Understanding the Immunopathogenesis of Inflammatory Diseases of the Gastrointestinal Tract

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Understanding the Immunopathogenesis of Inflammation

Dinesen's Textbook of "..."
Statement of Authorship

I, Elizabeth Eskdale Forbes, declare that the investigations of this thesis, are my own work, with exception to studies carried out in collaboration with others, and these are acknowledged in the text.

Acknowledgements

Many moons ago a great philosopher coined the phrase, “Our truest life is when we are in dreams awake”, Thoreau. I embarked upon this journey filled with the passion of being able to make that happen. I am so glad to be able to have come full circle, still with a love for what we do.

Having begun my PhD in August 2004, I have a multitude of people to thank from then to now. Just as well I’ll never make it to the Grammy’s or the Golden globes... As I’d definitely get cut short with the show’s theme music and politely carted off by some handsome men in sensational suits... I’ll ask you now to bear with me in this list of people who require a thank you. Each and every person on this list, in his or her own special way, made this moment possible and I’d like to take the time to acknowledge that.

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“Some people come into our lives and quickly go. Some stay for awhile and leave footprints on our hearts. And we are never, ever the same”. Eleanor Roosevelt.
Abstract

Numerous gastrointestinal diseases including food allergy, gastritis, cow's milk hypersensitivity and inflammatory bowel disease are associated with pronounced inflammation of the gastrointestinal (GI) tract. This inflammation leads to pathological and functional alterations to the GI tract, resulting in the clinical symptoms of these diseases. GI allergic disorders represent a vast range of inflammatory diseases that are increasing in both incidence and severity. However, the role of specific cells and mediators responsible for the key molecular and cellular responses, which characterise specific clinical manifestations, remains unknown. While the array of allergic diseases affecting the GI tract involves disease states with varying clinical presentation, the development of experimental models of GI allergy and inflammation has provided important insights into the immunological mechanisms regulating immunopathogenesis.

Eosinophilic inflammation is a common feature of numerous eosinophil-associated GI diseases. Central to eosinophil migration into the GI tract are integrin-mediated interactions with adhesion molecules. Although the mechanisms regulating eosinophil homing to the small intestine have begun to be elucidated, the adhesion pathways responsible for eosinophil trafficking into the large intestine are unknown. In the present study, the role of adhesion pathways in eosinophil recruitment into the large intestine during homeostasis and disease were investigated. Colonic eosinophilic recruitment during disease (experimental colitis) occurs via a b7-integrin-independent pathway. Eosinophils express both the MAdCAM-1- (a4b7) and ICAM-1- [MAC-1 and LFA-1 (b2, a5, and aM)] ligands and the expression of these molecules are upregulated by in vitro stimulation with eotaxin-1. Antibody blockade of a1, aM and b2 integrins during chemotaxis assays inhibits eosinophil chemotactic responses to eotaxin-1. Employing a well-established model of experimental colitis associated with the administration of DSS it was shown that ICAM-1 adhesion pathway (but not VLA-4/VCAM-1 and a4b7/MAdCAM-1 pathways) is important for the development of eosinophilic inflammation and during experimental colitis. These studies demonstrate that ICAM-1-dependent and not b7-integrin/MAdCAM-1-dependent pathways are integral to eosinophil recruitment into the colon during GI inflammation. A pathogenic role for eosinophilic inflammation via ICAM-1 mediated transmigration in the development of experimental colonic injury was demonstrated. These investigations suggest that
antagonism of ICAM-1 transmigration pathways may provide significant therapeutic approaches for the treatment of UC and/or allergic colitis; and highlights a previously unappreciated compartmentalization of eosinophil transmigration into the small versus large intestine.

To investigate in more depth the specific cells and mediators responsible for the key molecular and cellular responses involved in GI inflammation, the route by which oral sensitisation occurs and the mechanism driving mast cell responses during intestinal anaphylaxis was investigated. Previous clinical and murine studies have demonstrated a link between Th2-intestinal inflammation and induction of the effector phase of food allergy, and IL-9 has been significantly linked with allergic responses.

IL-9-deficient mice failed to develop experimental oral antigen-induced intestinal anaphylaxis - these studies support previous findings of associations of IL-9 and allergic inflammation. This is directly mediated by attenuation in mature mast cell numbers and subsequently the biomediators released from jejunal resident mast cells via antigen cross-linking of IgE on the surface. Moreover, this protection was elicited even in the presence of antigen-specific IgG1, IgE and the generation of a Th2 milieu (IL-4, -5 and -13). Intestinal IL-9 overexpression induced an intestinal anaphylaxis phenotype - intestinal mastocytosis including re-distribution and activation; gene profile; intestinal permeability and intravascular leakage. Importantly, this occurred in the absence of dysregulation of other immune parameters. Collectively, these findings indicate that overexpression of IL-9 in the small intestine induces an experimental intestinal anaphylaxis transcriptome and phenotype. In addition, overexpression of IL-9 in the small intestine results in a more severe oral antigen-induced intestinal anaphylactic reaction, and was sufficient to predispose to oral antigen-induced intestinal anaphylaxis, that is antigen-specific, in the absence of systemic priming. These results demonstrated a central role for IL-9 in the oral-antigen intestinal sensitisation phase of mast cell-mediated intestinal anaphylaxis.

To investigate the interactions between known Th2 mediators we assessed mice deficient in these factors. IL-4Ra-, STAT-6-dependent and partial IL-13-dependent intestinal anaphylaxis in iFABPp IL-9 Tg mice was established and this suggests that IL-9 may drive the generation of an antigen-specific CD4+ Th2-cell response. Remarkably, intestinal IL-9 overexpression predisposes to oral antigen sensitisation,
even in the absence of systemic priming. We demonstrate this requires mast cells and intestinal permeability and our findings suggest this occurs via a CD4⁺ Th₂-driven immune reaction. Our studies and previous investigations linking IL-9 with atopy identify IL-9/mast cell-regulated pathways as a critical determinant in the induction of food allergic reactions and these findings highlight the critical demand for assessment of IL-9 expression and its function in patients presenting with food allergic reactions.

These investigations focus towards identifying specific cellular and molecular targets for therapeutic approaches in GI allergy and inflammation. Further investigations into the role of cellular sources of eotaxin-1, the potent and specific chemokine for eosinophil transmigration and the mechanisms underlying increased intestinal permeability will not only enhance our understanding of these debilitating GI diseases but also bring us one step closer in our search for the cure.
Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title page</td>
<td>i</td>
</tr>
<tr>
<td>Statement of authorship</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Abstract</td>
<td>vi</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xvi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xvii</td>
</tr>
</tbody>
</table>

Chapter One: General Introduction

1.1 Gastrointestinal Immunomodulation                                    2

1.2 Aeitiology of food allergy                                           3

1.3 Eosinophil associated gastrointestinal diseases                       4

1.4 Inflammatory Bowel Disease                                           4

1.5 Clinical evidence of eosinophils in the pathogenesis of Ulcerative colitis 5

1.5.1 Experimental models of Ulcerative Colitis                          6

1.6 Regulation of tissue eosinophil accumulation                          7

1.7 Mast cells                                                            9

1.7.1 Mast cells and anaphylaxis                                         10

1.7.2 IgE                                                                 15

1.8 IL-9                                                                  16

1.8.1 IL-9 and mast cells                                                17

1.8.2 IL-9 and the gastrointestinal tract                                18

1.9 Intestinal anaphylaxis                                               19

1.10 Aims of this study                                                  20
Chapter Two: The role of $\beta_\tau$-integrin during inflammation of the large intestine

2.1 Introduction

2.2 Materials and Methods
   2.2.1 Mice
   2.2.2 Fluorescent-activated cell sorter (FACS) analysis
   2.2.3 Induction of colonic injury
   2.2.4 Disease Activity Index
   2.2.5 Body weight
   2.2.6 Diarrhoea
   2.2.7 Rectal bleeding
   2.2.8 Histopathological examination
   2.2.9 Detection and quantification of eosinophils by Immunohistochemistry
   2.2.10 Eosinophil peroxidase activity assay
   2.2.11 In vitro stimulation of eosinophils with eotaxin-1
   2.2.12 Fluorescent-activated cell sorter (FACS) analysis on colonic eosinophils
   2.2.13 Statistical analysis

