-treated mice yields highly pure DC from spleen and PLN.

CD11c<sup>+</sup> cells from spleen and PLN of FL treated mice were purified using anti-CD11c antibody conjugated microbeads as described in Materials and Methods. After selection, cells were washed and labeled with monoclonal antibodies against CD11c, MHC Class II, and CD8α. Proportions of LDC were maintained throughout the purification process. Purity of CD11c<sup>+</sup> cells was routinely ~95% or greater in these experiments. Similar results were obtained in the MLN.
Figure 3.11 Purification of DC using CD11c magnetic selection does not alter the surface expression of multiple costimulatory molecules.

CD11c+ DC were purified as described in Materials and Methods. Concurrent with purification, unfractionated cells were processed identically to those purified except for the magnetic bead addition. Purified and unfractionated cells were then cultured for 2 hours at 37°C, washed, and labeled with monoclonal antibodies to CD11c and various co-stimulatory/accessory molecules involved with antigen presentation. Overlays were generated by gating on live CD11c+ cells and assessing the relative expression of the indicated molecules. The black line represents accessory molecule expression on purified cells while the gray shaded areas represent expression on non-fractionated cells. Data are from a single experiment of pooled purified cells from 2 mice/group.
To compare DC from FL-treated and control mice, the spleen was the organ of choice because of the number of cells that can be obtained from non-FL treated mice and literature precedent. An initial feasibility experiment was conducted using purified DC from the spleens of FL-treated mice to confirm that purified DC alone were not capable of activating T cells in the absence of MBP. As shown previously in Figure 3.9, non-fractionated spleen cells from FL treated and control mice did not induce proliferation of MBP specific T cells. We hypothesized that the influence of purification on the capacity of splenic DC to initiate T cell proliferation in the absence of MBP would be negligible based on our earlier observations that purification per se does not activate T cells to proliferate. Figure 3.12 illustrates that purified T cells, when co-cultured with purified DC in the absence of antigenic stimulation, do not upregulate the surface expression of either of two activation molecules, namely CD69 and CD25 (IL-2Rα), nor do T cells downregulate their expression of CD62L (L-selectin) 18 hours after co-culture. Only when exogenous antigen is added at or above 0.1 μg/ml are there detectable levels of activation molecules on the cell surface and demonstrable T cell proliferation.

Finally, to test the stimulatory capacity of DC from FL treated mice relative to controls, CD11c+ cells from FL treated and control mice were cultured with naïve CD4+ T cells from MBP TCR Tg mice. FL derived and control DC induced identical levels of proliferation in the Tg T cells the peptide antigen NAc1-11 and comparable levels when presenting the soluble protein antigen MBP, as shown in Figure 3.13 (open and closed triangles). This results indicated that at the level of initiation of proliferation in naïve T
cells, that FL administration had no obvious effect on at the level of DC function. When comparing other DC populations from FL treated mice to splenic DC, it is interesting to note that LNC DC induced lower proliferative responses than those from the MLN. The significance of this observation is currently unknown. In summary, from the standpoint of inducing antigen specific proliferation, DC from the spleen of FL and control treated mice appear similar in their ability to stimulate naïve T cells to proliferate, a point consistent with other published reports.
Figure 3.12 The DC based antigen presentation assay using CD4+ T cells is sensitive over a variety of antigen concentrations.

Splenic DC from naïve B10.PL mice and LNC derived CD4+ Tg T cells were purified as described in Materials and Methods and cultured for 72 hours including a pulse of 3H-thymidine for the final 18 hours. Some wells were harvested for flow cytometric analysis of T cell activation markers 18 hours after cultures were initiated. Proliferation data are expressed as the mean CPM for 8 replicate wells. Proliferation above baseline was determined by ANOVA and Tukey’s post-hoc test. *=p<.01 for proliferation above background levels. Flow histograms were generated by gating on live Vβ8.2+ cells and overlaying resulting intensities of activation molecules (shaded area) on intensities of isotype controls (broken line).
Figure 3.13 DC purified from FL treated mice present both soluble protein and peptide antigens to CD4+ Tg cells.

DC from FL treated or control mice (spleen only) were purified from multiple lymphoid tissues MLN (MES), PLN (LNC), and spleen using CD11c positive selection as described in Materials and Methods. DC were plated with $4 \times 10^4$ T cells at multiple DC: T cell ratios with either media (not shown), MBP, or NAc1-11 peptide, and incubated for 72 hours as described in Materials and Methods. Data are displayed as mean antigen specific CPM – mean media CPM +/- SEM of quadruplicate wells. Cells were isolated from 2-5 mice/group.
3.3 FL treatment does not exacerbate spontaneous EAE but does differentially alter the EAE disease course depending upon the time of administration.

3.3.1 Expansion of DC with FL does not increase the rate of spontaneous EAE in either of two MBP specific TCR Tg mouse strains.

Treatment of mice with FL has been reported to cause adjuvant-like effects in the generation of anti-tumor immunity (177), the generation of alloantigen rejection responses in a model of hepatic allografts (185), and the elicitation of responses following soluble antigen exposure (206). These adjuvant-like effects have been attributed to the expansion of DC by FL. Based on these reports, it was necessary to assess the adjuvant properties of FL in the B10.PL mouse developing EAE. This would be an essential step in developing criteria whereby the hypothesis that DC expansion enhances oral tolerance could be tested. We chose examine these possible adjuvant-like effect in multiple ways. First in MBP TCR transgenic mice, where spontaneous EAE develops in some genetically susceptible mice under specific environmental conditions (93, 94, 223). We reasoned based on the findings in other systems, that FL administration might promote spontaneous EAE by increasing a potentially immunostimulatory APC population in an already self antigen reactive environment in these mice. We reasoned that this increase in APC numbers may augment the activation of the large population of autoreactive T cells in these mice, since mRNA for myelin antigens has been detected in
the thymus and in the peripheral lymphoid organs in other species. Second, we extended these studies into non-transgenic mice and assessed the adjuvanicity of FL on EAE in the B10.PL mouse.

When FL was administered to either of two strains of MBP TCR Tg mice, neither the frequency of disease occurrence nor the severity of spontaneous EAE increased compared to controls. This is illustrated in Figure 3.14, showing the group clinical scores of both FL treated and control mice. Mice were followed for 25 days during and after FL administration with no increase in disease. The most severe clinical sign observed was ataxia, occurring in a Vα2.3/Vβ8.2 mouse treated with FL. However, it should be noted that this animal displayed ataxia three days following initiation of FL treatment, and is unlikely that the observed signs were FL mediated. Mice in other groups demonstrated either no clinical signs or loss of righting reflex with no loss of tail tonus nor presence of ataxia. Although not exhaustive, this experiment indicated that FL did not have an effect on spontaneous EAE in either of two MBP TCR Tg strains of mice. In support of this, MBP specific CD4+ Tg T cells from FL treated mice maintain a naïve phenotype in vivo (Shown in Chapter 4).
Figure 3.14 FL administration does not exacerbate the development of spontaneous EAE in either of two strains of MBP TCR transgenic mice.

FL was administered as described in *Materials and Methods* to MBP TCR Tg mice for nine days. Mice were assessed for EAE clinical signs for an additional 15 days to monitor spontaneous disease development. Graphs indicate the mean daily clinical score for each group. 3-4 mice / group.
We next examined the effect of FL administration on EAE by expanding DC prior to or just following immunization with MBP in CFA. We reasoned that increasing the numbers of functional DC in mice just prior to immunization with a powerful adjuvant would lead to a more robust T cell response to MBP when compared to controls, resulting in both an increase in encephalitogenic T cell generation, as well as in EAE severity. By the same token, we expected that the administration of FL after MBP immunization would have adjuvant-like effects as well, since DC would be expanding in lymphoid tissues, in the presence of MBP and CFA. Quite unexpectedly, mice that received FL, and thus had expanded their DC numbers just prior to immunization with MBP/CFA showed marked and dramatic protection from EAE, relative to sham injected controls. These results are shown in Figure 3.15 and summarized in Table 3.1. Mice that received FL immediately after MBP immunization displayed enhanced disease characteristics marked by an increase in mortality and accelerated disease course when compared to sham injected controls. Interestingly, mice that received FL both prior to and after MBP immunization were protected from EAE development. Although surprising, we learned that these findings are similar to those obtained in SJL mice immunized with PLP peptide (Mary Kennedy, personal communication, Immunex Corp.)
Mice were injected with FL or MSA as described in Materials and Methods either before, after, or both before and after immunization with MBP/CFA. Mice were scored for EAE clinical signs daily for 60 days. Mice from MSA control groups were pooled into one composite group as the timing of sham injections did not alter the EAE disease course. Clinical scores are shown as the daily mean score for each group including those mice dying of EAE. N=7-21 mice / group. See Table 3.1 for a summary of additional disease parameters.

Figure 3.15 FL variably affects EAE in B10.PL mice depending on the time of administration.
<table>
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<th>Group</th>
<th>Incidence</th>
<th>Mean Max Clinical Score</th>
<th>Mean Day of Onset</th>
<th>Mortality</th>
</tr>
</thead>
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<td>FL -9 -0</td>
<td>1/8</td>
<td>0.44 +/- 0.44*</td>
<td>18 +/- 0.00*</td>
<td>0/8</td>
</tr>
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<td>14.6 +/- 1.69</td>
<td>3/7</td>
</tr>
<tr>
<td>FL -9 +9</td>
<td>2/7</td>
<td>1.21 +/- 0.80*</td>
<td>18 +/- 0.00*</td>
<td>1/7</td>
</tr>
<tr>
<td>Vehicle Controls</td>
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<td>2.38 +/- 0.43</td>
<td>17.3 +/- 2.40</td>
<td>6/21</td>
</tr>
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Table 3.1 - FL administration protects mice from EAE if administered before MBP/CFA immunization but not after.

FL administration after immunization may lead to a more severe disease course in relation to MSA controls (Group FL 0 → *9). Flt-3L, if administered for 18 days before and after immunization (Group FL 9 → "9), also gave protection from EAE. Groups FL 9 → 0 and FL 9 → "9 were significantly (*p < 0.01) different from controls and the group FL 0 → "9 in both Mean Max Clinical Score and Mean Day of Onset. Group differences were determined by ANOVA.
We assessed the immune response to MBP in FL treated and control mice 10 days after immunization, repeating the timing of FL administration in the aforementioned experiment. It was possible that mice that received FL prior to immunization were somehow ignorant of MBP and generated weak or no immune response \textit{in vivo}. Proliferative responses to MBP, as shown in Figure 3.16, were similar in all groups of mice in both the spleen and the LNC compartments, irrespective of whether they would later exhibit EAE clinical signs or not. went on to get EAE. This suggests that lack of an immune response to MBP, clonal ignorance, or general immunosuppression, were not factors in the observed protection from EAE.
Figure 3.16  Spleen and LNC from FL treated and control mice exhibit similar proliferative responses to MBP regardless of disease protection.

Mice were injected with FL or MSA as described in Materials and Methods either before or after immunization with MBP/CFA. 10 days after immunization, proliferative responses to MBP and OVA were measured in both LNC and spleen populations. Data shown as mean CPM's of 3 mice / group +/- the SEM. No significant differences were seen between treatment groups.
3.5 Summary

The results shown in this chapter demonstrate that FL administration to B10.PL mice is comparable to other strains where FL administration for nine days expands MHC Class II+ / CD11c+ DC in multiple lymphoid and non-lymphoid tissues. This expansion appears exclusive to CD11c+ cells, as there is no detectable increase in CD3ε+ T cells and / or CD19+ B cells. The populations of DC expanded include both myeloid DC and lymphoid DC, two populations reported to have distinct functions in vivo. These expanded cells have significant antigen presenting capabilities when cultured with naïve T cells in vitro. This activity is manifested by their ability to induce a T cell proliferative response over a variety of antigen concentrations presenting either the soluble protein MBP or the MBP peptide NAc1-11. The expanded DC from the spleens of FL treated mice induce comparable levels of proliferation stimulation in naïve T cells when compared to splenic DC from control mice. Taken together, these results indicate that DC from FL treated mice are mature, functional APC, and that other cell types involved with antigen presentation are not expanded.

Technically, the results described in this chapter demonstrate that magnetic positive selection of both CD4+ T cells and CD11c+ DC is a rapid and efficient method to purify cells for analysis ex vivo or for use in subsequent in vitro experiments without altering their activation state. CD4+ T cells purified using this method do not proliferate spontaneously nor do they exhibit evidence for cellular activation based on the surface expression of T cell activation markers, namely (CD69, CD25, and CD62L). Likewise,
purified DC fail to exhibit activation as measured by upregulation of accessory / co-stimulatory molecules following purification.

We considered the adjuvant properties of FL in vivo since DC are widely known to be initiators of primary immune responses in vivo. Multiple reports have shown that FL administration does have adjuvant like effects in several systems in vivo. We found that FL administration did not cause the development of spontaneous EAE in two different MBP TCR Tg mouse strains, despite both of these strains showing exquisite susceptibility to EAE. We additionally considered the adjuvanicity of FL on EAE in B10.PL mice since ultimately EAE would serve as the readout for later oral tolerance studies. Suprisingly, both adjuvant-like and protective effects of FL were observed in these experiments depending on when FL was administered. Importantly, regardless of when FL was administered in relation to MBP challenge or disease protection status, all groups exhibited similar anti-MBP proliferative responses as measured in vitro. The mechanisms operating in vivo to explain these counter-intuitive are not immediately obvious, but possibilities are raised in the Discussion section.
CHAPTER 4

PROTECTION AND TREATMENT OF EAE BY EXPANSION OF DC PRIOR TO FEEDING MBP IN B10.PL MICE

4.1 FL administration enhances oral tolerance to MBP resulting dramatic protection from EAE

4.1.1 Expanding Dendritic Cells prior to the oral administration of MBP enhances protection from EAE

Based on the results reported in Chapter 3 that showed a lack of adjuvant effect from FL in EAE induction when DC are expanded prior to immunization, we could now test our hypothesis that expanding DC with FL would enhance the development of oral tolerance. The possibility that FL-mediated DC expansion could abrogate the establishment of oral tolerance was still possible based on literature reports. Two reports had recently indicated that FL mediated DC expansion could ablate otherwise tolerogenic responses in two different in vivo models of tolerance induction;
a hepatic transplant model and IP administration of soluble antigen (206) (185).

Since FL had been shown to be protective in mice when DC were expanded prior to MBP/CFA immunization in Chapter 3, the effects of FL on oral tolerance in EAE were assessed. The timing of FL administration relative to immunization in an oral tolerance experiment was different from that of previous experiments; there was a seven day delay between the last FL injection and MBP/CFA immunization. The seven day period represented the normal induction period for tolerance to MBP in B10.PL mice following a 20 mg feed (90). It was therefore important to determine the effects of FL treatment on EAE induction using this particular experimental design.

To assess the effects of DC expansion on the disease course of acute EAE, mice were treated with either FL or MSA for nine days. Immediately following the last FL treatment, both groups of mice received PBS orally to control for the stress of handling and feeding mice. Seven days after termination of FL treatment, mice were challenged with MBP/CFA/PT as in other experiments. As shown in Figure 4.1A, the effects of FL administration on EAE were no longer apparent when mice were immunized seven days after cessation of FL treatment. Importantly, these results indicated that the EAE clinical course could be used as an in vivo indicator for oral tolerance to MBP in FL treated mice.

To test whether expanding DC would affect oral tolerance induction, male B10.PL mice were injected daily for nine days with either FL or MSA. After the last injection, mice were fed either PBS, 2 mg MBP, or 20 mg MBP via intra-gastric tube followed 7 days later by EAE challenge with MBP in CFA/PT. Mice were monitored daily for clinical signs of EAE.
As is illustrated in Figure 4.1B and 4.1C, feeding either 2 or 20 mg MBP in combination with FL administration produced a profound enhancement of protection from EAE compared to animals fed either 2 or 20 mg MBP alone. The oral administration of 2mg MBP alone produced no observable protection from disease, while a 20mg dose produced modest protection that in the three experiments shown, was not significantly different from controls (by ANOVA). Table 4.1 summarizes the results from all treatment groups and illustrates the enhanced protection in FL-treated MBP-fed mice for a variety of EAE disease parameters. FL-treated, MBP-fed animals displayed a lower incidence of clinical signs and a delayed time of disease onset. Moreover, both groups receiving FL and oral MBP showed significant decreases in maximum clinical score, mean score / day, and cumulative clinical score relative to all control groups. Thus, FL treatment not only enhanced oral tolerance using a tolerogenic dose of MBP, but also induced oral tolerance with a sub-tolerogenic dose.
Figure 4.1 FL enhances oral tolerance to MBP at both a low and high dose of oral MBP, but does not alter EAE.

Mice were treated with FL or MSA for 9 days as described in Materials and Methods. Mice were then fed either vehicle, 2 mg, or 20 mg oral MBP. Seven days later mice were assessed for EAE susceptibility by immunization with MBP/CFA/PT as shown on the graphs. Clinical scores were assessed daily for 30 days. Disease statistics are summarized in Table 4.1.
Table 4.1 Enhanced protection from EAE when FL is administered prior to feeding 2 or 20 mg of oral MBP

EAE was induced in B10.PL mice that received FL or MSA for nine days prior to receiving PBS, 2 mg or 20 mg MBP via the oral route. Results are pooled from 2-3 independent experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Feed</th>
<th>Incidence Of EAE</th>
<th>Mean Time to Onset&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mean Maximum Clinical Score&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Mean Cumulative Score&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Mean Score / Day&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSA</td>
<td>PBS</td>
<td>18/18</td>
<td>11.4 +/- 0.4</td>
<td>3.6 +/- 0.3</td>
<td>49 +/- 6</td>
<td>1.8 +/- 0.1</td>
</tr>
<tr>
<td>FL</td>
<td>PBS</td>
<td>17/17</td>
<td>12.7 +/- 0.8</td>
<td>3.8 +/- 0.3</td>
<td>45 +/- 4</td>
<td>1.8 +/- 0.1</td>
</tr>
<tr>
<td>MSA</td>
<td>2 mg MBP</td>
<td>10/10</td>
<td>11.8 +/- 1.0</td>
<td>3.7 +/- 0.4</td>
<td>50 +/- 9</td>
<td>1.9 +/- 0.3</td>
</tr>
<tr>
<td>FL</td>
<td>2 mg MBP</td>
<td>7/9</td>
<td>14.9 +/- 1.5</td>
<td>1.9 +/- 0.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13 +/- 4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.5 +/- 0.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>MSA</td>
<td>20 mg MBP</td>
<td>19/19</td>
<td>12.6 +/- 0.6</td>
<td>3.2 +/- 0.2</td>
<td>37 +/- 4</td>
<td>1.6 +/- 0.2</td>
</tr>
<tr>
<td>FL</td>
<td>20 mg MBP</td>
<td>10/17</td>
<td>16.5 +/- 1.8&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.3 +/- 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>12 +/- 4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.5 +/- 0.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>b</sup>Mean of time to onset of disease signs in individual animals

<sup>c</sup>Group mean of the highest clinical score by individual animals within that group

<sup>d</sup>Group mean of the cumulative clinical score for each animal within a group

<sup>e</sup>Group mean of the cumulative clinical score divided by the number of days observed

<sup>*</sup>= P<0.05 by ANOVA with Tukey's post-hoc test for differences between groups from controls.
4.1.2 Th1, Th2, and TGF-β cytokine responses are suppressed in protected mice despite no observable reduction in proliferative responses to MBP in vitro.

As described previously, multiple mechanisms operate in the induction and maintenance of oral tolerance, namely active suppression and T cell anergy and/or deletion. A third possibility includes the polarization of the oral antigen specific immune response to a Th2 cytokine response \textit{in vivo}. This latter possibility is observed in other systems of tolerance induction and is often referred to as immune deviation (163, 224, 225). As illustrated in Figure 4.1, FL-treated, MBP-fed mice were markedly protected from EAE, and we explored which of the three potential mechanisms were operating \textit{in vivo}.

We hypothesized that because the protection observed was achieved after relatively high doses of oral MBP, the mice would display hypo-responsiveness to MBP restimulation \textit{in vitro}, since the induction of T cell anergy / deletion would be favored following a single administration of high doses of oral antigens (31, 37). Initially, the proliferative response to MBP \textit{in vitro} ten days following MBP immunization was assessed. Protected mice showed no consistent significant differences in MBP-stimulated proliferation \textit{in vitro} in either the PLN or spleen compartments (data not shown). This result led us to consider the possibly that other mechanisms other than a total loss of responsiveness were involved, such as Th2 immune deviation or release of Th1 antagonistic / immunosuppressive cytokines. To address these possibilities, cytokine ELISPOT determinations were performed using PLN cells 10 days after MBP challenge. Figure 4.2 illustrates that mice protected from EAE (groups that received FL plus 2 or 20
mg MBP orally) exhibited cytokine secreting cell frequencies that were significantly reduced for Th1 (IL-2 and IFN-γ), Th2 (IL-4, IL-5), and TGF-β producing cells. This reduction was statistically significant when compared to responses in animals fed MBP alone or treated with FL alone for IL-2, IFN-γ, and IL-4. It is notable that FL treatment alone reduced the frequency of IL-5 and TGF-β secreting cells. The oral administration of 2 or 20 mg of MBP lowered the IL-2 and IL-5 secreting cell frequency in mice that did not receive FL when compared to PBS fed, MSA injected mice. However, these same mice did not display reduced levels of IFN-γ secreting cells and were not protected from EAE either. In summary, there is no apparent shift to either a Th2 or TGF-β secreting cell response in the PLN after oral exposure to MBP. Therefore, the decrease in frequency of all cytokine secreting cells observed with combined FL and oral MBP administration argues for MBP specific T cell inactivation or elimination. The cause of the apparent incongruity between the cytokine and proliferative response in protected mice is unknown but is considered later in the discussion.
A. Th1 Cytokine Responses

B. Th2 Cytokine Responses

C. TGF-β Responses

Figure 4.2 Th1, Th2, and TGF-β cytokine responses are suppressed in FL treated mice fed 2 or 20 mg oral MBP prior to sensitization.

Mice were treated with FL or MSA for 9 days as described in Materials and Methods. Mice were then fed either vehicle, 2 mg, or 20 mg oral MBP. Seven days later mice were immunized with MBP/CFA/PT. Ten days later mice were sacrificed and PLN cells were assayed by ELISPOT for cytokine responses. Results are representative of two experiments of 2 mice/group for pooled cells. *= different from vehicle-fed control and FL only group at p<0.01 by ANOVA. # = different from MSA/oral PBS group at p < 0.01 by ANOVA. @ = different from MSA-treated groups p<0.01 by ANOVA.
4.1.3 CD11c⁺ DC from FL treated mice display low levels of accessory molecules on their surface but can upregulate their expression in the presence of a pro-inflammatory stimulus

In other systems where DC have been shown to induce antigen-specific hypo-responsiveness in vivo, a common property of these DC is that they express low levels of co-stimulatory molecules such as CD80 and/or CD86 (166, 167). We illustrated in Figure 4.2 that cytokine responses in mice protected from EAE were significantly reduced when compared to controls that were not protected. This led us to speculate that at some point in the induction of oral tolerance, MBP specific T cells were either being inactivated through the development of clonal hypo-responsiveness (anergy) or through the deletion of MBP reactive T cells. To assess these possibilities, the expression of multiple APC co-stimulatory molecules was determined on CD11c⁺ cells from control mice, from FL-treated mice, and from FL-treated mice that received a systemic pro-inflammatory stimulus, bacterial lipopolysaccharide (LPS) administration. LPS was chosen as a positive control since its administration in vivo has been shown to increase the expression of multiple accessory molecules on DC (176). As illustrated in Figure 4.3, the expression of MHC Class II, CD40, ICAM-1, CD80 (B7.1) and CD86 (B7.2) on CD11c⁺ spleen cells is similar in FL treated (gray lines) vs. and control-treated mice (small population dotted line). Generally, when compared related to isotype labeling (shown in figure by thin lack lines), the expression of CD40, CD80 and CD86 are relatively low, while ICAM-1 and MHC Class II are decidedly positive. In summary, the phenotype of CD11c⁺ DC in the spleens of FL treated mice can be described as MHC Class II⁺ / ICAM-1hi /B7.1lo / B7.2⁺ / CD40lo. Six hours following systemic
administration of LPS, the expression of all of these molecules is markedly increased in FL treated mice (labeling represented by the dark black lines). This result suggests that DC from FL treated mice in vivo in the absence of pro-inflammatory stimuli such LPS or adjuvants are relatively low expressers of co-stimulatory molecules. Analysis of another lymphoid compartment, the MLN showed similar results, i.e. DC in FL treated mice (represented by gray line) when compared to isotype labeling (represented by thin black line) express low levels of CD40, CD80, and CD86, although they are decidedly positive for MHC Class II and ICAM-1 as in the spleen. It is interesting to note that with LPS treatment (labeling represented by dark black lines), the relative (to isotype), increases in surface expression of these markers does not appear as marked as it did in the spleen, with the exception of CD86, which increased the most. Direct comparisons based on Figure 4.3, although impossible since the cells were analyzed differently via flow cytometry, may suggest a resistance of APC in the MLN for increasing the expression of these markers. This may reflect the difference between a lymphoid compartment that receives drainage from a tissue that is regularly associated with tolerogenic responses and a site such as the spleen which is vital to the generation of anti-microbial responses (160). It is interesting to note the sometimes synergistic and other times antagonistic effects of LPS administration on oral tolerance induction depending on the route administered (62, 67). The abrogating effects of systemic LPS administration on oral tolerance, described in the literature, correlate with our observed increases in co-stimulatory molecule expression. On the other hand, results also point to the importance of low costimulatory molecule expression for oral tolerance induction.
Spleen MLN

Class II

CD40

ICAM-1

CD80

CD86

Figure 4.3 CD11c⁺ DC from FL mice express low levels of accessory molecules in the absence of pro-inflammatory stimuli

Mice were treated with FL or MSA for nine days as described in Materials and Methods. 24 hours after the last injection (FL treated mice only), mice received either PBS or 100 µg LPS IP. Mice were sacrificed 6 hours later, and single cell suspensions were labeled with fluorochrome conjugated monoclonal antibodies to multiple APC accessory molecules. Data shown are representative of a single experiment of 2 mice / group. Histograms were generated by gating on CD11c⁺ cells for expression of Class II, ICAM-I, CD40, CD80, and CD86. Thin black tracings represent isotype labeling. Gray lines represent labeling on CD11c⁺ cells from FL treated mice. Dotted lines represent labeling on CD11c⁺ cells from MSA treated mice. Dark black lines represent labeling on FL treated -LPS injected mice.
4.2 FL used in conjunction with oral MBP suppresses established EAE.

4.2.1 FL alone during REAE does not change the EAE disease course and augments the therapeutic efficacy of oral MBP in REAE

The possibility that FL could serve as an adjunct to treatment for mice with established EAE was assessed based on the work of Meyer et al. and Benson et al. where mice with established REAE received multiple oral administrations of MBP. In these studies, mice receiving oral MBP biweekly for seven weeks (14 feeds total) displayed a marked reduction in the clinical severity of EAE relative to mice fed PBS or whole myelin (90, 92).

To explore the feasibility of treating EAE with oral MBP in combination with ET administration, the effects of FL on ongoing EAE disease activity were examined. A pilot experiment was necessary since it was not known whether FL could successfully expand DC during REAE nor was it known whether FL administration would lead to exacerbation of EAE when given to mice with an ongoing disease process. This study was conducted by administering FL for nine days after the acute phase of EAE had remitted but before the mice relapsed into chronic disease. As shown in Figure 4.4, mice receiving FL did not experience an exacerbation nor did they experience an alleviation from disease compared to controls, reinforcing two important points. First, FL does not appear to be overtly immunosuppressive in this setting and second, FL does not appear to have any additional adjuvant effects. As shown in Figure 4.5, FL administration was shown to expand CD11c+/MHC Class II+ DC in multiple compartments in a similar
manner to that observed in naïve mice, indicating that EAE has no discernable effect on the hematopoietic activity of FL in expanding DC. Interestingly, co-stimulatory molecule expression on DC from mice with EAE was nearly identical to that observed on DC from naïve mice (data not shown). This indicates that neither the ongoing immune response to MBP nor the presence of residual CFA had any effect on the activation state of DC in the peripheral lymphoid organs, as measured by the surface expression of APC accessory molecules.
Figure 4.4 FL administration does not exacerbate EAE when administration is begun after acute disease has remitted.

Male B10.PL mice were immunized with MBP/CFA/PT as described in Materials and Methods. Mice were divided into groups based on the onset of disease so that mice in either group had similar days of onset. Mice received daily injections of FL or MSA vehicle for 9 days starting when their acute disease had reached a nadir. Mice were scored daily for clinical signs of EAE. N=6 mice group.
Figure 4.5 FL expands CD11c⁺/ MHC Class II⁺ DC in mice with EAE similarly to naïve mice.