2.3 Results
   2.3.1 Role of $\beta_\tau$-integrin in eosinophil recruitment into the colon and experimental colitis
   2.3.2 Expression of $\alpha$ and $\beta$-integrins on splenic eosinophils from CD2-IL-5Tg and CD2-IL-5Tg $\beta_\tau$-integrin$^{-/}$ mice
   2.3.3 The effect of eotaxin-1 and IL-5 on $\alpha$- and $\beta$-integrin expression on eosinophils
   2.3.4 The effect of blockade of $\alpha_1$, $\alpha_M$ and $\beta_2$ integrins on eosinophil chemotaxis
   2.3.5 Characterisation of the $\beta_2$ integrin/ICAM-1 pathway expression in the colon

2.4 Discussion

Chapter Three: The role of ICAM-1 dependent pathways in eosinophil recruitment into the large intestine

3.1 Introduction

3.2 Materials and Methods
   3.2.1 Mice
   3.2.2 Induction of colonic injury
   3.2.3 Disease activity index
   3.2.4 Body weight
   3.2.5 Diarrhoea
   3.2.6 Rectal bleeding
   3.2.7 Detection and quantification of eosinophils by immunohistochemistry
   3.2.8 Eosinophil peroxidase (EPO) activity assay
   3.2.9 Monoclonal Antibody treatment
3.2.10 Myeloperoxidase (MPO) activity 51  
3.2.11 Statistical analysis 52  

3.3 Results 53  
3.3.1. The effect of anti-ICAM-1 treatment on eosinophil recruitment into colon of CD2-IL-5Tg mice 53  
3.3.2 Anti-ICAM-1 treatment blocks DSS-induced experimental colitis and eosinophil recruitment into colon 53  
3.3.3 DSS-induced experimental colitis and eosinophil recruitment into colon occurs via an ICAM-1 dependent mechanism 53  
3.3.4 ICAM-1 deficiency results in suppression of DSS-induced experimental colitis by specific inhibition of eosinophil recruitment 58  

3.4 Discussion 63  

Chapter Four: The role of IL-9 in the development of oral antigen-induced intestinal anaphylaxis  

41. Introduction 67  

4.2 Materials and Methods 69  
4.2.1 Mice 69  
4.2.2 Protocol 69  
4.2.3 Ribonuclease protection assay 70  
4.2.4 Quantitative PCR 70  
4.2.5 ELISA measurements 71  
4.2.6 Intestinal mast cell quantification 72  
4.2.7 Activated CD4+ Th2 response 72  
4.2.8 Proliferation Assay 73  
4.2.9 Mononuclear cell preparation and MCp assessment 73  
4.2.10 Statistical Analysis 74  

4.3 Results 75  
4.3.1 Elevation of mRNA IL-9 expression during oral antigen-induced intestinal anaphylaxis 75  
4.3.2 Elucidation of the role of IL-9 in oral antigen-induced intestinal anaphylaxis 75  
4.3.3 Evaluation of the OVA-CD4+ Th2 response 78  
4.3.4 Effect of IL-9 deficiency on mature mast cells 78  

4.4 Discussion 89  

Chapter Five: The role of IL-9 in intestinal anaphylaxis, employing a transgenic approach  

5.1 Introduction 94  

5.2 Materials and Methods 96  
5.2.1 Reagents 96  
5.2.2 Generation of Fabpi IL-9 Transgenic Mice 96  
5.2.3 Intestinal mast cell quantification 97
7.2.2 Mice 154
7.2.3 Protocol 154
7.2.4 ELISA measurements 154
7.2.5 Activated CD4+ Th2 response 154
7.2.6 Proliferation assays 154
7.2.7 Mononuclear cell isolation from jejunal tissue 154
7.2.8 In vitro stimulation of jejenum mononuclear cells 155
7.2.9 Fluorescent-activated cell sorter (FACS) analysis 155
7.2.10 Lightcycler PCR 155
7.2.11 Passive anaphylaxis model 156
7.2.12 Anaphylaxis 156
7.2.13 Statistical Analysis 156

7.3 Results 157
7.3.1 Intestinal expression of IL-9 predisposes to oral antigen sensitisation 157
7.3.2 Role of CD4+ T cells in the regulation of oral antigen sensitisation 157
7.3.3 Intestinal IL-9/mast cell mediated intestinal permeability predisposes to oral antigen sensitisation in iFABPp IL-9 Tg mice 160

7.4 Discussion 168

Chapter Eight: General Discussion

8.1 Chemokines, cytokines, cells and their receptors: The roles they play in the induction and exacerbations of gastrointestinal inflammation 171

8.2 The role of chemokine signalling, adhesion molecules and integrin receptors in transmigration of eosinophils into the colon during injury and inflammation 171
8.2.1 Eosinophil recruitment in the intestine is compartmentalised 172

8.3 IL-9 is a critical factor regulating experimental oral antigen-induced intestinal anaphylaxis 172
8.3.1 IL-9 regulates intestinal mastocytosis 172
8.3.2 IL-9/mast cells and ion transport 176
8.3.3 IL-9 is sufficient to predispose to oral antigen-induced intestinal anaphylaxis 177

8.4 Scope for further investigation 178

Appendix 200
List of Figures

Chapter One

Figure 1.1 Mast cells and anaphylaxis 12
Figure 1.2 Schematic of mechanisms of anaphylaxis 14

Chapter Two

Figure 2.1. DSS-induced experimental colitis occurs via a β7-integrin independent mechanism 32
Figure 2.2. Characterisation of surface expression of LFA-1 and MAC-1 integrin chains on eosinophils 36
Figure 2.3. Characterisation of surface expression of α6β7, LFA-1 and MAC-1 integrin chains on eosinophils following IL-5 and/or eotaxin-1 stimulation 38
Figure 2.4. Blockade of LFA-1 and MAC-1 / ICAM-1 pathways inhibit Eosinophil chemotaxis in vitro 41
Figure 2.5. β2-integrin+ CCR3+ and ICAM-1+ cells in the colon during DSS-induced colonic inflammation 43

Chapter Three

Figure 3.1. Anti-ICAM-1 treatment blocks eosinophil recruitment into colon of CD2-IL-5Tg mice 55
Figure 3.2. Anti-ICAM-1 treatment blocks DSS-induced experimental colitis and eosinophil recruitment into colon 57
Figure 3.3. DSS-induced experimental colitis and eosinophil recruitment into colon occurs via an ICAM-1 dependent mechanism 60
Figure 3.4. MPO activity in the colon of DSS-treated WT and ICAM-1−/− mice 62

Chapter Four

Figure 4.1 Intestinal expression of IL-9 is elevated during oral antigen-induced intestinal anaphylaxis 77
Figure 4.2 Oral antigen-induced intestinal anaphylaxis is attenuated in IL-9 deficient mice 80
Figure 4.3 Th2 immune response in IL-9 deficient mice is comparable to BALB/c WT mice 84
Figure 4.4 IL-9 plays a role in intestinal mast cell levels  

Figure 4.5 Homeostatic maintenance of intestinal mast cell progenitor numbers occurs independently of IL-9  

Chapter Five  

Figure 5.1 Generation of iFABPp IL-9 Tg mice  

Figure 5.2 Intestinal expression of IL-9 induces intestinal mastocytosis and mast cell activation  

Figure 5.3 Mast cell activation is associated with the release of pro-inflammatory mediators  

Figure 5.4 Overexpression of IL-9 does not alter mast cell progenitor levels  

Figure 5.5 Serum cytokine and immunoglobulin levels are unaltered in iFABPp IL-9 Tg mice  

Figure 5.6 Intestinal expression of IL-9 is sufficient to induce a gene profile similar to that observed in mice with oral antigen-induced intestinal anaphylaxis  

Figure 5.7 Overexpression of IL-9 in the small intestine induces features of intestinal anaphylaxis phenotype including mast cell dependent intestinal permeability and intravascular leakage  

Chapter Six  

Figure 6.1 Intestinal expression of IL-9 is sufficient to predispose to intestinal anaphylaxis  

Figure 6.2 Spontaneous intestinal anaphylaxis in iFABPp IL-9 Tg mice is an antigen-specific acute immunological response  

Figure 6.3 Oral allergen-induced intestinal anaphylaxis is mediated by an IgE-dependent pathway  

Chapter Seven  

Figure 7.1 Overexpression of IL-9 in the intestine increases local Th2 responses after OVA i.g. challenge  

Figure 7.2 IL-9 predisposes to oral antigen CD4+ Th2-type sensitisation  

Figure 7.3 Treatment with mast cell stabilising agent Cromolyn sodium protects against antigen sensitisation
Chapter Eight