Mice were immunized with MBP/CFA/PT (EAE mice) or not (naïve mice) as described in Materials and Methods. At the nadir of acute disease, EAE mice received FL for nine days while naïve B10.PL received FL without prior immunization. After nine injections, expansion of DC in MLN, PLN, and SPL were compared via flow-cytometry as described in Chapter 2.
Capitalizing on these studies, FL administration was combined with oral MBP in the setting of established REAE. Mice were administered oral MBP or vehicle after recovery from the acute phase of EAE. Mice were divided into treatment groups based on the severity of the acute phase of disease such that all groups displayed similar disease characteristics (i.e. mean day of onset, cumulative clinical score, mean score / day). Subsequently, we began expanding DC at the time of the administration of the oral loading dose of MBP, so that by the third dose of MBP, DC were fully expanded. As shown in Figure 4.6, the oral administration of MBP during and after DC expansion with FL had a marked effect on the disease course in mice with REAE when compared to mice receiving either oral vehicle, FL alone, or oral MBP alone, after as few as 7 administrations of MBP. We administered FL every other day until seven bi-weekly oral MBP administrations had been given. At that time, we saw little evidence that DC were still expanded (data not shown). The reason for this is unknown but the speculation is that 10 μg / day dosed every other day is not sufficient to maintain DC numbers or that the DC progenitors are depleted in these mice after greater than 20 days of FL administration, a notion supported by other reports (205). Regardless, it is apparent from figure 4.6 that the dichotomy between groups that improved with treatment and those that did not, is probably due to the effect of the feeds taking place when FL had expanded DC the most (i.e. the earliest time points). The cumulative clinical score and mean score / day, shown in Figure 4.7 for mice receiving both FL and oral MBP were significantly
lower when compared to all other groups, while administration of oral MBP alone gave modest protection from REAE.
Figure 4.6 Multiple oral administrations of MBP in conjunction with FL decrease the severity of EAE to a greater extent than feeding MBP alone.

B10.PL mice were immunized and randomly assigned to treatment groups based on disease course so that all 4 treatment groups were similar. After the majority of mice recovered from acute EAE, mice were fed MBP and injected daily with FL as noted in Materials and Methods. Mice receiving FL in conjunction with oral MBP displayed lower clinical scores about 7 days after treatment initiation on day 20 compared to all other groups. Data displayed are group mean clinical score for N=8-16 mice / group.
Cumulative Clinical Score and Mean Clinical Score / Day were improved in FL treated MBP fed mice

Cumulative clinical score and mean clinical score/day were significantly lower in mice receiving oral MBP and FL when compared to controls. *For cumulative clinical score, FL + oral MBP treatment was significantly different from FL + oral vehicle and MSA + oral vehicle groups at p<0.005 by ANOVA. **For clinical score /day, FL + oral MBP was significantly lower than all other groups at p<0.001 by ANOVA. N=8-14 mice/group.
We examined the myelin antigen specific proliferative responses in a subset of mice from the experiment illustrated in Figures 4.6 and 4.7. As shown in Figure 4.8, following restimulation in vitro of both PLN and spleen cells from these mice, we observed a trend of decreased proliferative capacity in mice that had received oral MBP when compared to mice that had received vehicle alone. Although the results from this single experiment were not significant by ANOVA, it is worth pointing out that the MBP responses in mice fed MBP were noticeably reduced when compared to non fed mice, a finding seen on other studies (92). Interestingly, the expansion of DC in mice treated with FL did not seem to affect the development of immune responses to other myelin antigens (PLP 139-151) or MBP epitopes (MBP 43-67), a phenomenon known as determinant spreading (226-229). This is seen most clearly in the PLP 139-151 induced response. No significant increases in proliferation was seen in FL treated mice, which may explain why FL administration does not lead to an increase in disease severity when administered during REAE. It should be noted that although PLP responses in the spleen of MBP fed mice were lower than all other groups, the suppressive effect on PLP determinant spreading was abrogated by FL.
Figure 4.8 Proliferative responses to MBP are decreased in mice receiving 7 doses of oral MBP as therapy for REAE.

A subset of mice from the experiment illustrated in Figure 4.7 were sacrificed 2 days following the seventh oral administration of MBP or vehicle. PLN and spleens were assayed for myelin protein peptide specific proliferation in vitro as described in materials and methods. Data shown are mean antigen specific CPM's - media CPM's +/- SEM for 2 mice /group. * = P<0.05 from PBS fed by ANOVA.
4.3 Summary

The results presented in this chapter indicate that, although FL has dramatic adjuvant and tolerogenic effects on EAE as shown in Chapter 3, this effect is transient and the number of CD11c+ cells declines within seven days after termination of FL treatment. This observation allowed us to test the effects of DC expansion in the establishment of oral tolerance. When DC were expanded with FL prior to the induction of oral tolerance, the protection afforded from a single 20 mg oral dose of MBP was dramatically enhanced. Moreover, whereas a single 2 mg feed alone produced protection, a 2 mg feed combined with prior FL treatment decreased the incidence and severity of EAE.

While the data presented in this chapter indicate that oral tolerance to MBP, and hence EAE protection, is enhanced by prior expansion of DC, the mechanism(s) of tolerance unknown. It is possible that since two different doses of MBP are protective in FL treated mice, different mechanisms could be operating. However, although 2 mg vs. 20 mg represents an order of magnitude difference, these doses are still relatively "high" by most standards (25). Protected mice exhibited fewer cytokine producing cells in the lymph node compartment when restimulated with MBP. The decrease in responding cell frequency was seen not only for the Th1 cytokines IFN-γ and IL-2, but also for the Th2 cytokines IL-4 and IL-. TGF-β responses in the lymph node were neither increased nor decreased as a consequence of feeding MBP in both FL treated and MSA treated mice. Thus, from the standpoint of cytokine responses, protected mice exhibit lower frequencies of Th1 and Th2 cytokine producing cells in
the lymph nodes. Together with the lack of an increase in TGF-β producing cells, these data suggest that MBP reactive cells are not responding in vitro due to induction of anergy or through deletion. Protection from EAE is likely a result of these self-reactive cells not expanding in response to MBP/CFA. These possibilities will be addressed in more detail in Chapter 5.

We also examined the general activation state of DC in FL treated mice, in light of the decreases in cytokine producing cells observed in protected mice. We observed that DC from FL treated mice express low levels of the APC accessory molecules MHC Class II, CD40, ICAM-1, CD80, and CD86, similar to DC from control mice. This further reinforces the concept that expanded DC are mature, but resting (or naïve) APC. Compared to DC activated by a strong bacterial adjuvant such as LPS, these resting DC express relatively low levels of co-stimulatory molecules. If these low co-stimulatory molecule-expressing DC present MBP in vivo, the outcome may be induction of antigen specific hypo-responsiveness, a documented phenomenon with immature / low co-stimulator expressing DC (164, 166). How FL may enhance oral tolerance in this model is thought producing more resting APC in vivo that can interact with and tolerize a limited number of naïve MBP specific CD4+ T cells. The apparent requirement for low co-stimulatory molecule levels on DC for tolerance induction in vivo has been recently reported (185, 230).

During the execution and completion of the studies presented in Figures 4.1, 4.2, and 4.3, similar results were published by Viney et al, in which oral tolerance to OVA was enhanced by prior expansion of dendritic cells with FL. In these studies,
oral tolerance was measured by inhibition of DTH responses, inhibition of in vitro proliferation, and lack of expansion of OVA specific T cells following OVA/CFA challenge. The enhancement of oral tolerance to OVA was achieved at multiple oral doses, ranging from 25 mg to 0.01 mg, an impressive dose range. Proliferation and DTH responses were both suppressed in mice receiving both oral OVA and FL. Further, OVA reactive cells failed to expand following immunization with OVA/CFA and were no longer detectable via flow cytometry in OVA-fed FL treated mice. Together with our observations this report supports the hypothesis that the enhancement of oral tolerance by FL leads to the induction of anergy in reactive cells and the eventual deletion of these cells in vivo. Although these studies are based in a non-autoimmune model, they do serve to reinforce our findings.

Importantly, we used FL in combination with oral MBP to treat EAE in mice that had established disease. Treating an ongoing autoimmune disease is more relevant to treatment of human disease with oral self-antigens. We have already reported that multiple oral administrations of MBP to mice with REAE can limit the severity of disease in these mice when compared to feeding vehicle alone or feeding whole myelin (90, 92). We illustrate in this chapter that FL expands DC in mice with ongoing EAE, and that the expansion of DC under these conditions does not exacerbate EAE. Multiple oral administrations of MBP significantly limited REAE severity in mice treated with FL after only seven feeds, approximately half those required in other studies (92). It is unknown which mechanism(s) of oral tolerance are operating following the repeated high doses of MBP. Based on previous studies, the decrease seen in proliferation would argue for the
inactivation of autoreactive cells either by anergy or deletion. Regardless of the mechanism(s) operating in this setting, the optimization of a FL based treatment protocol using either oral MBP or DC based "vaccine" should be explored based on these initial encouraging results.
CHAPTER 5

FATE OF MBP SPECIFIC T CELLS IN VIVO IN FL TREATED MBP FED MICE

5.1 Rapid Activation of MBP specific T cells in vivo after feeding MBP

We have established that when FL is used to expand DC in mice prior to the oral administration of MBP, protection from EAE is dramatically enhanced relative to mice fed MBP alone. The T cell cytokine responses in mice protected from EAE were consistent with either the induction of T cell anergy or the deletion of MBP specific cells. Upon restimulation with MBP in vitro, the number of cells capable of responding to MBP by secreting Th1 and Th2 cytokines was decreased. Distinguishing between anergy and deletion in vivo is difficult since one is monitoring populations of antigen specific T cells which are present in extremely low frequency in non-immunized
animals. The use of T cell receptor transgenic mice where the antigen specificity of a large proportion of the CD4+ T cells is known gets around this problem. It is possible to monitor changes in antigen specific T cells as a consequence of feeding the specific antigen. Recent studies of peripheral tolerance induction have used either adoptive transfer of antigen specific T cells or TCR transgenic mice themselves as models to study tolerance mechanisms. A conclusion resulting from several of these studies is that tolerance to an exogenous soluble antigen or tissue antigen expressed as a transgene requires priming and proliferation of antigen specific T cells (212, 231, 232). Hence activation and proliferation of antigen specific cells precedes the establishment of the tolerant state in multiple tolerance systems, including oral tolerance. We hypothesized based on the results shown in Figures 4.1 and 4.2 that MBP specific CD4+ T cells were either being anergized or eliminated in vivo. We reasoned that oral tolerance was not due to the deemed the possibility of the generation of a regulatory T cell population based on the high antigen doses administered to FL treated mice and the lack of TGF-β production (Figure 4.2).

To distinguish between anergy and deletion in FL treated mice, studies were first performed on the response of MBP specific CD4+ T cells to oral antigen stimulation in MBP-specific T cell receptor (TCR) transgenic mice. For these studies, we utilized the TCR Tg mouse developed by Lafaille et. al (Vα4/Vβ8.2) (94). These mice have 95% of their CD4+ T cell repertoire directed against the encephalitogenic epitope of MBP (NAc1-11). Consequently, this transgenic mouse and other similar strains have enabled
investigators to assess changes to antigen-specific T cells following oral antigen administration (45, 95, 233-236).

5.1.1 Oral administration of MBP to FL treated TCR Tg mice leads to CD4+ T cell activation in multiple lymphoid organs in vivo.

We examined the responses of MBP specific T cells following a tolerogenic dose of oral MBP in FL treated TCR transgenic mice. As shown in Figure 5.1, feeding 20 mg of MBP to FL treated Tg mice leads to the activation of a large proportion of the transgenic T cells in the mesenteric lymph node as soon as 18 hours after antigen feeding. This rapid activation of the MBP specific CD4+ T cells was manifested by a marked upregulation in the expression of the early T cell activation marker CD69. It is interesting to note that the Tg T cells brightest for CD69 appear to be larger in size when analyzed by forward scatter. T cell blasting in vivo has been reported elsewhere and correlates with future cell divisions (212). The T cell activation shown in the mesenteric lymph node was also seen in other tissues from FL treated TCR transgenic mice, including the PLN, PP, and spleen, indicating a rapid systemic response to MBP following a 20 mg oral dose of MBP. It should be noted that increases in cell size were not observed in every experiment. This may relate to the amount of time between sample preparation and analysis; with time we have observed a decrease in cell size following fixation with 1% paraformaldehyde (data not shown).

Since a 2 mg oral dose of MBP was nearly as effective as 20 mg in protecting FL treated mice from EAE (Figure 4.1 and Table 4.1), we next examined the responses to 2 mg of oral MBP in comparison to feeding vehicle or 20 mg MBP in FL treated mice.
Since we had observed widespread antigen specific priming and activation of CD4^+ T cells after a 20 mg feed, we predicted that intermediate levels of activation would be seen following a 2 mg feed. As shown in figure 5.2, oral administration of 2 mg or 20 mg MBP to FL treated TCR Tg mice produced widespread T cell activation in the PLN whereas vehicle treatment did not. A large percentage of Vβ8.2^+ TCR Tg T cells were positive for CD69 18 hours after oral antigen treatment. A larger percentage of CD69^+ Tg T cells was seen in mice fed 20 mg MBP relative to those fed 2 mg, suggesting a relationship between the number of activated antigen specific T cells and the antigen dose administered. The intensity of staining of CD62L^+ (L-selectin) TCR Tg cells diminishes with feeding MBP when compared to vehicle. Again, as seen with CD69 expression, feeding 2 mg MBP produced an intermediate effect as judged by MFI between high levels observed with vehicle treatment and lower levels with 20 mg MBP feeding. It is worth mentioning that although the potential upregulation of multiple activation and accessory molecules was examined on TCR Tg T cells following the oral administration of MBP did not consistently affect the expression of IL-2Rα, α4β7 integrin, or CD40L (data not shown) on Tg T cells. This is notable since other routes of antigen administration (i.e. IV) did cause increased expression of these the markers.
Figure 5.1 Vβ8+ T cells are activated in the MLN of FL treated mice within 18 hours after oral MBP administration.

TCR Tg mice were treated with FL for 9 days as described in Materials and Methods. Mice were fed either vehicle or 20 mg MBP and sacrificed 18 hours later. Single cell suspensions were subsequently labeled with fluorochrome conjugated monoclonal antibodies specific for the Tg TCR and the T cell activation marker CD69. Dot-plot histogram data presented above were generated by gating on live cells based on forward and side scatter, and are representative of results seen in the PLN and spleen of FL treated Tg mice 18 hours following oral MBP administration.
Figure 5.2 The oral administration of 2 mg or 20 mg oral MBP activates a large proportion of Vβ8.2+ / CD4+ TCR Tg T cells in the peripheral lymph nodes.

TCR Tg mice were treated with FL for 9 days as described in Materials and Methods. Mice were fed vehicle, 2 mg, or 20 mg MBP and sacrificed 18 hours later. Single cell suspensions were subsequently labeled with fluorochrome conjugated monoclonal antibodies specific for the Tg TCR and the T cell activation markers CD69 as well as CD62L. Histogram data presented above were generated by gating on live cells based on forward and side scatter, and are representative of results seen in the MLN and spleen of FL treated Tg mice 18 hours following oral MBP administration. N=1-2 mice / group
5.1.2 Development of an adoptive transfer model to study the fate of MBP specific T cells after their initial activation in vivo

Although TCR Tg mice have been widely used to study the mechanisms of oral tolerance, there are some drawbacks to this approach. First, the T cells understudy are present in an extremely high frequency, which may exert effects on the behavior of individual cells through mass action. Second, TCR Tg mice continuously produce large numbers of naïve Tg T cells from the thymus, making long-term analysis of changes to antigen specific populations difficult because of the constant influx of naïve cells. We initially sought to track oral antigen induced changes in CD4+ T cells in FL treated TCR Tg mice for at least three days after feeding. This time period had been used by others to examine tolerance after feeding antigen in vivo (212). Because of the aforementioned limitations of studying T cell responses in TCR Tg mice, we sought to develop other approaches.

Alternative approaches first described by Kearney and modified by others take advantage of the known antigen specificity of TCR Tg T cells by adoptively transferring a known quantity of naïve Tg T cells to syngeneic recipient mice prior to experimental manipulation of the recipients (237-239). This technique represents a major advance in visualizing antigen specific immune responses in a naturally occurring environment in vivo. The original system was developed in the D011.10 OVA TCR Tg mouse with transfer of cells Tg cell into syngeneic Balb/C recipients (237, 238, 240). Typically prior to cell transfer, donor cells were characterized for CD4+ content so that recipient mice would receive a consistent number of transgenic T cells. In other cases CD4+ TCR Tg T
cells were purified. A monoclonal antibody (KJ1-26) specific for the transgenic TCR was in the identification of transferred cells and their direct progeny. Following experimental manipulation of the recipients, OVA specific cells could be tracked via flow cytometric or immunohistologic means using this monoclonal reagent. In the MBP TCR Tg mouse, there is no clontypic antibody suitable for the detection of the MBP specific TCR. To compensate for this we employed purification of CD4\(^+\) Tg T cells and fluorescent cell labeling with two different fluorescent dyes, CMFDA and CFSE, both having been used extensively in other reports for flow-cytometric and immunofluorescent evaluation of transferred cells (211, 212, 231, 241).

The development of an adoptive transfer method in B10.PL mice for these studies was first performed using the fluorescent dye CMFDA. Initial experiments were carried out by transferring purified CMFDA labeled CD4\(^+\) Tg T cells intravenously and recovering the lymphoid organs of recipient mice 1 day later. As shown in figure 5.3, transferred cells were detectable in the MLN of recipient mice 24 hours after transfer, as visualized by CMFDA. CMFDA\(^+\) cells were shown to Tg T cells, since they labeled with an antibody directed against the TCR and not with anti-CD19 or isotype reagent. This was an important consideration since it was possible that donor T cells could have been phagocytized following transfer, thus labeling phagocytic cells. The phenotype of transferred cells \textit{in vivo} was clearly V\(\beta8.2^+\) and not positive for other lymphocyte markers such as CD19. This led us to conclude that if we labeled and transferred pure Tg T cells, then CMFDA\(^+\) cells represented transgenic T cells. This assumption was borne
out in virtually every experiment that was performed and permitted two-color analysis of transferred cells (see below Figure 5.4)

Although transferred cells were detectable via flow cytometry 24 hours after transfer, it was not known whether they maintained their naïve phenotype, nor was it known if they could respond to MBP stimulation. To test this, purified CMFDA labeled cells were transferred into sex matched naïve B10.PL mice, and 24 hours after transfer recipient mice were either fed PBS, 20 mg MBP, or were immunized with MBP/CFA/PT. Cells from recipient mice were harvested 24 hours later. Figure 5.4 shows that CMFDA^ cells maintain their naïve state in vivo after transfer based on lack of CD69 expression. Cells from mice fed 20 mg MBP or mice immunized with MBP/CFA/PT showed expression of CD69, while all groups maintained high levels of surface Tg TCR expression. It is of interest that immunization with MBP resulted in a higher percentage of CD69^ / CMFDA^ cells and a higher intensity of CD69 expression relative to oral MBP administration. Clearly, immunization with MBP/CFA/PT produces a much different outcome than feeding MBP, namely the induction of EAE vs. protection. This observation led us to compare immunization with MBP to feeding MBP in recipient mice in selected future experiments. This important set of studies indicated that not only can purified TCR Tg cells labeled with CMFDA maintain a naïve phenotype post-transfer in vivo, but can also respond to MBP and express T cell activation molecules.
Figure 5.3  CMFDA labeled cells are detectable *in vivo* as a discrete Vβ8.2<sup>+</sup> / CMFDA<sup>+</sup> population.

CD4<sup>+</sup> Tg T cells were purified, labeled, and transferred as described in *Materials and Methods* to sex matched B10.PL mice. Mice were sacrificed 1 day later, and single cell suspensions from MLN, PLN, and spleens were analyzed by flow cytometry for the presence of CMFDA<sup>+</sup> cells. Histograms were generated by gating on live cells and analyzing CMFDA<sup>+</sup> cells for expression of CD19, isotype antibodies, and the Tg TCR Vβ8.2. CMFDA positive threshold was determined using cells from non-fed B10.PL mice that did not receive CMFDA labeled cells (not shown). Dotplots shown are from the PLN of mice receiving CMFDA labeled cells and are representative of MLN and spleen as well.
Figure 5.4 CMFDA labeled cells remain naïve but can respond to antigen in vivo in B10.PL mice.

CD4$^+$ Tg T cells were purified, labeled, and transferred as described in Materials and Methods to sex matched B10.PL mice. Twenty-four hours following transfer, mice were either fed vehicle, 20 mg MBP, or were immunized with MBP/CFA/PT. 18 hours later PLN, spleen, and MLN cells were harvested and analyzed via flow cytometry for the expression of CD69 and Vβ8.2. Single parameter histograms were generated by double gating on live CMFDA positive cells. Filled regions represent expression of the indicated marker while the clear regions represent isotype labeling. 1-2 mice group.
For technical reasons, the green fluorescent dye CFSE was substituted for CMFDA in all subsequent transfer experiments. This was largely due to the more widespread and accepted use of this dye for long-term tracking of cells in vivo. Whereas CMFDA was suitable for tracking cells up to 3 days post-transfer, turnover of cellular constituents and cell division had been shown result in the loss of fluorescence over time (correspondance with Molecular Probes). CFSE is chemically similar to CMFDA and has been more widely used in in vivo in studies, especially for monitoring cell division in vivo following antigenic stimulation.

We undertook similar experiments to those described above for CMFDA using CFSE and achieved nearly identical results (data not shown). The parameters for long-term detection of CFSE labeled cells were then determined over a period of 8 days post transfer. This time period was chosen since we had established earlier that tolerance to MBP was effected within 7 days after oral aministration of MBP fed mice based on the results reported in Chapter 4. Since antigens were administered 1 day after transfer, we would need to be able to track cells for a total of 8 days. Figure 5.5 illustrates that although cells lose some CFSE intensity over the course of 8 days in vivo, they still represent a distinct and easily detectable CD4+ /CFSE+ population.
<table>
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<th>No Cells</th>
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<th>5 days</th>
<th>8 days</th>
</tr>
</thead>
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<td><img src="image.png" alt="Graph" /></td>
<td><img src="image.png" alt="Graph" /></td>
<td><img src="image.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

**Figure 5.5 CFSE labeled cells persist in vivo for up to eight days as a distinct Tg population.**

CD4$^+$ Tg T cells were purified, labeled, and transferred to sex matched B10.PL mice as described in *Materials and Methods*. Mice were sacrificed 1, 5, or 8 days later, and single cell suspensions from MLN, PLN, and spleen were analyzed by flow cytometry for the presence of CD4$^+$ /CFSE$^+$ cells. Histogram dotplots were generated by gating on live cells and analyzing CD4$^+$ cells for CFSE intensity. CFSE positivity was determined by analyzing non-fed FL treated B10.PL mice that did not receive CFSE cells. Dotplots shown are from the PLN of mice receiving CFSE labeled cells and are representative of MLN and spleen as well.
5.1.3 MBP specific T cells are activated to a higher degree in FL treated mice than in control mice 18 hours following the oral administration of MBP.

We next examined the activation of MBP specific CD4^ T cells in FL treated and MSA treated mice following the oral administration of MBP. We reasoned that since FL treated MBP fed mice displayed a markedly enhanced protection from EAE compared to controls then cells from these mice would likely display T cell activation. As shown in Figure 5.6 and summarized in Table 5.1, adoptively transferred MBP specific T cells in both the MLN and PLN compartments are activated, as evidenced by CD69 expression in both FL and control treated mice. FL treated MBP fed mice display a markedly and consistently higher percentage of activated MBP specific T cells after a 20 mg feed relative to MSA treated controls. Moreover, activated MBP specific T cells from FL treated mice are larger as measured by forward scatter, presumably in preparation for cell division. This increase in cell size was described above in FL treated Tg mice and has been reported elsewhere (159). The T cell activation observed in the MLN and LN was also seen in the spleen, PP, and IEL, but not in the LPL of recipient mice (data not shown). Activation after a 20 mg feed of MBP was seen in all compartments noted above, or no compartments.
Figure 5.6 Different degrees of MBP specific T cell activation in FL treated vs. MSA treated mice after a 20 mg feed of MBP.

MSA or FL treated B10.PL mice received approximately 5x10⁶ purified CFSE labeled CD4⁺ TCR Tg T cells as described in Materials and Methods. Recipients were fed either PBS or 20 mg MBP. 18 hours later PLN, spleen (not shown), and MLN cells were harvested and analyzed via flow cytometry for the expression of CD69. Single parameter histograms were generated by double gating on live CFSE positive cells. Filled regions represent expression of the indicated marker while clear regions represent isotype labeling. Data are representative of 3 experiments of with 1-4 mice group. Selected experiments are summarized below in Table 5.1.
### Experiment 1 - PLN

<table>
<thead>
<tr>
<th>Group</th>
<th>CD69 Response</th>
<th>CD69 %</th>
<th>CD69 MFI</th>
<th>FS Response</th>
<th>FS Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSA Vehicle</td>
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<td>11</td>
<td>0.8</td>
<td>0/1</td>
<td>300</td>
</tr>
<tr>
<td>MSA MBP</td>
<td>1/1</td>
<td>19</td>
<td>1.1</td>
<td>0/1</td>
<td>305</td>
</tr>
<tr>
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<td>0.8</td>
<td>0/1</td>
<td>320</td>
</tr>
<tr>
<td>FL MBP</td>
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<td>76</td>
<td>3.8</td>
<td>1/1</td>
<td>340</td>
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</table>

### Experiment 1 - MLN

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<th>Group</th>
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<th>CD69 %</th>
<th>CD69 MFI</th>
<th>FS Response</th>
<th>FS Mean</th>
</tr>
</thead>
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<tr>
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<td>0.8</td>
<td>0/1</td>
<td>273</td>
</tr>
<tr>
<td>MSA MBP</td>
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<td>FL Vehicle</td>
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<td>0/1</td>
<td>283</td>
</tr>
<tr>
<td>FL MBP</td>
<td>1/1</td>
<td>76</td>
<td>4.3</td>
<td>1/1</td>
<td>305</td>
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</table>

### Experiment 2 - PLN

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<th>Group</th>
<th>CD69 Response</th>
<th>CD69 %</th>
<th>CD69 MFI</th>
<th>FS Response</th>
<th>FS Mean</th>
</tr>
</thead>
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<td>1.1</td>
<td>0/1</td>
<td>273</td>
</tr>
<tr>
<td>MSA MBP</td>
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<td>47 +/- 15</td>
<td>2.05 +/- 0.5</td>
<td>1/2</td>
<td>283 +/- 8.5</td>
</tr>
<tr>
<td>FL Vehicle</td>
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<td>18</td>
<td>1.1</td>
<td>0/1</td>
<td>284</td>
</tr>
<tr>
<td>FL MBP</td>
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<td>4.6 +/- 0.7*</td>
<td>4/4</td>
<td>363 +/- 11.5*</td>
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</tbody>
</table>

### Experiment 2 - MLN

<table>
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<tr>
<th>Group</th>
<th>CD69 Response</th>
<th>CD69 %</th>
<th>CD69 MFI</th>
<th>FS Response</th>
<th>FS Mean</th>
</tr>
</thead>
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<td>MSA MBP</td>
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<td>1.36 +/- 0.2</td>
<td>2/2</td>
<td>274 +/- 0.5</td>
</tr>
<tr>
<td>FL Vehicle</td>
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<td>12</td>
<td>0.9</td>
<td>0/1</td>
<td>296</td>
</tr>
<tr>
<td>FL MBP</td>
<td>4/4</td>
<td>72 +/- 6*</td>
<td>4.6 +/- 0.7*</td>
<td>4/4</td>
<td>376 +/- 14*</td>
</tr>
</tbody>
</table>

### Table 5.1 Different degrees of activation of transferred Tg T cells after oral MBP administration to FL or MSA treated mice.

MSA or FL treated B10.PL mice received approximately 5x10^6 purified CFSE labeled CD4^+ TCR Tg T cells as described in Materials and Methods. Recipients were fed either PBS vehicle or 20 mg MBP. 18 hours later LNC, spleen (not shown), and MLN cells were harvested and analyzed via flow cytometry for the expression of CD69 and Forward scatter. Data shown are from 2 of 3 independent experiments that showed similar results of 1-4 mice / group. *=P<0.05 by students T-Test for differences between FL treated MBP fed mice and MSA treated MBP-fed mice.
5.2 Proliferation of MBP specific T cells in vivo after feeding