Figure 8.1 Transmigration of eosinophils to the small and large intestine 175

Figure 8.2 IL-9 promotes oral sensitisation and is a critical factor in the development of experimental oral antigen induced intestinal anaphylaxis 179
List of Tables

Chapter Four

Table 4.1 CD4+ cytokine productions in IL-9 deficient mice is comparable to BALB/c WT mice 82

Chapter Five

Table 5.1 IL-9 in the small intestine promotes mastocytosis and mast cell activation independently of CD4+ T-cell, B-cell and DC-cell dysfunction 118

Chapter Six

Table 6.1 Role of Th2 molecules in the development of oral antigen-induced intestinal anaphylaxis 143

Table 6.2 Role of Th2 molecules in the development of oral antigen-induced intestinal anaphylaxis in iFABPp IL-9 Tg mice 145

Chapter Seven

Table 7.1 OVA-challenge of iFABPp IL-9 Tg mice does not predispose to a Th2 response in the draining mesenteric lymph nodes or the spleen 162
Abbreviations

GI gastrointestinal
Th₂ T-helper 2
MAdCAM-1 mucosal addressin cell adhesion molecule -1
ICAM-1 intercellular adhesion molecule -1
VCAM-1 vascular cell adhesion molecule -1
LFA-1 lymphocyte function-associated antigen-1
MAC-1 macrophage associated cell surface marker-1
VLA-4 very late antigen -4
UC ulcerative colitis
IBD inflammatory bowel disease
IL- interleukin
TNF tumour necrosis factor
DSS dextran sulfate sodium
Tg transgenic
-/ knock out
DAI disease activity index
MBP major basic protein
EPO eosinophil peroxidase
OPD o-phenylenediamine
CTAB cetyltrimethylammonium bromide
WT wild type
HPF high power field
Ig immunoglobulin
mAb monoclonal antibody
FSC forward scatter
SSC side scatter
2-ME 2-mercaptoethanol
CCR CC chemokine receptor
CCL CC chemokines ligand
RANTES Regulated on Activation Normal T Cell Expressed and Secreted
MCP monocyte chemotactic protein
MPO myeloperoxidase
H/E haemotoxylin and eosin
IFN interferon
OVA ovalbumin
SAL saline
alum AIK(SO₄)₂·12H₂O
i.p. intraperitoneal
i.g. intragastric
RPA ribonucleases protection assay
RNA ribonucleases acid
DNA deoxyribonuclease acid
GAPDH glyceraldehyde-3-phosphate dehydrogenase
HPRT Hypoxanthine-guanine phosphoribosyltransferase
PCR poly chain reaction
RT-PCR realt time PCR
mRNA messenger RNA
cDNA copy DNA
mMCP murine mast cell protease
<table>
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<tr>
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</tr>
</thead>
<tbody>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>MNC</td>
<td>mononuclear cells</td>
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<td>FCS</td>
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<td>FBS</td>
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<td>HBSS</td>
<td>Hank's buffered saline solution</td>
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<td>SCF</td>
<td>stem cell factor</td>
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<td>mast cell progenitor</td>
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<td>CTMC</td>
<td>connective tissue mast cell</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>iFABPp</td>
<td>intestinal fatty acid binding promoter protein</td>
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<td>MIAME</td>
<td>minimum information about a microarray experiment</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>MLN</td>
<td>mesenteric lymph node</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>horseradish peroxidase</td>
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<td>ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>OD</td>
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<tr>
<td>BMMC</td>
<td>bone marrow-derived mast cells</td>
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<tr>
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<td>dendritic cell</td>
</tr>
<tr>
<td>RELM</td>
<td>resistin like molecule</td>
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<td>SPRA</td>
<td>small proline rich-protein 2A</td>
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<td>IVL</td>
<td>intravascular leakage</td>
</tr>
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<td>STAT</td>
<td>signal transduction and activation</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>PAF</td>
<td>platelet activation factor</td>
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<td>TNP</td>
<td>3,5,6,8-tetramethyl-n-methyl phenanthrolinium</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>CMF</td>
<td>calcium and magnesium free</td>
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<td>GC</td>
<td>golgi complex</td>
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<td>endoplasmic reticulum</td>
</tr>
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<td>SD</td>
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</tr>
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<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance between groups</td>
</tr>
</tbody>
</table>
Chapter One

General Introduction
Chapter One: General Introduction

1.1 Gastrointestinal Immunomodulation

The gastrointestinal (GI) tract is a mucosal organ capable of eliciting a powerful immune response to invading pathogens and luminal antigens whilst maintaining an amicable microenvironment for commensal bacteria. Homeostasis is maintained by mechanisms including exclusion of the mucosal barrier, phagocytosis and clearance of translocating bacteria and associated macromolecules, immunological tolerance to ubiquitous antigens and coordinated, self-limited inflammatory responses leading to clearance of pathogens whilst limiting tissue injury (Sartor 2006). The deleterious effects of salivary lysosome, gastric acid, pancreatic digestive enzymes and the detergent effects of bile acids on both pathogenic and environmental bacteria along with a relatively resistant mucosal barrier to dietary and bacterial antigens is the primary luminal and epithelial barrier defence. This is characterised as the extrinsic GI barrier. Further limitation occurs via antibacterial peptides secreted by Paneth cells and the epithelium. Mucous then shields the epithelium and tight junctions prevent macromolecule transportation.

The intrinsic GI barrier excludes paracellular transport of macromolecules through tight junctions, located proximal to the apical surface of the lateral plasma membranes and function by circumferentially fusing adjacent epithelial cells (Van Itallie and Anderson 2004). Rapid rearrangement of tight junctions and desmosomes maintain barrier function when epithelial permeability has been enhanced, for instance, during Fas-mediated apoptosis (Abreu, Palladino et al. 2000). Mucosal dendritic cells sample luminal antigens with pseudopodia through intestinal tight junctions and do so without disrupting tight junction integrity as they express occludin-1, claudin-1 and the peripheral membrane protein zonula occludens-1 (Rescigno, Rotta et al. 2001). Goblet cells in the intestine and gastric antral mucous cells selectively produce and secrete mucin glycoproteins and trefoil peptides; and by means of interaction safeguard mucosal epithelium from harsh luminal conditions, non-motile bacteria and luminal antigens (Wright 2001). Despite its robust and multi-faceted nature the GI barrier can be breached, activating the innate and possibly the subsequent adaptive, immune response.
The immune response provides host protection and each class of immune cells will secrete a characteristic profile of cytokines, proinflammatory mediators and antimicrobial peptides. These inflammatory signals stimulate inhibitory responses to downregulate the inflammatory response: This management of the immune response is key to mucosal homeostasis, if unregulated, can lead to chronic GI inflammation. Defects in mucosal immunity leading to chronic GI inflammation is one of the most common inflammatory processes and is diverse in aetiology, pathogenesis and manifestation (Bamford 1999). Conserved and variable features of inflammation at differing sites of the GI tract is a major focus of this study and these investigations have established differential regulation of adhesion pathways for inflammatory cell recruitment into the large versus the small intestine (Forbes, Hulett et al. 2006).

1.2 Aetiology of food allergy

Food allergy is increasing in incidence and severity throughout the Western World. It is estimated approximately 2-6% of people in Western society suffer from inappropriate immune reactions to food, with children representing the higher end of the scale at 6%, most of whom will seemingly outgrow their burden with 2% of adults living with food allergy. The molecular basis regulating the pathogenesis of food allergy remains to be elucidated. However, investigations have shown mast cells and food-specific immunoglobulin (Ig)–E are required for type I hypersensitivity reaction, the immediate allergic response. Despite the sophisticated GI barrier approximately 2% of ingested food antigens are absorbed and transported, intact and thus retaining antigenicity (Husby, Svehag et al. 1987). However, most individuals will acquire tolerance to these penetrating food antigens. Tolerance has been broadly defined as a state of unresponsiveness and it is suggested to be as a result of T-cell anergy or induction of regulatory T-cells.