5.2.1 MBP specific T cells proliferate in vivo after oral administration of 20 mg MBP but fail to accumulate.

As shown in Figure 5.6 and summarized in Table 5.1, a higher percentage of MBP specific T cells from FL treated mice become activated with a 20 mg dose of oral MBP. Moreover, the degree of oral antigen induced activation in FL treated, MBP fed-mice as measured by an increase in T cell size was higher as well. It is tempting to associate this higher degree of activation in FL treated mice with the enhancement of oral tolerance to MBP observed in Chapter 4, based on other observations linking tolerance to T cell activation and proliferation in vivo. We tested this notion by examining the in vivo proliferation and expansion of MBP specific T cells after oral MBP administration in both MSA and FL treated mice. To accomplish this, CFSE labeled cells were transferred into naïve MSA and FL treated mice which were subsequently fed 20 mg MBP. Mice were sacrificed 18 or 72 hours later and transferred cells were assessed for proliferation by measuring the diminution of the CFSE dye. Figure 5.7 shows that in the PLN and MLN of both MSA and FL treated mice fed vehicle, the CFSE⁺/CD4⁺ cells are present as a discrete population. However, in the PLN and MLN of mice fed 20 mg MBP 72 hours earlier, CFSE⁺/CD4⁺ cells display evidence that multiple cell divisions have taken place in vivo. Interestingly, despite the observed differences in degree of activation between FL treated and MSA treated MBP fed mice(Figure 5.6), there were no apparent differences exist between these two groups when considering the degree of in vivo proliferation. It is also interesting to note that despite the obvious evidence for cell division in both groups
of MBP fed mice in both the MLN and the PLN, there is little evidence for accumulation of MBP specific cells within these sites. For instance, if cells have divided two times, we would expect a four fold increase, three times should yield an eight-fold increase. It is estimated based on the analysis of flow-cytometric data from experiments like that shown in Figure 5.7, that the majority of MBP specific T cells have divided between 1 and 2 times during the 72 hours after feeding MBP. We would expect to see at least a 2-4 fold increase in cell number, but rather we observe a less than 2 fold increase as percent of the total. As shown in Figure 5.8, comparisons of total CFSE+ cell recovery from the PLN, MLN, and spleen of MBP-fed mice, reveals that at 72 hours after feeding, although CFSE+ cells are dividing in vivo to some extent, they fail to accumulate in these tissues, suggesting that these dividing cells may be short lived in vivo. A potential caveat to this interpretation is that some cells could have divided so rapidly that they could no longer be distinguished from background CFSE staining. Another caveat to this interpretation is that CFSE+ cells have trafficked to some other peripheral tissue after becoming activated. It is important to note that similar cell division is observed in the spleen of both FL and MSA treated MBP fed mice. Since there are currently no suitable monoclonal anti-clonotypic reagents available for use in this system, the relative percentage of cells that have divided to that extent in vivo is not known. It is expected to be negligible though as the histograms in Figure 5.7 appear to discrete CD4+ /CFSE- and CD4+/CFSE+ populations. It is clear however, that because of the rapid in vivo cell divisions after oral MBP treatment, analyses using CFSE as a marker of Tg T cells after the 72 hour time point would be impossible due to the limitations imposed by the dilution of CFSE with cell division.
It is necessary to point out that not all proliferative responses in MBP fed mice display such marked dilution of the CFSE signal 72 hours after oral MBP administration, as that shown in Figure 5.7. Based on the 3 independent experiments performed in this manner, it is possible to subdivide MBP fed mice into hi responders and lo responders based on the degree of proliferation in vivo. Relative proliferation is expressed as the mean fluorescence intensity (MFI) of CD4^+/CFSE^+ cells. Table 5.2 summarizes the results from the experiment from where Figure 5.7 was generated. All vehicle-fed mice, regardless of whether the received FL or MSA treatment, are nearly identical for the MFI of their CFSE population. As expected, when FL-treated MBP-fed and MSA-treated MBP-fed mice are compared to vehicle-fed mice, the mean MFI's for the former groups are much lower than for the vehicle group. Mice immunized with MBP/CFA/PT are included in this analysis as a positive control. We observed a wide distribution of MFI values (as exemplified by the large SEM) for the fed groups. It is important to point out that MBP fed mice appeared to proliferate using the criterion of CFSE MFI diminution, but that this proliferation in vivo was quite variable. Because of such a wide distribution in response, MFI values from the MBP fed groups were not significantly different from the controls by ANOVA. Careful analysis of histogram data reveals that MBP fed mice, regardless of pre-treatment, when assayed for in vivo proliferation based on CFSE dilution, can be grouped into two distinct groups: hi responders where the CFSE MFI is less than 50% of the vehicle value and lo responders where CFSE MFI is greater than 50% but less than 100%. When using these criteria for classifying responses, it is clear that there is a bimodal distribution of responses. When grouping based on hi and lo
proliferative responses \textit{in vivo}, distinct group means can be calculated and all groups are then different from vehicle-fed mice. Although somewhat arbitrary, this grouping clearly shows that it cannot be assumed that all MBP fed mice are going to respond to MBP in an identical manner, making generalization about the \textit{in vivo} responses to oral MBP difficult. It is clear that \textit{lo} responders do exhibit some proliferation \textit{in vivo} of \textit{CFSE}^+ cells, but they do not have the same flow cytometric profile as the \textit{hi}-responding mice.
Figure 5.7  MBP specific CD4$^+$ T cells proliferate in vivo after 20 mg oral MBP.

MSA and FL treated B10.PL mice received approximately 5-8 x 10$^6$ CFSE labeled Tg T cells as described in Materials and Methods. Mice were then fed either vehicle or 20 mg oral MBP and sacrificed 18 or 72 hours later. Single cell suspensions were analyzed by flow cytometry using fluorochrome conjugated monoclonal antibodies to CD4. Histogram dotplots were generated by gating on live cells and analyzing CD4$^+$ cells for CFSE intensity following feeding. CFSE positivity was determined by comparison to non-fed FL treated B10.PL mice that did not receive CFSE cells.
Figure 5.8 CFSE* cells do not accumulate in the PLN, MLN, or spleen of MBP fed mice after feeding despite proliferation in vivo.

MSA and FL treated B10.PL mice received approximately 5-8 x 10^6 CFSE labeled Tg T cells as described in Materials and Methods. Mice were then fed either vehicle or 20 mg oral MBP and sacrificed 72 hours later. Single cell suspensions were analyzed by flow cytometry. CFSE* cells numbers were measured as % of total cells as multiplied by the total number of cells in each organ. CFSE positivity was determined by comparison to non-fed FL treated B10.PL mice that did not receive CFSE cells. Data are pooled from 3 independent experiments for a total of 3-7 mice / group.
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<tr>
<th></th>
<th>Responders</th>
<th>MFI CFSE'/CD4'</th>
<th>% of Vehicle MFI</th>
<th>Hi Responders</th>
<th>Hi Resp. MFI</th>
<th>Hi Resp. % of Vehicle</th>
<th>Lo Responders</th>
<th>Lo Resp. MFI</th>
<th>Lo Resp. % of Vehicle</th>
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<tr>
<td><strong>Vehicle Fed</strong></td>
<td>0/4</td>
<td>31.1 +/- 1.0</td>
<td>100 +/- 1%</td>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>FL MBP Fed</strong></td>
<td>4/4</td>
<td>15.6 +/- 6.7</td>
<td>50 +/- 21%</td>
<td>2/4</td>
<td>3.9 +/- 0.4*</td>
<td>13 +/- 1%*</td>
<td>2/4</td>
<td>27.2 +/- 0.8*</td>
<td>87 +/- 3%*</td>
</tr>
<tr>
<td><strong>MSA MBP Fed</strong></td>
<td>3/3</td>
<td>19.1 +/- 7.3</td>
<td>61 +/- 24%</td>
<td>1/3</td>
<td>4.7*</td>
<td>15%*</td>
<td>2/3</td>
<td>26.3 +/- 1.7#</td>
<td>84 +/- 5%#</td>
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<td><strong>MBP/CFA/PT</strong></td>
<td>2/2</td>
<td>4.9 +/- 0.1</td>
<td>16 +/- 1%</td>
<td>2/2</td>
<td>4.9 +/- 0.1*</td>
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<td><strong>MLN</strong></td>
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<td>100 +/- 2%</td>
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<td><strong>FL MBP Fed</strong></td>
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<td>12.8 +/- 3.7</td>
<td>49 +/- 15%</td>
<td>2/4</td>
<td>6.4 +/- 0.4*</td>
<td>24 +/- 2%*</td>
<td>2/4</td>
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<td>74 +/- 5%</td>
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<td>16.2 +/- 4.8</td>
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<td>2/2</td>
<td>10.4 +/- 0.8</td>
<td>41 +/- 3%</td>
<td>2/2</td>
<td>10.4 +/- 0.8*</td>
<td>41 +/- 3%*</td>
<td>0/2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 5.2 *In vivo* proliferative responses to MBP in FL and MSA treated mice are variable but can be grouped into hi and lo responders. *p<0.001 as different from vehicle and all other groups by ANOVA.
5.2.2 *Tg T cells are hypo-responsive to in vitro restimulation 72 hours after the oral administration of MBP*

We next assessed antigen specific T cell function in mice that had received TCR Tg T cells followed by oral MBP. As described above, MSA and FL treated mice were fed vehicle, 20 mg MBP, or immunized with MBP in CFA 24 hours following adoptive transfer of $5 \times 10^6$ purified CD4$^+$ Tg T cells. The immunized group was included as a positive control for T cell activation. An additional control group was included wherein cells from sham-transferred mice (receiving IV saline only) served to indicate the activity of endogenous T cells were during an anti-MBP immune response. At 72 hours after feeding, MLN and PLN cells were harvested and re-stimulated *in vitro* with MBP and/or NAc1-11 peptide over a range of antigen doses. This experimental design was modeled after studies by Van Houten et al. and Sun et al. who reported antigen specific T cell activation and proliferation following feeding of OVA (212, 242).

Despite the injection of a comparable number of transgenic cells into recipients, there are differences between individual mice in the proportion of CFSE$^+$ Tg cells within the lymphoid compartment. This observation makes it difficult to compare proliferative responses in mice of different groups, where different numbers of reactive cells are present. The ability to track individual CFSE$^+$ Tg T cells, circumvents this problem since the actual number of Tg T cells present within a lymphoid organ can be calculated. In multiple experiments, we have shown that in naïve non-transferred mice, proliferation in response to MBP and NAc1-11 is negligible. Therefore, the proliferative response can be normalized on a per transgenic cell basis in recipient mice, since transgenic cells
represent the source of functional activity. This example illustrates the point: mice that receive treatment #1 exhibit an average PLN response of 100,000 CPM while mice receiving treatment #2 show an average response of 90,000 CPM, appearing similar. However, mice that receive treatment #2 have 4,000 transgenic cells / well while group #1 mice have 8,500 transgenic cells / well. If these responses are normalized to the number of Tg T cells present, there is a very different result. Group #1 normalizes to ~11.8 CPM/ Tg cell and Group #2 normalizes to 22.5 CPM / Tg cell, indicating that the responses in group #2 were actually more robust when compared to group 1 on a per cell basis. This method has been used in an effort to demonstrate in vivo hyporesponsiveness (212, 242).

In these studies, 72 hours after feeding vehicle, feeding 20 mg MBP, or immunization with MBP/CFA, proliferative responses to MBP are easily demonstrated (total CPM's / well in the 20,000-100,000 range). As shown in Figure 5.9, restimulation of PLN and MLN from MBP fed or immunized B10.PL mice was carried out 72 hours after primary antigen exposure in vivo. Cells from immunized mice display a markedly elevated response to NAcl-11 after in vitro stimulation, while cells from vehicle fed or MBP fed mice show markedly lower responses on a per cell basis, particularly in the PLN. In this particular experiment, Although the cells from MBP immunized and MBP fed mice have both divided significantly as to the results from a different experiment documented in Table 5.2, they display different restimulation profiles. If cells from vehicle fed mice are considered to be naïve, then cells from MBP fed mice retain a similar naïve response upon restimulation, despite having undergone significant proliferation in vivo.
The restimulation responses in MBP-fed FL-treated recipient mice yields similar result to those seen in non-FL-treated mice (Figure 5.10). MLN and PLN cells from FL treated MBP fed mice, display a less robust recall response to both MBP and NAc1-11 as compared to vehicle fed mice. Figure 5.9 illustrates that 72 hours after feeding, although CFSE^+ Tg^+ T cells have undergone activation and some proliferation in vivo, when MLN or PLN cells are restimulated with differing concentrations of either MBP or NAc1-11, these cells respond less than cells from vehicle treated mice. These results indicate that MBP reactive cells that are detectable are less responsive than naïve cells on a per cell basis, suggestive of T cell anergy in this population.
Figure 5.9 MBP specific T cells are less responsive to restimulation in vitro 72 hours after oral administration of MBP.

Naïve B10.PL mice received 5 x 10^6 CFSE labeled Tg T cells as described in Materials and Methods. Mice were then fed either vehicle or 20 mg MBP or were immunized with MBP/CFA/PT and sacrificed 72 hours later. Single cell suspensions were then restimulated with either MBP or NaCl-11 and tested for proliferation. Data displayed are (mean MBP specific CPM or NaCl-11 specific proliferation minus the media proliferation) / divided by the number of CFSE^+ Tg^+ T cells / well +/- SEM for 2-3 mice group. This calculation for CPM/ Tg cell has been described elsewhere (212,242).
Figure 5.10 MBP specific CD4⁺/CFSE⁺ cells display hypo-responsiveness when restimulated in vitro 72 hour after 20 mg oral MBP administration to FL treated mice.

FL treated B10.PL mice received 5 x 10⁶ CFSE labeled Tg T cells as described in Materials and Methods and shown above in Figure 5.8. Mice were then fed either vehicle or 20 mg MBP and sacrificed 72 hours later. Single cell suspensions from MLN and PLN were then restimulated with either MBP or NAc1-11 and tested for proliferation. Data displayed are (mean MBP specific CPM or NAc1-11 specific proliferation minus the media proliferation) / divided by the number of CFSE⁺/Tg⁺ T cells / well +/- SEM for 2-4 mice group and are representative of two independent experiments. This calculation for hyporesponsiveness has been described elsewhere (212,242). *=p<0.05 by Student's T-Test.
5.2.3 CD11c+ DC are pulsed with orally administered antigens soon after feeding antigen and can present it to T cells in vitro.

We have shown both in transgenic mice and in mice adoptively transferred with MBP specific Tg T cells that specific T cells are activated rapidly after feeding antigen in multiple compartments, and this activation is more widespread in FL treated mice. Based on the report by Gutgemann et al., we hypothesized that the appearance of activated antigen specific cells in peripheral lymphoid organs is the result of antigen presentation on APC in those compartments (159). We reasoned that the activation of antigen specific T cells soon after feeding would serve as a marker for the sites of that antigen presentation.

We tested this hypothesis in FL treated mice employing a similar strategy to Gutgemann et al. Within 6 hours after feeding MBP to naïve FL treated mice, we isolated cells from spleen, PLN, MLN, PP, and LP and used these cells as antigen presenting cells to stimulate purified MBP specific CD4+ T cells without the addition of exogenous antigen. We reasoned that since these Tg T cells only proliferate in the presence of MBP peptides, as demonstrated in Chapter 3, that any significant proliferation above baseline would indicate the presence of processed MBP peptide on these APC. We fed varying doses of MBP to FL treated mice (12, 25, 50, 100, or 200 mg) and sacrificed the fed mice 6 hours later. As illustrated in Figure 5.11, cells from the MLN and PLN of MBP-fed mice stimulated substantial proliferation of Tg T cells when compared to cells from vehicle fed mice. Results in the spleen were confounded by high levels of proliferation in
the vehicle-fed group. Interestingly, when the PP and LPL cells were used as APC, they
did not stimulate proliferation above baseline in these studies.
Figure 5.11 APC from FL-treated-MBP-fed mice induce proliferation in MBP specific T cells *ex vivo*.