Food hypersensitivity occurs in susceptible individuals when tolerance fails to either develop normally, or breaks down (Husby, Svehag et al. 1987).
1.3 Eosinophil-associated gastrointestinal diseases

Eosinophil-associated GI diseases (EGID) encompasses a numerous array of GI diseases, presenting with varying clinical manifestations unified by a rich eosinophilic inflammation of the GI tract, existing in the absence of known causes for eosinophilia or other GI disorders (including but not limited to drug reactions, parasitic infections or malignancy) (Rothenberg 2004; Hogan and Rothenberg 2006). Clinical studies suggest pathogenicity as the major role of eosinophils in EGID but the function of this leukocyte in these disorders remains unexplained (Hogan and Rothenberg 2006). EGID, including eosinophilic eosophagitis (EE), eosinophilic gastroenteritis (EG), eosinophilic gastritis, mucosal eosinophilia, protein enteropathy, classic IgE mediated food allergy (FA) and IBD, present with a direct correlation between disease severity and the accumulation and activation state of GI eosinophils (Hogan, Mishra et al. 2001). EGID also manifest with a variety of symptoms and may be mediated by allergic IgE responses or as non-IgE associated disease.

1.4 Inflammatory bowel disease

Crohn’s disease (CD) and ulcerative colitis (UC), together referred to as inflammatory bowel disease (IBD), are chronic, relapsing, remitting GI diseases characterised by chronic inflammation of the intestine, leading to long term and sometimes irreversible impairment of the GI structure and function (Blumberg, Saubermann et al. 1999; Russel 2000; Strober, Fuss et al. 2002; Bouma and Strober 2003). Although both CD and UC do share certain clinical and pathological features of disease, there is also evidence of markedly different features, suggesting the pathophysiologic moieties in these two diseases are vastly distinct (Bouma and Strober 2003). CD and UC are associated with intestinal and extraintestinal clinical manifestations of disease including weight loss, diarrhoea accompanied by blood and/or mucous, fever and gastric dysmotility (Fiocchi 1998; Hendrickson, Gokhale et al. 2002). However, CD most commonly affects the terminal ileum, caecum, peri-anal area and colon. Conversely, UC remains restricted to the colon, involving the rectum and continuing proximally. UC seems to present with uniform lesions through the affected area, whereas CD is characterised with the presence of normal bowel sections in between lesions. Finally CD presents with transmural dense infiltration of inflammatory cells whereas UC presents with
inflammation of the superficial mucosa layers (Bouma and Strober 2003). Recent advances in scientific discovery have expanded our understanding of the immunopathogenesis of IBD and several novel pathways and specific targets have been identified to focus new therapeutic approaches. Currently, the approach is to avoid chronic steroid usage and eliminate the need for surgery (Targan 2006). Absolute benefit of developed biologies (percentage difference between effectiveness of treatment among patients receiving active compound versus placebo (Targan 2006)) ranges from 15-32%, and UC treatments ranged anywhere from 15-60% (Hanauer, Feagan et al. 2002; Mannon, Fuss et al. 2004; Feagan, Sandborn et al. 2005; Korzenik, Dieckgraefe et al. 2005; Rutgeerts, Sandborn et al. 2005; Sandborn, Colombel et al. 2005; Schreiber, Rutgeerts et al. 2005; Targan, Landers et al. 2005; Hommes, Mikhajlova et al. 2006). Thus, any efficacy is limited despite recent development of more potent biologics. This thesis describes investigations into the pathways regulating colonic eosinophilic inflammation in an experimental model of colonic injury with disease characteristics resembling UC in patients.

1.5 Clinical evidence of eosinophils in the pathogenesis of ulcerative colitis

The development of experimental models of EGID has provided important insights into the immunological mechanisms regulating eosinophilic inflammation of the GI tract. Eosinophils are multifunctional leukocytes possessing the capacity to initiate or potentiate inflammatory reactions through the release of a range of inflammatory cytokines, chemokines, and lipid mediators (Gleich and Adolphson 1986; Gleich, Adolphson et al. 1993; Rothenberg 1998). In addition, eosinophils may induce GI dysfunction through the release of lipid mediators (platelet-activating factor and leukotriene C4) and eosinophilic granular proteins (major basic protein (MBP), eosinophil peroxidase (EPO), and eosinophil-associated ribonucleases, i.e., eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN)). Clinical investigations of bowel biopsy specimens from UC patients have demonstrated a correlation between the eosinophil numbers in the mucosa, the levels of MBP, EPO, ECP, and EDN in perfusion fluid samples, and disease severity (Carlson, Raab et al. 1999; Saitoh, Kojima et al. 1999; Jeziorska, Haboubi et al. 2001; Sangfelt, Carlson et al. 2001) creating interest in the involvement of eosinophils in the pathogenesis of UC (Jeziorska, Haboubi et al. 2001). Elevated levels of eosinophils have been observed in
colonic biopsy samples from UC patients and increased numbers of this cell have been shown to correlate with morphological changes to the GI tract, disease severity, and gastrointestinal dysfunction (Bischoff, Mayer et al. 1999; Carlson, Raab et al. 1999; Saitoh, Kojima et al. 1999; Sangfelt, Carlson et al. 2001). A number of inflammatory mediators (platelet-activating factor, interleukin (IL-) 5), and chemokines (Regulated upon Activation, Normal T-cell Expressed, and Secreted RANTES), macrophage chemoattractant protein (MCP), macrophage inhibitory protein (MIP), eotaxin-1 and -2, and 3) have chemotactic activity for human eosinophils and are candidates for the control of colonic eosinophilia in UC (Chen, Paulus et al. 2001; Lampinen, Carlson et al. 2001). In particular, IL-5 regulates eosinophil growth, differentiation, and activation and the chemokine eotaxin is primarily involved in the regulation of eosinophil chemotaxis and effector functions including respiratory burst (Elsner, Petering et al. 1998).

1.5.1 Experimental models of ulcerative colitis

Several animal models have been developed that mimic certain pathophysiologic features of disease processes in IBD. These include chemically induced, adoptive transfer and genetically susceptible induction of disease. Two well-established models of colonic injury resulting in Th2-type inflammation resembling that of UC involve the administration of oxazolone or dextran sulfate sodium (DSS) and result in diarrhoea, epithelial cell damage, intestinal inflammation and dysfunction. Evidence suggests oxazolone induced colitis is mediated by NK T cells and requires IL-13 (Heller, Fuss et al. 2002; Fuss, Heller et al. 2004). Administration of DSS has been demonstrated to involve eosinophilic inflammation dependent on eotaxin-1 (Forbes, Murase et al. 2004). As such, we chose to again employ the DSS model of experimental UC – a previously identified robust model of colonic eosinophilic inflammation.

The similarities between DSS-induced colonic eosinophilic inflammation and UC suggest that both diseases could be explained by common underlying pathological mechanisms. The administration of DSS induces a prominent colonic eosinophilic inflammation and GI dysfunction (diarrhoea with blood and shortening of the colon) that resembles UC in patients. In addition, a direct link between eosinophilic inflammation and the pathogenesis of colonic eosinophilic inflammation has been
demonstrated; an important role for eotaxin-1 in the recruitment of eosinophils into the colon during colonic eosinophilic inflammation; infiltrating eosinophils degranulate by a process of cytolysis releasing protein-laden granules; eosinophil-derived EPO is an important mediator in the development of the colitis and that blockade of EPO activity can attenuate the development of colonic eosinophilic inflammation (Forbes, Murase et al. 2004).

This demonstrates that GI dysfunction is associated with evidence of eosinophilic cytolytic degranulation and identifies EPO as a key mediator of disease in colonic eosinophilic inflammation.

1.6 Regulation of tissue eosinophil accumulation

Eosinophil trafficking into sites of inflammation is a tightly regulated process involving a number of cytokines, predominantly of the Th₂ phenotype including IL-4, IL-5 and IL-13, expression of ligands for E and P selectins, the adhesion molecules β₁-, β₂-, and β₇- integrins, chemokines including RANTES and eotaxin and other identified molecules such as acidic mammalian chitinase (Rothenberg, Owen et al. 1987; Sher, Coffman et al. 1990; Bochner and Schleimer 1994; Horie, Okubo et al. 1997; Rothenberg, Mishra et al. 2001; Zimmermann, Hershey et al. 2003; Zhu, Zheng et al. 2004).

The eosinophil is formed in the bone marrow under haematopoietic conditions involving transcription factors GATA-1, GATA-2 and c/EBP (Rothenberg 2004; Hogan and Rothenberg 2006). IL-3, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) perform their role as growth factors, with IL-5 being specific to the eosinophil and allows for the selective expansion and release of eosinophils from the bone marrow (Rothenberg 2004). Eosinophils then become part of the peripheral circulation and finally traffic to specific tissues, predominantly the GI tract due to homeostatic immune regulation, where they reside for at least a week (Rothenberg 1998).

Numerous inflammatory mediators have been implicated in regulating tissue eosinophilia: cytokines IL-1, -3, -4, -5, -13, tumour necrosis factor (TNF) -α and GM-
Chapter One: General Introduction

CSF; the chemokines RANTES, MCP-3 and -4, MIP 1α, eotaxin-1, -2 and in the human, eotaxin-3 and lipid mediators platelet-activating factor and cysteinyl leukotriene C4 (Rothenberg 1999; Hogan, Mishra et al. 2000; Hogan, Foster et al. 2002; Hogan, Rothenberg et al. 2004; Rothenberg 2004; Hogan and Rothenberg 2006). IL-1, -4, -13 and TNFα have been shown to promote adhesive interactions between eosinophils and the endothelium (Rothenberg 1998; Rothenberg 2004). Chemokines and lipid mediators in the presence of IL-5 promote chemotraction, however, only IL-5 and the eotaxins are eosinophil specific - several studies have now identified IL-5 as a critical for the rapid proliferation required for mounting proper immune response and the eotaxins as tissue recruitment factors (Foster, Hogan et al. 1996; Kopf, Brombacher et al. 1996; Tachimoto, Ebisawa et al. 1997; Matthews, Friend et al. 1998; Hogan, Mishra et al. 2001).

Recruitment begins with margination of eosinophils in the circulation, due to shear forces encountered from blood flow. Adhesion molecules, specifically selectins, L- and P-selectin and their carbohydrate-containing counterligands mediate tethering and rolling along the endothelium. The levels of surface expression of selectins and their counterpart ligands is believed to be the mechanism of preferential leukocyte recruitment during inflammation (Bochner 2000). The process of firm eosinophil adhesion and transendothelial migration involves different subsets of adhesion molecules, termed integrins. Integrins are heterodimeric surface molecules consisting of an α- and a β- chain. Eosinophils are known to express members of the β1 (α4β1 and α6β1), β2 (α1β2, αMβ2, αXβ2 and αDβ2) and β7 (α4β7) integrin families (Georas, McIntyre et al. 1993; Grayson, Van der Vieren et al. 1998; Tachimoto and Bochner 2000; Tachimoto, Ebisawa et al. 2002; Brandt, Strait et al. 2003). These various integrin molecules selectively interact with adhesion receptors and these receptors are part of the Ig gene superfamily including vascular cell adhesion molecule (VCAM) -1, mucosal addressin cell adhesion molecule (MAdCAM) -1 and intercellular cell adhesion molecules (ICAM) -1, -2 and -3 (Kunkel and Butcher 2002; Hogan, Rothenberg et al. 2004). α4β1 binds to VCAM-1 and fibronectin, α6β1 binds to laminin, α4β7 binds selectively to MAdCAM-1, α1β2 and αXβ2 bind to ICAM-1 and α1β2 can bind to ICAM-2 and -3 whilst αMβ2 can bind to fibrinogen, αXβ2 binds fibrinogen also and αDβ2 has been shown to bind to VCAM-1 and ICAM-1. Priming via IL-5 and upregulation of the eotaxin receptor CCR-3 on eosinophils markedly potentiates
responses to eotaxin and shifts integrin-mediated events, influencing recruitment to tissue compartments (Grayson, Van der Vieren et al. 1998; Bochner 2000; Shahabuddin, Ponath et al. 2000; Tachimoto, Burdick et al. 2000).

Eosinophil accumulation in the lung and skin during allergic inflammation is known to occur via $\alpha_4\beta_1$/VCAM-1 dominant interaction (Weg, Williams et al. 1993; Abraham, Sielczak et al. 1994; Nakajima, Sano et al. 1994; Pretolani, Ruffie et al. 1994; Gonzalo, Lloyd et al. 1996). Pretreatment of mice with either $\alpha_4$ or $\beta_1$ antibodies or mice deficient in VCAM-1 attenuates eosinophil accumulation and subsequent inflammation of the lung during allergic airways disease (Weg, Williams et al. 1993; Abraham, Sielczak et al. 1994; Nakajima, Sano et al. 1994; Pretolani, Ruffie et al. 1994; Gonzalo, Lloyd et al. 1996). Recruitment of eosinophils into the GI tract is thought to occur via a MAdCAM-dependent pathway and thus the most important integrin for GI eosinophils has been shown to be $\alpha_4\beta_7$. MAdCAM-1 is expressed on high endothelial venules in the intestinal lamina propria, lymph nodes and Peyer’s patches (Shaw and Brenner 1995; Butcher, Williams et al. 1999). Indeed, studies have shown eotaxin-induced GI eosinophilia is dependent on eosinophil expression of $\alpha_4\beta_7$ and MAdCAM-1 expression on endothelial cells in the small intestine (Mishra, Hogan et al. 2002). However, other investigations show that leukocyte recruitment into the large intestine can also occur via a $\beta_7$-integrin-independent mechanism, suggesting different adhesion systems could be possible for trafficking to the various GI compartments (Artis, Humphreys et al. 2000; Sydora BC 2002). Knowing that eosinophils can utilize various integrin/receptor adhesion systems to transmigrate to different tissue compartments and that leukocytes can migrate to the large intestine via a $\beta_7$-integrin-independent mechanism, we were interested in identifying the dominant integrin/receptor adhesion mechanism in eosinophil recruitment into the large intestine.

1.7 Mast cells

Paul Ehrlich first described mast cells in 1878 on the basis of their unique staining characteristics and large granules. These granules also led him to the mistaken belief that they existed to nourish the surrounding tissue, and he named them "mastzellen," meaning "feeding-cells." Investigations have since shown mast cells as versatile mediators of the immune response, from innate immune responses and the regulation of
tissue homeostasis to allergy. Mast cells are located strategically throughout the vascular and mucosal tissues and in the skin. This is believed to be due to the role mast cells play in innate immunity to bacteria and parasite infections, releasing cytokines and other mediators to recruit inflammatory leukocytes to body barriers (Malaviya and Georges 2002; Marshall 2004).

Mast cells facilitate allergic reactions through IgE surface receptor FcεRI crosslinking. This results in degranulation and release of vasoactive, pro-inflammatory and nociceptive mediators, encompassing a vast array of biologically potent mediators (Figure 1.1) (Galli, Kalesnikoff et al. 2005; Galli, Nakae et al. 2005; Rottem and Mekori 2005; Theoharides and Kalogeromitros 2006; Bischoff 2007).

1.5.1 Mast cells and anaphylaxis

Murine models have demonstrated two pathways responsible for the development of anaphylaxis in mice (Finkelman, Rothenberg et al. 2005). Mediation via IgE, FcεRI, mast cells, histamine and platelet activating factor (PAF) is most likely the pathway involved in anaphylaxis in the human and this cell-mediated reaction involving GI and systemic anaphylaxis can be experienced in mice as in humans, to ingested antigen (Finkelman, Rothenberg et al. 2005). In comparison to the pathophysiology of other allergic reactions (e.g. asthma, allergic rhinitis, dermatitis), anaphylaxis seems relatively simple – antigen crosslinks antibodies activating immunoglobulin receptors causing the release of mediators that result in increased vascular permeability, smooth muscle contractions and resulting in urticaria, hypotension, dyspnea, abdominal cramping and diarrhoea (Finkelman, Rothenberg et al. 2005). However, experimental models of anaphylaxis demonstrate two distinct pathways, the latter of being IgE-independent and regulated by IgG and the macrophage FcγRIII. This data belies the simplicity believed to operating in these models of anaphylaxis (figure 1.2) (Finkelman, Rothenberg et al. 2005).

The investigations of this thesis involve a model of intestinal anaphylaxis mediated via an IgE-dependent mechanism. The IgE/mast cell-mediated food allergies are considered serious and life threatening, and the underlying molecular basis of food allergy and anaphylaxis are unknown.
Figure 1.1 Mast cells and anaphylaxis.