FL treated mice were fed varying doses of MBP six hours prior to harvesting cells from MLN, PLN, spleen, lamina propria, and PP. Single cell suspensions were used as APC without exogenous antigen addition and purified CD4⁺ T cells were overlaid for proliferation as described in Materials and Methods. N= 2 mice / group. * indicates significant proliferation above baseline (vehicle-fed APC + T cell CPM) by Student’s T-Test p<0.05.
Although we demonstrated above that antigen was present on APC from FL treated mice shortly after feeding, whether or not the DC themselves were loaded with antigen after feeding was unknown. If DC were functioning as APC in vivo, we would expect to be able to detect processed antigen on their surface following isolation from the peripheral lymphoid organs of MBP fed mice. To test whether DC were actually functioning as APC following the oral administration of MBP, CD11c⁺ DC were rapidly purified from lymphoid compartments of mice fed PBS or MBP, and these DC were assayed for the ability to present antigen to MBP TCR Tg T cells. MBP doses for feeding were 100, 50, or 25 mg based on the results from the MLN and PLN shown in Figure 5.10. Figure 5.12 illustrates that DC purified from both the PLN and MLN of mice 6 hours after feeding 100, 50, or 25 mg MBP but not vehicle stimulated significant proliferation of purified transgenic T cells. This proliferation, was significantly higher than background stimulation of T cells by vehicle fed DC from vehicle fed-fed mice. Although CD11c⁺ DC appear to contain processed peptides on them capable of simulating proliferation in vitro, other APC types cannot be ruled out entirely since other MHC Class II⁺ populations were not examined. This proliferative response demonstrates the presence of MBP derived peptide on the surface of the DC.
Figure 5.12 DC from FL-treated-MBP-fed mice induce proliferation in MBP specific T cells \textit{ex vivo}.

FL treated mice were fed varying doses of MBP six hours prior to harvesting cells from MLN and PLN. Single cell suspensions were subjected to CD11c+ magnetic selection as described in \textit{Materials and Methods}. Purified DC were used as APC without exogenous antigen addition and purified CD4+ T cells were overlaid for proliferation as described in \textit{Materials and Methods}. \(N=2\) mice / group. * indicates significant proliferation above baseline by Students T-Test, where \(p<0.05\). ** \(p<0.01\).
5.3 Summary

The studies described in this chapter were performed in part to further define the mechanisms responsible for the enhanced protection from EAE in mice where DC were expanded prior to the oral administration of MBP. A common cascade of events has been described in T cell tolerance in vivo for both CD8+ T cells and CD4+ T cells. Following administration of antigen by a tolerogenic route, antigen is presented to antigen specific T cells that subsequently become activated and proliferate in vivo. As a consequence of this activation and proliferation, progeny T cells display diminished recall responses when restimulated ex vivo with the same antigen. In addition to this period of hypo-responsiveness in specific T cells, there appears to be a component of antigen-specific T cell deletion as well. In other systems, although significant cell division takes place following exposure to the tolerogen, antigen specific cell activity does not increase with cell division and over time diminishes to near undetectable levels. We hypothesized based on our results from Chapter 4, that FL-treated mice exhibit the activation sequence more quickly or would do so more completely following exposure to 20 mg oral MBP administration. To test this, we used two well accepted methods for analyzing the kinetics and dynamics of T cell tolerance in vivo: antigen feeding of MBP specific TCR Tg mice directly or feeding recipients of adoptively transferred T cells from MBP TCR Tg mice.

It was established in FL treated TCR Tg mice that a single 20 mg dose activates MBP specific T cells in multiple lymphoid compartments in vivo including the MLN, PLN, spleen, and PP. Activation of these cells was detected by an increase in the T cell
activation marker CD69, decrease in the lymph node homing receptor CD62L (L-selectin), as well as an increase in forward scatter on activated cells (predicting future in vivo T cell division). Subsequently, other studies demonstrated activation in these same compartments following oral administration of 2 mg MBP to FL treated TCR Tg mice. Activation in this setting was less extensive, i.e., fewer T cells showed signs of activation, indicating an effect of antigen dose on the number T cells activated.

Subsequent studies examining the fate of MBP specific T cells in FL treated MBP fed mice were carried out using an adoptive transfer system to track oral antigen induced changes to naïve T cells. To do this, we used two fluorescent dyes, CMFDA and CFSE, to identify MBP specific cells in vivo via flow-cytometry. Once parameters were established for the transfer and detection of MBP specific T cells, it was shown that antigen specific T cells were activated in FL treated MBP fed recipients 18 hours after feeding a 20 mg oral dose of MBP. However, as shown in Figure 5.6 and Table 5.1, FL treated mice had a higher percentage of CFSE^+Tg^+ cells expressing CD69, compared to controls. In addition, activated T cells in FL treated MBP fed mice display an increase in T cell size as well, while control recipients did not despite the presence of activated T cells. Together, these data argue that in FL treated MBP fed mice, antigen specific T cells undergo a more complete activation than in MSA treated control mice, which is likely related to the enhanced oral tolerance in FL treated mice.

To determine the short-term consequences of the T cell activation observed in Figure 5.6, the fate of antigen specific cells was determined 3 days following feeding. As shown in Figure 5.7 and summarized in Table 5.2, although significant differences were observed on day 1 between FL-treated and MSA-treated MBP fed mice, virtually no
differences were seen in the degree of cell division between these two groups when measured as a dilution of CFSE labeling. It is interesting to note that cell division was widespread in fed mice 72 hours after feeding and was observed in the MLN, PLN, and spleen as well. Furthermore, the degree of cell division within a similar treatment group was variable and responses in individual mice could be classified as being either hi or lo based on dilution of the CFSE label. This is an especially observation since on day 1, virtually all mice display some degree of antigen specific T cell activation, but only a subset of mice go on to demonstrate proliferation *in vivo* following antigen administration. The effect of this variability on tolerance induction *in vivo* is unknown at present. It should be pointed out that in hi responders, although many cells have divided at least twice based on the dilution of the CFSE dye, the cell numbers have not increased accumulated to a high degree as would be expected if parent cells are dividing. This observation has been made in other systems as well and strongly suggests that antigen-specific T cells are reaching proliferative senescence due to a lack of survival signals or because of repeated restimulation (231).

MBP specific T cells from mice fed MBP 72 hours earlier, are shown to be hypo-responsive on restimulation when compared to controls (Figure 5.9 and 5.10). In FL treated mice, this phenomenon is also observed. When compared to naïve (vehicle fed) T cells, MBP specific T cells from FL treated MBP fed mice are markedly less responsive on a per cell basis to restimulation *ex vivo*, suggestive of anergy.

The data presented in Figures 5.1, 5.2, 5.4, 5.6, and 5.7, when put into the context of other reports, strongly suggest that processed antigen is on the surface of the APC in the compartments where activation is observed. We tested this hypothesis by feeding a
range of MBP doses to FL treated mice, isolating cells from multiple lymphoid compartment and overlaying antigen specific CD4+ T cells. The results indicated that multiple doses of antigen were capable of loading APC from the MLN and PLN with MBP for presentation ex vivo as shown in (Figure 5.11). In a subsequent experiment, DC were rapidly purified from FL-treated MBP-fed mice and vehicle-fed mice. Stimulation of MBP specific T cells is seen only in DC purified from the MLN and PLN of MBP fed mice (Figure 5.12). These studies strongly suggest that the initial activation of antigen specific T cells in vivo is likely a result of the interaction between a naïve antigen specific T cell and an antigen loaded DC in vivo.
CHAPTER 6

DISCUSSION AND CONCLUSIONS

Discussion and Conclusions

The oral administration of protein antigens is widely recognized for inducing systemic tolerance in a variety of animal models. With the application of oral tolerance to the prevention and treatment of inflammatory autoimmune diseases in multiple animal models and in selected human diseases, elucidating the mechanisms of oral tolerance is a primary goal of immunotherapy (243). Identifying the key cellular and molecular events that lead to the induction of non-responsiveness to orally administered antigens would help to optimize current and formulate new protocols for treating specific autoimmune conditions and other disease states where down-regulating a specific immune response is desirable. In designing the studies described here, we considered the induction of oral tolerance from a different level, i.e. from the perspective of the antigen presenting cell. In doing so, we assumed, based on the role of CD4⁺ T cells in oral tolerance induction, that there was a requirement for MHC restriction with MHC class II⁺ cells acting as APC.
Several APC populations have been implicated in the presentation of orally administered antigen, such as IEC, B cells, Mϕ, and DC. IEC are the most numerous of these cell types, but remain fixed within the gut. They have been shown to acquire orally introduced antigen, to express MHC Class II, and to exhibit low levels of co-stimulatory molecules (160). IEC are not inherently mobile, since they adhere to the gut wall. The recent identification of dead IEC within migratory DC in vivo suggests that IEC could be classified as passive containers of orally administered antigens, not as APC (244). Moreover, Blanas et al. (245) reported that bone marrow derived APC acquire oral antigens for presentation to both CD4+ and CD8+ T cells in vivo. APC that fit this description can be narrowed to B cells, Mϕ, and DC. All are present in both the GALT and periphery, and all three cell types have been shown to promote immunologic tolerance in several experimental animal models (163-169). However, studies in B cell deficient mice have shown that B cells are not required for the establishment of either peripheral tolerance or more relevant to this study, oral tolerance (170). Vella et. al. (170) reported that T cell tolerance and antigen-induced T cell death occurred in the absence of B cells. In other studies, Alpan et. al. (246) similarly found that oral tolerance, as measured by decreased DTH, and suppression of lymphocyte proliferation and cytokine secretion, proceeded unabated in B cell deficient mice. Collectively, the studies in B cell deficient mice argue that the B cell is not the principal APC in either form of tolerance. A role for Mϕ in the induction of oral tolerance has not yet been examined, but these cells are not positioned optimally in secondary lymphoid organs to participate in tolerogenic responses to soluble antigens (247).
DC are widely recognized for their ability to initiate T cell activation both in vivo and in vitro and for their unique relationship with the circulating pool of naïve T cells in secondary lymphoid tissues (213, 247-249). Two functionally distinct subsets of DC, MDC and LDC, distinguished by expression of CD8α and tissue localization in the steady state, have been reported to differentially direct T cell responses towards either immunity (MDC) or tolerance (LDC) (186, 187, 214, 250-252). New results suggest that this may be an oversimplification as both cell types are capable of stimulating responses in vivo. The outcome of any DC / T cell interaction is therefore thought to depend on a multitude of factors including DC type, maturation state, tissue of origin, and cytokine milieu (190, 253-255). Because of obvious phenotypic and functional heterogeneity among DC subsets, it has been proposed that DC may also be involved in the induction and maintenance of peripheral tolerance to self antigens as well as in the initiation of immune responses to infectious organisms. This is based on their variable expression of surface co-stimulatory and death molecules, their capacity for limiting the expansion of naïve T cell expansion, and their central role in presentation of soluble antigens in the T cell areas of secondary lymphoid organs (187, 188, 190, 211, 253).

The aforementioned studies prompted us to purpose a major role for DC as the APC in oral tolerance. We hypothesized that expanding this population of APC using the hematopoietic growth factor FL would enhance oral tolerance to MBP in B10.PL mice since FL had been described to expand functionally mature DC in vivo. The results described here support that hypothesis by demonstrating that oral tolerance to the
autoantigen MBP and protection from as well as treatment of EAE is enhanced in B10.PL mice following expansion of DC. Our results reported in Chapter 3 show that FL expands CD11c+/MHC Class II+ DC in the GALT and the peripheral lymphoid organs. The degree of expansion and observed phenotype of the expanded population confirms studies conducted in other strain of mice (176, 184, 198-200, 256). To the limits of our detectability, we observed only the expansion of CD11c+ cells, the majority of which are also MHC Class II+. Others have identified an expansion of with NK expanding activity \textit{in vivo} for expanding as well, especially in the blood, bone marrow, liver, and spleen (205). In our hands however, such an expansion was not seen; no significant increases were seen in the percent of NK cells in the spleens of mice treated for nine days with FL as defined by CD122 (IL-2Rβ) and DX5 dual positivity (data not shown). Expansion of cells bearing NK markers was not assessed in other compartments and this represents a potential caveat to the interpretation of the results reported in Chapter 3, where in Figure 3.15 FL administration was linked to early but not lasting protection from EAE, possibly due to the protective effects of NK cells (257). Although speculative, this notion could be easily tested \textit{in vivo} depletion of NK cells from FL treated mice prior to induction of EAE (Todd Fehniger, personal communication).

The DC expanded by FL, exhibit markers consistent with a resting phenotype. When analyzed directly \textit{ex vivo}, the expression of surface costimulatory molecules such as MHC Class II, CD40, ICAM-1, CD80, and CD86 is relatively low and can be greatly increased in the presence of a strong adjuvant such as LPS. This pattern of expression,
shown in Figure 4.3, is consistent with the results of others in FL treated mice. The importance of these results will be discussed later in the context of our studies in oral tolerance, but the expression of low levels of costimulatory molecules on DC has obvious implications for these cells inducing tolerance in vivo. Importantly, FL expanded DC when purified, present both soluble protein (MBP) and peptide (NAc1-11) antigens in similar fashion to purified DC from the spleens of mice not treated with FL. Although studies comparing DC populations from FL treated mice to those from non FL-treated mice were not exhaustive, our results are consistent with published showing that, DC from FL treated mice are functionally mature but not dissimilar from control DC (198). More extensive comparisons between all DC subtypes from FL treated and control mice may yield important mechanistic insights and targets for in vivo antagonism in future studies, since many reports do show functional heterogeneity among DC based on tissue of origin (254, 255).

In Figure 3.5, FL administration is shown to expand both MDC and LDC lineages in the GALT (PP and MLN) and in the peripheral lymphoid organs (PLN and spleen) in B10.PL mice, in agreement with previous reports (176, 198). Expansion of both lineages is significant importance in the interpretation of our results, since it is the LDC population that has received the most attention as the tolerogenic APC in vivo.(186-188, 190). Had FL expanded only MDC or LDC, insights into which population is vital to oral tolerance could have been obtained. Alternatively, such a study could be performed done in the B10.PL EAE system if DC are expanded with GM-CSF, since this cytokine has been described to expand only MDC. Related experiments have been performed in
other mouse strains to distinguish MDC from LDC functionally in vivo in the generation of immune responses rather than tolerance (215). Similar studies of oral tolerance induction with GM-CSF in B10.PL mice might be useful in the future for testing whether the lineage of expanded DC has any significant effects on tolerance to oral antigens.

Since it was our ultimate goal to assess oral tolerance induction in FL treated mice, it proved necessary to assess the effects of FL alone on the EAE disease course. Despite the similarities between control and FL-expanded splenic in antigen presenting capabilities in vitro, we undertook experiments to assess the effects of FL administration on the EAE disease course, since others had reported adjuvant effects following FL administration in vivo (177, 185, 206). We first administered FL to two different strains of MBP TCR Tg mice to assess the de novo adjuvant properties of FL and hence DC expansion, as these strains differ in their expression of spontaneous EAE (93, 94, 223). It was conceivable that the massive expansion of DC could result in a cascade of MBP specific T cell activation causing autoimmune pathology (258). As shown in Figure 3.14, neither strain of mice was observed to develop spontaneous EAE during the test period as a consequence of receiving FL for nine days. Consistent with this lack of disease induction, when CD4+ T cells from FL treated Vα4Vβ8.2 TCR Tg mice are examined directly ex vivo, they maintain a naïve phenotype consistent with being naïve, as shown in Figure 5.2.