Mast cell degranulation results in the release of vasoactive, pro-inflammatory and nociceptive mediators, encompassing a vast array of biologically potent mediators, resulting in secretion and epithelial permeability; wound healing and fibrosis; neuroimmune interactions, peristalsis, bronchoconstriction and pain; immune cell recruitment and activation and blood flow, coagulation and vascular permeability, as depicted schematically in this figure. Adapted from Bischoff *Nature Reviews Immunology* 7, 93-104.
Chapter One: General Introduction

Bacteria, Allergens

Immune cell recruitment and activation

Epithelium

Histamine, LTC₄, and PGD₂

Secretion and epithelial permeability

Neutrophil

Blood vessel endothelium

Blood flow, coagulation and vascular permeability

Mast cell

Histamine, LTC₄, chymase, VLA₄, and heparin

IL-5, IL-9, IL-13

Histamine, PGD₂ and proteases

IgE

TGF-β and FGF

Wound healing and fibrosis

Fibroblast

Neuroimmune interactions, peristalsis, bronchoconstriction and pain

Nerve cell

Smooth muscle cell
Two pathways of anaphylaxis exist in the mouse. One involves antigen crosslinking of IgE to its receptor, FceRI on the mast cell, the other antigen crosslinking of IgG to its receptor FcγRIII on the macrophage resulting in degranulation and the release of histamine and/or PAF resulting in alterations to smooth muscle and vascular endothelium and subsequent anaphylactic reactions can occur. Adapted from Finkelman, F.D., et al. *J Allergy Clin Immunol*, 2005. 115(3): p. 449-57.
Chapter One: General Introduction

Antigen

Mast cell

Histamine + PAF

Smooth muscle
Vascular endothelium

Anaphylaxis

Macrophage

PAF
1.7.2 IgE

As previously described, antigen crosslinking of IgE on its receptor, FcεRI, on the surface of mast cells resulting in degranulation and release of immunomodulating mediators is a well accepted mechanism of the allergic reaction. In 1966-67 the Ishizaka group in Denver, Colorado, USA, reported on an antiserum that could interfere with reaginic activity. This factor, postulated to represent a new immunoglobulin, was provisionally called γE-globulin (Ishizaka and Ishizaka 1967; Ishizaka, Ishizaka et al. 1967; Ishizaka, Ishizaka et al. 1967) and attaches to cell membranes causing the release of histamine and other substances responsible for the local inflammatory response. Despite many attempts, the group was unable to purify the γE-globulin, which is now known to be present in extremely low concentrations in serum.

Independently of this work, Bennich and Johansson, in 1965, discovered a new class of immunoglobulins, provisionally called IgND (Johansson 1967; Johansson and Bennich 1967; Stanworth, Humphrey et al. 1967). An atypical myeloma protein was found that shared the known physicochemical properties of reagin. It was shown that the PK reaction (Prausnitz Küstner hypersensitivity skin prick test for allergens) could be blocked in a dose-dependent way with isolated IgND or Fc fragments of the ND protein. Using radioimmunoassays, a normal counterpart could be detected in serum of healthy individuals and it was found that patients with allergic asthma had on average a six-fold higher concentration of IgND than normals or patients with non-allergic asthma. A new radioimmunoassay was developed, the radioallergosorbent test (RAST), capable of detecting allergen-specific IgND antibodies to allergen, and their presence in serum correlated with skin test results.

Reagents were exchanged between the laboratories in the USA and Sweden in 1967. It was found that antiserum to γE-globulin reacted with isolated ND protein, and that purified ND protein could block the reaction of anti-γE-globulin in a biological test system for reaginic activity. At the WHO Immunoglobulin Reference Centre in Lausanne, Switzerland in February 1968 the researchers from the two groups met, and it was agreed that the data available on the unique structure, antigenic properties and biological activity of IgND, supported by data on γE-globulin, would allow for the declaration of a new immunoglobulin, which was called IgE (Johansson 1997).
The past 40 years since the discovery of IgE has lead to a significant knowledge bank in the field of allergy. Now available are reliable procedures for allergy diagnosis, standardised allergen preparations for diagnosis and treatment and improved knowledge about mechanisms and tests to monitor efficacy of therapy (Johansson 1997). However, the prevalence and incidence of allergy is ever increasing and the elucidation to how sensitisation occurs and possible prevention of the allergic reaction still remains to be achieved. In the words of a team member responsible for the discovery of IgE “Allergy is a serious problem for hundreds of millions of people and no effort should be spared to improve their quality of life”, S.G.O Johansson.

1.6 IL-9

IL-9 is characterised as a pleiotropic cytokine for its diverse array of influences on immunogenic activities. IL-9 is produced by activated Th2 clones in vitro and during Th2-type responses in vivo (Renauld, Kermouni et al. 1995). IL-9 has been shown is several models to enhance mast cell proliferation in vitro in synergy with IL-3 or IL-4 (Hultner, Moeller et al. 1989; Moeller, Hultner et al. 1989; Eklund, Ghildyal et al. 1993; Yanagida, Fukamachi et al. 1995; Toru, Kinashi et al. 1997; Lorentz, Schwengberg et al. 2000). This activity termed mast cell growth-enhancing activity (MEA) was also discovered in the supernatants of murine T-helper cell lines that produced a T cell growth factor (Moeller, Hultner et al. 1989; Renauld, Kermouni et al. 1995). Finally, in vitro investigations suggest IL-9 has the capacity to target haemopoietic progenitors (Yang, Ricciardi et al. 1989; Donahue, Yang et al. 1990), B cells (Dugas, Renauld et al. 1993; Petit-Frere, Dugas et al. 1993) and immature neuronal cell lines (Mehler, Rozental et al. 1993). In vivo, IL-9 has been shown to play a role in asthma and in nematode infections (Knoops and Renauld 2004).

The human IL-9 gene is a single copy and has been mapped on chromosome 5, in the region, 5q31->q35 (Modi, Pollock et al. 1991; Renauld, Kermouni et al. 1995). This region also contains various growth factor and growth factor receptor genes and has been shown to be deleted in various haematological disorders (Renauld, Kermouni et al. 1995). In the mouse, IL-9 is localised on chromosome 13 (Mock, Krall et al. 1990). The human and murine IL-9 genes share structure homology, with five exons and four introns with a 4kb span (homology levels ranging from 56 to 74%) (Renauld, Goethals
The role of IL-9 in CD4+ Th2-cell function, mastocytosis, eosinophil survival and IgE production has led investigators to hypothesise that IL-9 is central to allergic inflammatory processes (Levitt, McLane et al. 1999; Hauber, Bergeron et al. 2004). Clinical investigations have demonstrated increased expression of IL-9 in biopsies from asthmatics and increased expression of this cytokine correlated with disease severity (Ying, Meng et al. 2002; Erpenbeck, Hohlfeld et al. 2003; Erpenbeck, Hohlfeld et al. 2003). Furthermore, genetic mapping studies in both humans and mice showed linkage between the atopic phenotype and the IL-4 and IL-9 gene, identifying IL-9 as a candidate gene for asthma (Postma, Bleecker et al. 1995; Nouri-Aria, Pilette et al. 2005). Animal models support the clinical investigations, demonstrating that IL-9 has important roles in the development of the allergic asthma phenotype including eosinophilic and lymphocytic inflammation, mucous accumulation, mast cell hyperplasia, subepithelial collagen deposition and airways hyperresponsiveness (McLane, Haczku et al. 1998; Temann, Geba et al. 1998; Townsend 2000; Kung, Luo et al. 2001; Temann, Ray et al. 2002; Temann, Laouar et al. 2007).

The IL-9 receptor is a member of the haemopoietin receptor superfamily (IL-9 specific chain, IL-9R) and is formed in combination with the γ-common chain (γc), shared with IL-2, -4, -7, -15 and –21 receptors collectively and the heterodimeric receptor allows for signal transduction (Knoops and Renauld 2004). The IL-9R is sufficient to bind IL-9 with high affinity but alone cannot mediate an effective signal (Renauld, Druez et al. 1992; Knoops and Renauld 2004). Receptor ligation, initiating juxtaposition and conformational change of the heterodimeric chain results in phosphorylation of JAK (Janus protein tyrosine) kinases and the receptor, activating signaling (Knoops and Renauld 2004).