Although we saw no effects of FL administration on spontaneous EAE in TCR Tg mice, we did observe markedly different effects on EAE depending upon whether in FL
treatment occurred either immediately before or immediately after EAE challenge. A shown in Figure 3.15, when FL was administered for nine days prior to disease induction, mice did not develop EAE. We were surprised by these given the expansion of such a large number of immunogenic and immunocompetent APC, especially since DC have been described as the primary APC to interact with naïve T cells in vivo following immunization in other systems (259, 260). In contrast to this result, mice receiving FL for nine days after disease induction had a markedly more severe disease course as summarized in Table 3.1 and Figure 3.15. These latter were more the expected outcome since DC stimulatory capacity is highly dependent on the environment and milieu in which the DC develop (261-263). We predicted that DC maturing in peripheral tissues with ongoing immune response would lead to enhanced effector T cell generation and worsening of the acute disease course. That outcome was observed in the latter group of FL recipients as summarized in Table 3.1. It should be pointed out that despite the lack of EAE in mice treated with FL prior to immunization with MBP, protected mice did mount an MBP-specific T cell proliferative response, shown in Figure 3.16, indicating that FL did not produce a generalized immunosuppression. Further experiments are required to examine the cytokine producing phenotype in vivo in these mice, as this may be more relevant to disease than proliferative responses.

In contemplating the mechanism underlying these results in mice that received FL prior to EAE induction, it is worth noting that FL has been reported to expand functional NK cells in multiple tissues including the blood, liver, and bone marrow when administered for 10 days or greater. Although we did not see expansion in the spleen in
B10.PL mice, we cannot rule out that these cells were expanded in other tissues. If NK cells are expanded in FL treated mice, their presence may explain the protection from acute EAE observed in FL treated mice. It has been shown that depletion of NK cells alters the course of EAE making it more severe (257). Going further, NK cells have been identified as "possible innate protectors" from autoimmune conditions based on their activity against encephalitogenic T cells in vitro (264). This possibility for the mechanism of protection from EAE could be tested both in vivo and in vitro by NK depletion, adoptive transfer of NK cells from FL treated mice, and/or, in vitro assessment of NK-mediated killing of MBP specific encephalitogenic T cell targets.

FL administration had significant effects on EAE induction when mice were immunized directly before or directly after FL mediated DC expansion. However, the design of oral tolerance experiments is fundamentally different from those depicted in Figure 3.15. Our goal was to test oral tolerance in FL treated mice, and we therefore expanded DC directly prior to feeding with the timing in relation to sensitization being EAE challenge occurring seven days after feeding. We went on to test the effects of FL administration on oral tolerance aware of the possibility that FL effects on EAE may make studies of oral tolerance difficult or impossible. As shown in Figure 4.1, FL administration had no effect on EAE when DC were expanded seven days prior to MBP immunization, suggesting that protective effects seen in Figure 3.15 were short-lived. This opened the door for studies comparing oral tolerance in FL treated versus control-treated mice since EAE was the same in both groups.
When testing oral tolerance in FL treated mice, enhanced protection from EAE was achieved following a single dose of 2 mg of MBP only when DC were expanded with FL, whereas a single 2 mg feed alone did not suppress disease as shown in Figure 4.1 and Table 4.1. This result is quite impressive when considering the gap between the effects of 2 mg oral MBP, alone that provided no protection, and 2 mg oral MBP in the presence FL that provided significant protection from EAE. Oral tolerance using FL was enhanced in mice fed a larger 20 mg dose of MBP was fed (Figure 4.1C and Table 4.1). Surprisingly in these studies, a 20 mg feed of MBP did not provide statistically significant protection from EAE as we had observed in other studies (90). Despite this, oral MBP in the presence of expanded DC, regardless of the amount of MBP given orally, correlated with protection from EAE. These observations are consistent with oral tolerance to OVA in which DTH responses and anti-OVA proliferative responses were reduced in FL-treated animals fed as little as 0.25 mg OVA relative to animals fed OVA alone (176). Taken together, both of these reports suggest that oral tolerance in FL treated mice is made more efficient, i.e. better “tolerance” is observed and lower doses of antigen are effective in the presence of FL.

We considered the mechanism of protection in FL-treated mice by examining the cytokine secreting frequencies in the PLN of protected mice in relation to mice that were not protected via ELISPOT. T cell populations in FL-treated, MBP-fed mice are hypo-responsive when compared to controls as to the frequency of detectable cell secreting either both Th1 (IL-2 and IFN-γ) and Th2 (IL-4) cytokines following restimulation ex
In vivo shown in Figure 4.2. Furthermore, we did not observe an antigen-specific increase in production of TGF-β, at either dose of fed antigen and the frequency of cells secreting this cytokine did not change with feeding nor did it correlate with protection. These observations are consistent with tolerance due to anergy and/or deletion, and not immune deviation or active suppression. However, there still exists the possibility that suppressor cells secreting IL-10 and/or TGF-β reside in the CNS or some another compartment and have escaped detection. Again, as mentioned in Chapter 4, the results as to the induction of an anergic or deletion response in oral antigen reactive cells presented here are in general agreement with studies of oral tolerance to OVA in FL treated Balb/C mice, where mice were protected from DTH more completely and that anti-OVA proliferative responses were suppressed more completely in FL treated-OVA-fed mice. Although, we did not see an effect on proliferation, major cytokine secreting hypo-responsiveness was observed leading us to speculate that this may ultimately bear more relevance to protection from EAE. Ultimately we maintain based on these data that protection from EAE in FL treated MBP fed mice results from either a paucity of MBP reactive cells following a deletion of a critical number of these cells after feeding or in the induction of anergy in these cells rendering them unable to become encephalitogenic T cells upon immunization with MBP. In support of this point, data in the aforementioned OVA/FL system, adoptive transfer experiments reveal that after feeding and subsequent sensitization with OVA, FL treated-OVA fed mice fail to expand OVA specific T cells with immunization. However, despite our best efforts we are still unable to rule out the
population of a novel cell type that is acting as a suppressor population *in vivo*, possibly acting to limit the expansion of encephalitogenic cells by some undescribed means.

These results indicate that the oral tolerance achieved in the presence of an expanded DC network is capable of inhibiting an organ specific autoimmune disease and maintaining the protected state for several weeks. However, Pulendran et al. (206) have recently reported that expanding DC with FL abolishes peripheral tolerance induced by IP injection of soluble antigen. One possibility to explain the differing outcomes pertains to the route of tolerogenic exposure. By administering MBP via the oral route, antigen could be targeted to a different subset of expanded DC than when antigen is administered IP, a hypothesis consistent with other reports that show specific populations of GALT derived DC becoming loaded with orally administered antigens (174, 175). Whether these same APC are being loaded with antigen following an IP injection is unknown, but probably unlikely based on the total difference in routes of introduction.

An alternative explanation is that oral administration may cause more effective disaggregation of soluble antigen before uptake by APC. To discriminate between these possibilities however, it will be necessary to compare the two routes of antigen administration directly with identical experimental measures. The differences in outcome between these studies (tolerance vs. priming) using the same growth factor suggest that orally induced tolerance represents a unique situation relative to the induction of IP tolerance. This is of additional importance with regards to the induction of tolerance following the injection of soluble antigen via the IV route, as many have proposed based
on well established data that DC mediate tolerance to soluble IV antigens. The importance of this with regards to oral tolerance will be discussed further below.

Building on our initial studies that focussed on the enhancement of prevention of EAE with FL and oral MBP and those described in the OVA system, we have gone on to demonstrate that FL is effective in conjunction with oral MBP in the treatment of EAE in mice with ongoing chronic disease. We demonstrate that FL is not only effective in expanding DC during REAE, shown in Figure 4.5, but that doing so does not exacerbate the course of the disease. Interestingly, as shown in Figure 4.6, DC from FL-treated mice with REAE are nearly indistinguishable from those from FL-treated naïve mice data bit shown with respect to APC accessory molecule expansion. It should be noted that DC were expanded in mice with EAE similar to mice without EAE, and that co-stimulatory molecule expression was similar between the two groups of animals, despite the presence of an ongoing inflammatory response in the former group (data not shown). Figure 4.6 shows that significant reductions in clinical signs were observed after only seven feeds of MBP, when DC were first expanded in mice with REAE. Typically, a total of 14 feeds is necessary to achieve significant clinical benefit using this feeding regimen (92). How feeding is benefiting these mice is speculative at this point however. It is clear that in the periphery there is a general decrease in anti-MBP proliferation but this may not be sufficient to explain the suppression of EAE in the mice that received FL and oral MBP since oral MBP fed mice displayed decrease in MBP reactivity as well. Instead, there may be more to this protection immunologically than what is seen in the peripheral lymph nodes and spleen. It is quite possible that a population of suppressor cells has been
generated *in vivo* due to the multiple oral MBP administrations that are acting to suppress disease in the spinal cord or brain of FL treated / MBP fed mice. It is conceivable that such a population could have been generated via the multiple oral administrations of MBP, which have again escaped our attention. The protection in these mice may be quite distinct from that observed in mice from fed prior to challenge. Detailed histopathological studies of the CNS of these different groups should yield insights into these possibilities. What is most significant in these studies is probably that EAE is not exacerbated by FL opening the door to multiple antigen specific interventions in these mice with expanded DC.

Finally, Chapter 5 addresses the fate of antigen specific T cells in FL treated mice in order to better understand the enhancement of oral tolerance in FL treated mice. A common cascade of events has been described in multiple systems of T cell tolerance *in vivo* for both CD8+ T cells and more relevant to this work, CD4+ T cells. Contained within this cascade is the presentation of the test antigen to antigen specific T cells that subsequently become activated and proliferate *in vivo*. In these studies it has thematically been shown that following activation and some proliferation *in vivo*, progeny cells display diminished recall responses when restimulated *ex vivo* with the test antigen. In addition to the hypo-responsiveness in specific T cells, there appears to be a component of deletion of cells as well as they do not accumulate as predicted and fail to persist *in vivo* (212). Following soluble antigen exposure in the absence of adjuvant, although significant cell division takes place following challenge with test antigen, antigen specific cell activity does not increase with cell division and over time diminishes entirely to near
undetectable levels (239). We hypothesized based on our results form Chapter 4, that in FL treated mice subjected to similar analysis, MBP specific T cells would either go through the activation / tolerization process more quickly or would do so more completely following a 20 mg oral MBP administration, given that oral tolerance was better in these mice.

It was established in FL treated TCR Tg mice that a single 20 mg dose activates MBP specific T cells in multiple lymphoid compartments *in vivo* including the MLN, PLN, spleen, and PP. Activation of these cells was detected by noting increases in the T cell activation marker CD69, decreases in the lymph node homing receptor CD62L (1-selection), as well as antigen specific T cell blasting. Subsequently, other studies demonstrated activation in these same compartments following oral administration of 2 mg MBP to FL treated TCR Tg mice. Activation in this setting was less extensive as a percent of the response seen in mice fed 20 mg MBP, indicating an effect on antigen dose for the percentage of T cell activated.

Subsequent studies of the fate of MBP specific T cell in FL treated MBP fed mice were carried out using an adoptive transfer system to track oral antigen induced changes to naïve T cells using two fluorescent dyes CMFDA and CFSE. The dyes were used to mark and later identify MBP specific cells *in vivo* via flow-cytometry. The results from Figure 5.1 were confirmed in FL treated MBP fed recipients where antigen specific T cells were activated 18 hours after feeding a single dose of 20 mg oral MBP in multiple tissues. However, a potentially significant pattern emerged when MSA treated recipients were compared to FL treated recipients after oral administration of MBP. As shown in
Figure 5.6 and listed in Table 5.1, FL treated mice had a higher percentage of the detectable CFSE$^+$/Tg$^+$ population of cells expressing CD69, an early T cell activation marker when compared to controls in the MLN and PLN. In addition, activated T cells in FL treated MBP fed mice display T cell blasting as well, while control recipients did not despite the presence of activated T cells. Together these data argue that in FL treated MBP fed mice, antigen specific T cells undergo a more complete and more rapid activation than in MSA treated control mice, which may have some bearing on the enhanced oral tolerance in FL treated mice.

The functional consequences of the observed widespread activation discovered in Figure 5.6, was determined 3 days following feeding by restimulating recovered in vitro with MBP and NAc1-11 peptide. Results shown in Figure 5.7 shows and is summarized in Table 5.2, that, although significant differences were observed on day 1 between FL treated MBP fed mice and MSA treated MBP fed mice, virtually no differences were seen in the degree of cell division between these two groups when measured as a dilution of CFSE labeling. What is interesting is that cell division was widespread in fed mice 72 hours after feeding and was measured in the MLN, LNC, and the spleen. Furthermore, the degree of cell division within similar treatment groups was variable and responses in individual mice could generally be classified as being either hi or lo based on dilution of the CFSE label. This is especially interesting since 18 hours after feeding virtually all mice display some degree of antigen specific T cell activation, but only a subset of mice display vigorous proliferation in vivo following antigen administration. It is impossible to tell what impact, if any, this variability has on tolerance induction in vivo. More
experiments would be needed to sort this out. In mice that displayed extensive \textit{in vivo} proliferation of MBP specific T cells, it should be pointed out that although cells have divided at least twice based on the dilution of the CFSE dye, the cells have not accumulated or persisted to a high degree as might be expected when nearly 100% of parent cells are dividing. This observation has been made in other systems as well and strongly suggests that specific T cells are reaching proliferative senescence due to a lack of sufficient costimulatory survival signals or because of repeated restimulation (231).

Going further in examining the fate of MBP specific T cells, upon restimulation, MBP specific T cells from the MLN and PLN mice fed MBP 72 hours prior, are hypo-responsive to restimulation when compared to controls as shown in figure 5.8. In FL treated mice this is seen as well. As illustrated in Figure 5.9, when compared to naïve (vehicle fed) T cells, MBP specific T cells from FL treated MBP fed mice are markedly less responsive on a per cell basis to proliferation restimulation \textit{ex vivo}, a phenomenon suggestive of, but not exclusive to, the induction of anergy. This observation was made in two important lymphoid compartments, the MLN and PLN. Further analysis of other functional readouts such as MBP specific cytokine production will be critical in determining the properties of this hypo-responsiveness (i.e. do cells "tolerized" cells make any cytokines or are MBP specific T cells incapable of making any cytokines?)