1.5.1 IL-9 and mast cells

As described above, IL-9 was originally designated MEA for mast cell growth-enhancing activity (Moeller, Hultner et al. 1990) and leads to mast cell proliferation (Hultner, Druez et al. 1990) and upregulation of mast cell proteases (Eklund, Ghildyal et al. 1993). Furthermore, mast cells themselves have been shown to be potent
producers of IL-9. In cultured bone marrow derived mast cells, in the presence of IL-1 and ionomycin or IgE-antigen complexes induces IL-9 mRNA expression and an increase in IL-9 at the protein level (Hultner, Kolsch et al. 2000). The same group also found an increase in IL-9 mRNA and protein production in activated murine bone marrow derived mast cells after stimulation with c-kit ligand or IL-10 (Stassen, Arnold et al. 2000). Other investigations in vitro have shown recombinant IL-9 supported growth of bone marrow derived mast cell lines, the production of the pro-inflammatory cytokine, IL-6, and increased survival and proliferation of primary mast cell cultures (Uyttenhove, Simpson et al. 1988; Hultner, Druez et al. 1990; Renauld, Goethals et al. 1990; Soussi-Gounni, Kontolemos et al. 2001). Further studies demonstrate that IL-9 has the ability to enhance human mast cell development under stimulation with stem cell factor (SCF). Furthermore, mast cell progenitors of asthmatic patients responded to IL-9 in combination with SCF to a greater extent than the normal controls. Taken together this suggests IL-9 appears to act as a potent enhancer of SCF-dependent growth of mast cell progenitors in humans, in particular, asthmatic patients [116].

These studies provide a mechanism of the increased numbers of mast cells seen in the airway epithelium of lung specific IL-9 transgenic mice and in the intestine, kidneys and respiratory tract of mice constitutively overexpressing IL-9. Moreover, this suggests that the accumulation of mast cells in tissues of allergic patients and during helminth infection may be attributed to the recruitment, enhanced survival and proliferation and/or local differentiation of mast cells as a consequence of local production of IL-9 (Soussi-Gounni, Kontolemos et al. 2001).

1.5.2 IL-9 and the gastrointestinal tract

While there have been many studies examining the role of IL-9 in allergic inflammatory responses of the lung, little is known about the role of IL-9 in GI allergic inflammatory responses. Furthermore, studies that have investigated the role of IL-9 in GI inflammation have primarily focused on the role of IL-9 in parasitic infestations (Faulkner, Renaud et al. 1998; Fallon, Smith et al. 2000). Recently, employing models of parasitic infestations, investigators have demonstrated that IL-9 influences GI inflammation through the promotion of a detrimental Th2-type inflammatory response (Arendse, Van Snick et al. 2005). While IL-9 has been shown to regulate an array of
allergic inflammatory processes critically involved in mast cell and eosinophil effector function, we hypothesise that this Th2-cytokine orchestrates the allergic inflammatory reaction in food allergy and anaphylaxis through the polarisation of CD4+ Th2-type T cells. Surprisingly, while substantial evidence exists suggesting a role for IL-9 in Th2-inflammatory reactions, very little is known about the contribution of IL-9 to food allergy and anaphylaxis. One goal of this thesis is to elucidate the mechanism of IL-9 in food allergic reactions and anaphylaxis.

1.7 Intestinal anaphylaxis

Intestinal anaphylaxis is a term used to describe hypersensitivity resulting in local, specific anaphylactic reactions affecting the GI tract. These are associated with the development of diarrhoea, marked intestinal mucosal mastocytosis and degranulation, antigen-specific IgE and significant changes in intestinal transport (Perdue, Chung et al. 1984; Kweon, Yamamoto et al. 2000; Brandt, Strait et al. 2003).

My colleagues have developed an experimental model of oral allergen-induced intestinal inflammation associated with Th2 responses, intestinal eosinophilia and mastocytosis and the development of acute diarrhoea (Brandt, Strait et al. 2003). Diarrhoea was accompanied by increased permeability which persisted for at least 48 hours post-challenge, in contrast to diarrhoea, that was present only transiently post-challenge (Brandt, Strait et al. 2003). Furthermore, the presence of significant mastocytosis and evident mast cell degranulation (measured by increased levels of mouse mast cell protease (mMCP)-1) support a primary acute type I sensitisation. Brandt et al clearly show a critical role for mast cells in the development of allergic diarrhoea in this model, since by repeated administration of a mast cell depleting antibody (anti-c-kit), diarrhoea development was abolished. In addition, they were able to show that anti-IgE treatment similarly blocked diarrhoea. The results also suggest that mast cell derived serotonin in combination with platelet activating factor (PAF) (by way of antibody administration) play a central role in the mediation of oral antigen-induced diarrhoea.
1.8 Aims of this study

Our previous investigations have shown that Th$_2$ responses and the associated inflammatory infiltrate play a crucial role in the induction of inflammatory diseases of the GI tract. A central question still to be explored concerning GI allergic disorders is the role of specific cells and mediators responsible for the key molecular and cellular responses, which characterise the induction of disease pathogenesis. Thus, the overall aim of this project was to define the role of key cellular and molecular inflammatory mechanisms that regulate the pathological and functional alterations to the GI tract, leading to GI dysfunction in experimental UC and intestinal anaphylaxis.

Aim 1: To define the role of β7-integrin during inflammation and to identify possible target candidate integrins and their receptors in the recruitment of eosinophils to the large intestine.

Aim 2: To characterise the role of ICAM-1 dependent pathways in eosinophil recruitment into the colon during homeostasis and disease.

Aim 3: To identify the role of the Th$_2$ cytokine, IL-9, and its target in the development of experimental oral antigen-induced intestinal anaphylaxis.

Aim 4: To define the individual contribution of IL-9 in other discreet aspects of disease pathogenesis of intestinal anaphylaxis employing a transgenic approach.

Aim 5: To elucidate the mechanisms involved in the predisposition of intestinal anaphylaxis in the absence of systemic priming and assess the requirement of systemic Th$_2$ immunity and IL-9 in local vs. systemic immunity.

Aim 6: To establish the role of CD4$^+$ Th$_2$ cells in oral sensitisation in and intestinal anaphylaxis.
Chapter Two

The role of \(\beta_7\)-integrin during inflammation of the large intestine
2.1 Introduction

The transmigration of leukocytes, such as eosinophils, across the vascular epithelium into mucosal tissues, is regulated by coordinated interaction between networks involving chemokine and cytokine signalling, eosinophil adhesion molecules (e.g. selectins and integrins) and integrin receptors (e.g. vascular cell adhesion molecule -1 [VCAM-1], mucosal addressin cell adhesion molecule -1 [MAdCAM-1] and intercellular adhesion molecule 1 [ICAM-1]) expressed on vascular endothelial cells (Kunkel and Butcher 2002; Hogan, Rothenberg et al. 2004). Eosinophilic inflammation is a common feature of numerous eosinophil associated gastrointestinal diseases (EGID). Central to eosinophil migration into the gastrointestinal (GI) tract are integrin-mediated interactions with adhesion molecules. Although the mechanisms regulating eosinophil homing into the small intestine have begun to be elucidated, the adhesion pathways responsible for eosinophil trafficking into the large intestine are unknown.

Eotaxin-1 has been shown to drive eosinophil recruitment into mucosal tissues (Matthews, Friend et al. 1998; Rothenberg 1999; Mishra, Hogan et al. 2002), and eotaxin-1 is ubiquitously expressed in all segments of the GI tract (Mishra, Hogan et al. 1999). Clinical studies have demonstrated an association between increased expression of CCL11 (eotaxin-1) in eosinophil associated GI diseases including cows milk associated reflux eosophagitis and ulcerative colitis (UC) (Garcia-Zepeda, Rothenberg et al. 1996; Chen, Paulus et al. 2001; Jeziorska, Haboubi et al. 2001; Butt, Murch et al. 2002; Mir, Minguez et al. 2002). Integrins are heterodimeric surface molecules consisting of an α- and β-chain and eosinophils are known to express members of the β$_1$ ($\alpha_4\beta_1$ and α$_5$β$_1$), β$_2$ ($\alpha_4\beta_2$, $\alpha_5\beta_2$, $\alpha_6\beta_2$ and α$_7$β$_2$) and β$_7$ ($\alpha_8\beta_7$) integrin families (Georas, McIntyre et al. 1993; Grayson, Van der Vieren et al. 1998; Tachimoto and Bochner 2000; Bochner and Schleimer 2001; Tachimoto, Ebisawa et al. 2002). These various integrin molecules selectively interact with adhesion receptors (VCAM-1, MAdCAM-1, and ICAM-1, -2 and -3 and fibrinogen) expressed on the vascular endothelium. $\alpha_4\beta_1$ selectively binds to VCAM-1 and fibronectin, and $\alpha_4\beta_1$ is the primary ligand for the extracellular matrix protein laminin. The $\alpha_4\beta_7$ integrin selectively binds to MAdCAM-1 in the GI tract, and LFA-1 ($\alpha_5\beta_2$) and MAC-1 ($\alpha_5\beta_2$) bind to ICAM-1. In addition, LFA-1 ($\alpha_5\beta_2$) can also bind ICAM-2 and -3, whilst $\alpha_5\beta_2$ has been shown to bind both VCAM-1 and ICAM-3. Leukocyte integrin/intercellular adhesion molecule interactions
particularly LFA-1/ICAM-1 and VLA-4/VCAM-1, are regulated by cytokines and chemokines such as interleukin (IL-)1 (α and β), tumour necrosis factor (TNF) (α and β), IL-4 and IL-13 (Meager 1999). IL-1 and TNF stimulate VCAM-1 and ICAM-1 expression on a variety of cell types. By contrast, IL-4 and IL-13 differentially enhance the expression of VCAM-1, having little impact on ICAM-1. Chemokines alter the activation state and selectivity of adhesion molecules (Weber, Kitayama et al. 1996; Tachimoto, Burdick et al. 2000). For example, treatment of eosinophils with MCP-3, RANTES or eotaxin-2 switches eosinophils from a β₁-integrin/VCAM-1 dominant to a β₂-integrin/ICAM-1 dominant interacting cell (Tachimoto, Burdick et al. 2000). α₄β₇/MAdCAM-1 has been demonstrated to be the predominant adhesion system involved in eotaxin-1 mediated eosinophil recruitment to the GI tract (Mishra, Hogan et al. 1999; Hogan, Rothenberg et al. 2004).