The data presented in Figures 5.1, 5.2, 5.4, 5.6, and 5.7, when put into the context of the report by Gutgemann et al. where rapid T cell activation after feeding pigeon cytochrome-C (PCC) peptides was correlated with the presence of unidentified antigen pulsed APC capable of stimulating peptide specific T cells \textit{ex vivo}, strongly suggest that
processed antigens are on the APC themselves in the compartments where activation is observed (159). We tested this hypothesis in our system where we had observed activation in multiple compartments after feeding. Feeding a range of MBP to FL treated mice followed by overlaying antigen specific CD4⁺ T cell in culture, indicated that multiple doses of antigen were capable of loading APC form the MLN and PLN with MBP for presentation \textit{ex vivo} as shown in Figure 5.10. In subsequent experiments DC were rapidly purified from FL treated MBP fed mice and FL treated vehicle fed mice. Figure 5.11 demonstrates that with purification, the ability to stimulate MBP specific T cell is seen only in DC purified from the MLN and PLN MBP fed mice. Although not exhaustive, these studies indicate that the initial activation of antigen specific T cell \textit{in vivo} is likely a result of the interaction between a naïve antigen specific T cells and an antigen loaded DC \textit{in vivo}. Further immunofluorescent analyses of tissues from FL treated, MBP-fed mice visualizing CD11c⁺ DC interacting with CFSE⁺ T cells \textit{in vivo}, to definitively rule in DC and not some other cell type in FL treated MBP fed mice.

Summarily, antigen specific T cell activation in multiple lymphoid compartments including the GALT, spleen, and PLN shortly after feeding was observed. The route(s) by which antigen distributes to these site(s) of antigen specific T cell activation and subsequent tolerance induction is presently unknown. A pathway for the transport and transfer of antigen between migratory MDC and stationary LDC has recently been reported and has obvious implications for oral tolerance (265, 266). We have shown that cells of the DC lineage can be isolated from the MLN and PLN of mice fed MBP, but
not vehicle, and that these DC can stimulate MBP specific T cells \textit{in vitro} to proliferate (Figure 5.11). Importantly, these cells can be isolated in as little as six hours after feeding a single bolus of oral antigen. The quickness of the appearance of antigen in these sites taken with the fact that antigen appears in lymph nodes that do not directly receive the drainage of the gut to receive antigen pulsed DC suggest that there is an early intra-vascular (IV) phase of oral tolerance in which antigen traffics to the periphery for capture by resident APC. This hypothesis is not novel and has been proposed based on the results of other studies where antigen was detect via a similar antigen specific T cell readout using APC isolated from the spleen of fed mice (159). What is important to consider in the interpretation of these results is that the primary APC located in the T cell areas of unstimulated (steady state) secondary lymphoid organs are LDC, which are incapable of phagocytizing protein antigens and may only present peptide antigens (190). It is in these T cell areas where the majority of T cell priming occurs following IV administration of antigens (238), following which T cell anergy ensues, suggesting that the proliferation induced by DC \textit{ex vivo} when mixed with MBP reactive T cells may represent the initial T cell activation and proliferation seen in studies by Kearny et al, Sun. et al, and in our own studies. Together, the similarities to IV antigen administration and oral administration argue strongly for an initial spillover of oral antigen derived peptides in the circulation. Determining the precise route(s) (i.e. whether antigen alone or antigen on the surface of a migratory APC) by which antigens enter the T cell areas in the periphery following oral administration is therefore critical to understanding the development of oral tolerance. In either case, based on our results that detect antigen on DC isolated from Fl treated, MBP
fed mice, DC would be expected to play a pivotal role in the initial events of oral tolerance in FL treated mice, both for their ability to transport antigens from distant sites to the T cell areas of lymphoid organs and for their representation in these sites \textit{in vivo}.

Putting the results reported in this dissertation into the proper immunological context requires taking a step away from oral tolerance and exploring multiple and important current issues that are developing linking DC to the maintenance of peripheral tolerance to self \textit{in vivo}. Overwhelming evidence now exists implicating DC in the induction and maintenance of peripheral tolerance to self antigens. A series of studies performed in Tg mice using the neo-self antigen influenza hemagglutinin (HA) by Adler et al. demonstrated through the use of bone marrow chimeras that tolerance to HA in adoptively transferred HA specific CD4\(^{+}\) T cells required that bone marrow derived antigen presenting cells express the appropriate MHC molecules (267). Said another way, the T cell anergy induced in this system required that self-antigens be presented on bone marrow derived cells as opposed to parenchymal cells. When non-hematopoietic cells expressed the appropriate class II molecules but not the bone marrow derived cells, tolerance did not ensue. Subsequent studies using the HA self-antigen model combined with adoptive transfer of HA specific CFSE labeled CD4\(^{+}\) T cells have further shown that the tolerance achieved requires a subsequent activation and multiple cell divisions of transferred naïve self–antigen specific CD4\(^{+}\) T cells (231). It was interesting in these studies that neither the rate nor the number of cell division determined whether or not tolerance was achieved \textit{in vivo}. Instead in these studies it was the immunologic context in
which the antigen was presented. Antigens presented as a soluble neo-self antigen induced tolerance while antigens presented in the context of a viral antigen, tolerance was ablated. The results depicted in this latter study are very similar to those described in Chapter 5 following oral antigen administration, where cells divide rapidly after either oral antigen administration or sensitization, but with widely divergent outcomes. Studies in related systems of neo-self antigens have shown similar results in CD8^+ T cell tolerance where OVA was used as a self-antigen expressed by pancreatic β-cells. These experiments demonstrated conclusively that transferred naïve CD8^+ cells are tolerized after successive rounds of cell division and this tolerance was mediated by a cell of bone marrow origin contained only within the draining nodes of the pancreas (232, 268). Although these experiments do not indicate which type(s) of bone marrow derived APC are involved in this process, DC are the most likely candidates as predicted by close approximation with circulating naïve T cells (269). DC have been shown to express high levels of self-tissue and tumor derived antigens in the T cell areas of draining nodes, thereby forming a link between what proteins are present in tissues and what antigens are being presented in the draining nodes (265, 270).

The answer to the key question of how self-antigen is processed and presented by DC in draining lymph nodes is also coming to light. It is known that immature DC phagocytize and process apoptotic as well as necrotic cell debris in vitro. Immature DC are well suited to the task of engulfing apoptotic debris via a αvβ5 integrin mediated mechanism (271). Immature DC additionally possess FcγR for engulfment of immune complexes. Not limiting their diet, immature DC will engulf microbes, latex beads, as
well as very large amounts of extracellular fluid via the process of macro-pinocytosis (272). Intuitively however, what is engulfed seems to determine the outcome of the consequent immune response generated after processing and presenting engulfed material. For instance, necrotic cells when engulfed act as natural adjuvants by stimulating the maturation of DC, while apoptotic material is processed but does not induce final maturation of DC (273, 274). Collectively these studies suggest that DC present antigens based on the signals received from their initial environment confirming the role that “danger signals” may be playing in instructing the APC to present antigen appropriately (275). The problem with this finding is that based on recent findings of Inaba et al. indicating that immature DC do not express surface peptide MHC complexes for presentation to CD4^+ T cell without the presence of maturational stimuli such as TNF-α, LPS, or CD40 ligation (263). These stimuli, although important for inducing antimicrobial immunity, also cause DC to increase their expression of multiple costimulatory molecules and pro-inflammatory products such as IL-12 (213). The obvious problem here is that if in order for DC to be tolerogenic after apoptotic cell engulfment, based on the findings of Inaba et al., DC would require maturational (danger?) signals to present peptides that would at the same time cause them to be come more immunostimulatory. This catch-22 is a major obstacle in relating the studies of engulfment of apoptotic cell debris and neo-self antigen tolerization in vivo to the maintenance of peripheral tolerance to self in vivo. Demonstration of a pathway linking apoptotic cell engulfment or other self antigen transport to T cell areas of draining lymph nodes, and the appearance of detectable self-antigen on MHC Class II molecules on DC with low co-stimulatory
molecule expression would have to made to support the claimed relevance to tolerance via DC antigen presentation.

Many of aspects of this hypothesized pathway have been described. Work by Huang et al. has recently shown that debris from apoptotic IEC can be detected in a population of migrating DC in the rat (244). This report demonstrates beautifully the existence of a constitutive pathway taking apoptotic self cells from the lumen of the gut and being delivered to the T cell area of areas of the mesenteric lymph node. In other studies, Inaba et. al have demonstrated the appearance of processed antigen from phagocytized apoptotic cells on the MHC Class II molecules of dendritic cells (266). It is interesting to note that the DC on which high levels of MHC Class II / self antigen complexes have been detected are mature DC in that they express high levels of Class II, but fail to express high levels of CD86 (265). How this is occurring in light of the fact that Class II molecule expression is tied closely to maturational state and co-stimulatory molecule expression occurs presumably following some sort of antigen transfer mechanism that is described or implied and speculated upon in other tumor and DNA injection models (270, 276). These intriguing observations have lead to the speculation that certain lymph node DC can process other shorter lived transport DC; effectively transferring antigens from one cell to another (253). This is supported directly from multiple experiments that describe a population of short lived “transfer DC” that engulf antigens sampled from non-lymphoid tissues such as the gut as described by Huang et al., migrate via the lymphatic drainage, for eventual processing by other lymph node resident DC that do not turn over (266, 277)
Does any of this apply to the induction of oral tolerance? It appears based on multiple studies examining the roles of these cells in the processing of orally administered antigens that it does. Antigen-laden cells of the DC lineage can be detected in the LP, PP, and mesenteric lymph drainage following oral antigen administration, suggesting that cells at these sites acquire orally administered antigen in and subsequently traffic to the mesenteric lymph node (173-175). However, in tolerance transfer attempts these DC were demonstrated to cause immune activation and T cell priming \textit{in vitro} and \textit{in vivo}. It has been suggested that the immune priming observed is a result of DC activation induced during cell purification (160). It should be noted that on one occasion tolerance was induced in mice receiving oral antigen pulsed non-purified APC taken from the lamina propria (278). It may be that potentially tolerogenic DC, once isolated, lose their ability to promote tolerance \textit{in vivo} because of altered trafficking patterns to lymphoid organs and/or the expression of surface molecules and/or chemokines. This notion is supported by recent DC transfer studies where lymphoid DC when transferred failed to leave the injection site (279, 280). As mentioned, oral antigen has been detected indirectly on an unidentified APC in the spleen of peptide-fed mice in as little as six hours after feeding peptide antigen (159). Just how the antigen appears at this site so quickly is a matter of speculation however. It is possible that antigen pulsed cells arrive in the spleen within six hours, but a more likely possibility given the drainage patterns of the gut, is that the fed peptides in these studies by Gutgemann et al appear in the spleen via and intravascular route. It is vital to point out that when soluble antigens are given via
an IV route, T cell tolerance is the default with T cells interacting not with B cells in vivo, but with APC in the T cell areas of lymphoid organs (238). In fact B cells are not required in this process at all (170). This latter point is of utmost importance if oral antigens are getting to draining lymphoid tissues via an IV route, since DC as well as B cells acquire soluble antigens administered this way (171). Regardless of how the antigen got to APC in the spleen in studies by Gutgemann et al., these results when taken with the more recent observation that a subset of DC from the lamina propria of rats actively transport fragments of recently apoptosed IEC to the T cell areas of the MLN, these results strongly suggest that not only are GALT derived DC important to consider as APC of oral antigens, but DC in peripheral tissues are as well (244). The importance of this pathway is additionally reinforced in recent studies indicating that the APC that present oral antigens to CD8+ T cells and CD4+ T cells are of bone marrow origin (245).

Summarily, the bulk of the aforementioned studies are convincing, albeit somewhat circumstantial, in implicating DC in the induction and maintenance of peripheral tolerance. Despite the apparent differences between the two kinds of tolerance discussed (i.e. tolerance to "self" in the periphery vs. tolerance to oral antigens), we now hypothesize based on our findings a similar role for DC as APC in high-dose oral tolerance to MBP in FL treated mice. Initially, we hypothesized that expanding this normally trace population of APC using the hematopoietic growth factor FL, would enhance oral tolerance by virtue of increasing the numbers of potentially tolerogenic APC. It could very well be that FL has acted in simply increasing the numbers of DC capable of presenting oral antigens that in turn increases the chances a naïve MBP
specific T cell will interact with an oral antigen laden APC. Importantly, in these experiments the DC were in a resting state expressed low levels co-stimulatory molecules. What the actual tolerogenic mechanisms are that these DC are using are unknown but many possibilities exist. These possibilities are put forth based on the findings reported in Chapter 4 and 5 where the fate of T cells in FL treated MBP fed mice could best be described in terms of initial priming followed by proliferation without accumulation with surviving cells being hypo-responsive to antigen upon restimulation.

What we propose is happening based on our results and the results of other in similar systems is as follows: oral antigens by whatever means (IV or on a migratory DC) are presented by DC in multiple tissues in vivo to resting naïve T cells. These DC for their own part are resting in vivo and they express low levels of costimulatory molecules such as B7.1 and B7.2. This fact may be the most important aspect of the entire process as reversal of this resting state expression with adjuvants such as CT, IL1β, or systemic LPS ablates oral tolerance and increase costimulatory molecule expression on the APC (67). In FL treated mice, oral OVA given concomitantly with adjuvant actually acted as an oral vaccine and sensitized mice to OVA. (230). In fact, the low levels of surface costimulatory molecule expression by DC in FL treated mice may actually favor the preferential use of the T cell inhibitory molecule CTLA-4 (281, 282). Said another way, costimulation through CD28 is not augmented and signaling through the negative signaling molecule CTLA-4 may be preferred, disallowing the T cells from proliferating into effector cells, and forcing them into a "holding pattern" and ultimate state of anergy. The role of CTLA-4 in multiple models of tolerance is well documented including a role
in high dose oral tolerance (283). Although we have not examined the role of this molecule explicitly in this system, this could be tested via either in vivo antagonism of CTLA-4 using monoclonal antibodies or using MBP specific T cells from CTLA-4 knockout mice.

Other possibilities exist as well as to how MBP specific T cells may be rendered non-responsive including the breakdown of the amino acid tryptophan by indoleamine 2,3-dioxygenase secreted or produced by DC. In vivo this enzyme effectively starves T cells of a critical nutrient for T cell development (tryptophan) by eliminating it in the microenvironment. This enzyme has been identified as being critical for limiting T cell expansion and effector function and could be a way that resting DC limiting expansion of MBP reactive T cells once they are activated based on other reports (284). A critical role for this enzyme in suppressing T cells has been described elsewhere for the protection of fetal tissues from spontaneous abortion and this enzymes system has been described in DC (285,286). Definitive studies including well controlled inhibition in vivo of this enzyme would have to be performed to answer this question directly since expression of this enzyme is widespread in lymphoid tissues including DC (284). Still other DC phenotypic possibilities could account for our results, namely the autocrine production of TGF-β and / or IL-10 by the DC at the time of antigen presentation which may limit the expansion and effector development of oral antigen specific T cells in vivo (255). However, these possibilities are probably not as likely as high dose oral tolerance has been shown to be independent of both of these cytokines (39, 47). Ultimately however, a role for these two cytokines cannot be excluded as possibilities as until
adequate *in vivo* studies antagonizing these cytokines have been done either through antibody depletion or some other means of *in vivo* antagonism.


47. Aroeira, L. S., F. Cardillo, D. A. De Albuquerque, N. M. Vaz, and J. Mengel. 1995. Anti-IL-10 treatment does not block either the induction or the maintenance of orally induced tolerance to OVA. *Scand J Immunol* **41:**319.


176