The importance of eosinophilic accumulation into the colon during inflammation led us to investigate the integrin pathway responsible for the recruitment of eosinophils into the colon. In this chapter we examine the role of β₇-integrin during inflammation and investigate possible target candidate integrins and their receptors in the recruitment of eosinophils to the large intestine.
2.2 Materials and Methods

2.2.1 Mice

BALB/c and C57BL/6 mice (6-8 weeks of age) used in our experiments were obtained from the Specific Pathogen Free Facility or the Gene Targeting Facility of the John Curtin School of Medical Research (Australian National University, Canberra, Australia). CD2-IL-5 Tg $\beta_7^{-/-}$ mice were generated by crossing CD2-IL-5 Tg (BALB/c) mice into the $\beta_7^{-/-}$ (C57BL/6) background [kindly provided by Prof. N. Wagner, City Hospital of Dortmund, Germany]. The transgene positive offspring ($N_i$) were subsequently mated with $\beta_7^{-/-}$ (C57BL/6) to generate CD2 IL-5 Tg $\beta_7^{-/-}$ mice ($N_2$). The ($N_2$) CD2 IL-5 Tg $\beta_7^{-/-}$ (BALB/c x C57BL/6) mice were crossed with $\beta_7^{-/-}$ (C57BL/6) mice generating chimeric transgene positive and negative $\beta_7^{-/-}$ mice. To generate background-control mice the CD2-IL-5 Tg mice were crossed with $\beta_7^{-/-}$ (C57BL/6) mice and, the transgene positive offspring ($N_i$) were subsequently backcrossed with WT C57BL/6 mice generating background matched CD2-IL-5 Tg $\beta_7^{-/-}$ mice. Mice were treated according to Australian National University Animal Welfare guidelines and were housed in an approved containment facility. $\beta_7^{-/-}$ (C57BL/6) and CD2 IL-5 Tg $\beta_7^{-/-}$ (BALB/c x C57BL/6) mice were identified using following amplification primers and reaction conditions;

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th># TUBES</th>
<th>MASTER</th>
<th>CYCLES</th>
<th>H2O, +ve, -ve</th>
<th>Beta-7</th>
</tr>
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<tbody>
<tr>
<td>PCR REAGENTS</td>
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<tr>
<td>water</td>
<td>9</td>
<td>0</td>
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<tr>
<td>10 x ST buffer</td>
<td>2</td>
<td>0</td>
<td>2 x 35</td>
<td>94°C: 3 ’</td>
<td>95°C: 0’15”</td>
</tr>
<tr>
<td>5mM dNTP’s</td>
<td>0.8</td>
<td>0</td>
<td>3 x 1</td>
<td>63°C: 0’15”</td>
<td>25°C: 5 ’</td>
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<tr>
<td>Beta-7 P1</td>
<td>1</td>
<td>0</td>
<td></td>
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<tr>
<td>Beta-7 P2</td>
<td>1</td>
<td>0</td>
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<tr>
<td>SUBTOTAL</td>
<td>15</td>
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WT P1 and P3 ~600bp
mutant P2 and P3 ~300bp

| Beta-7 P1 | AgTcAccATgTgAgcAgTAc |
| Beta-7 P2 | ccTgcgTgcAATccATcTTg |
| Beta-7 P3 | gTcAccAggAAgcccAgcc |
2.2.2 Fluorescent-activated cell sorter (FACS) Analysis

CD2-IL-5Tg mice were exsanguinated and spleens were removed and prepared into single cell suspensions as previously described (Forbes, Murase et al. 2004). To analyze integrin expression on eosinophils, splenocytes were incubated with either PE-conjugated anti-α4β7 (Clone DATK32 BD Pharmingen) (1μg/10⁶ cells) or biotin-conjugated anti-αm (Clone M1/70 15.1 Biosource International) or biotin-conjugated anti-αL (Clone 121/7 Biosource International) or biotin-conjugated anti-β2 (Clone PS/2 BD Pharmingen) Alexa Fluor 647 rat anti-mouse CC chemokine receptor 3 (CCR3; 83103; BD PharMingen), or isotype-matched control immunoglobulin (Ig; rat IgG biotin, Southern Biotechnology, Birmingham, AL) in phosphate-buffered saline (PBS)/1% foetal calf serum (FCS) on ice for 30 minutes and washed twice in PBS/1% FCS. To visualise the binding of the anti-integrin antibodies, cells were incubated with streptavidin-PE (1:1600 dilution; BD Pharmingen) in PBS/1% FCS at 4°C for 30 minutes and washed twice in PBS/1% FCS. Cells were analyzed by flow cytometry on a FACSAdvantage SE cell sorter (Becton Dickinson Biosciences). Eosinophils were identified by forward scatter (FSC) vs side scatter (SSC) and polarizing light, as previously described (Shapiro 2003). Briefly, eosinophil granules are birefringent and act to depolarise the scattered light, thereby reducing the difference in scattering intensity between the two polarisations. The depolarisation was measured by impinging polarised light on a sample, collecting the large angle scattered light at a single large
angle, splitting the collected light into two beams and measuring the scattered light in the two beams using two detectors, one for orthogonal light scattering of all polarisations, and the second preceded by a polarising filter to measure depolarised orthogonal light scattering.

2.2.3 Induction of colonic injury

Dextran sulfate sodium (DSS) used for the induction of experimental colitis (ICN Biomedical Inc., USA) was supplied as the sodium salt with an average molecular weight of 41kDA. It was used as a supplement in the drinking water of the mice for 7 days as a 2.5% (w/v) solution.

2.2.4 Disease activity index

Disease activity index (DAI) was derived by scoring three major clinical signs (weight loss, diarrhoea and rectal bleeding)(Stevceva, Pavli et al. 2000). The clinical features were scored separately and then correlated with a histological score. DAI = (body weight change) + (diarrhoea score) + (rectal bleeding score).

2.2.5 Body weight

Changes in body weight were calculated as the difference between the predicted body weight and the actual weight on a particular day. The formula for predicted body weight was derived by simple regression using the body weight data for the control group. The following formula was used: \( Y = a + kx \), where \( Y \) = body weight change (loss or gain), \( k \) = daily increase in body weight, \( x \) = day, \( a \) = starting body weight.

2.2.6 Diarrhoea

The appearance of diarrhoea was defined as mucous/faecal material adherent to anal fur. The presence or absence of diarrhoea was scored as either 1 or 0, respectively. The presence or absence of diarrhoea was confirmed by examination of the colon following completion of the experiment (Stevceva, Pavli et al. 2000). Mice were sacrificed and the colon excised from the animal. Diarrhoea was defined by the absence of faecal pellet formation in the colon and the presence of continuous fluid faecal material in the colon.
2.2.7 Rectal bleeding

The appearance of rectal bleeding was defined as diarrhoea containing visible blood or gross rectal bleeding and scored as described for diarrhoea.

2.2.8 Histopathological examination

Animals were sacrificed on day 8 and the colon was excised. The length of the colon was measured using digimatic calipers (Mitutoyo, Kawasaji, Japan). Tissue specimens were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (H/E) and Masson's trichrome using standard histological techniques. The % colon length with mucosal ulceration was determined by performing morphometric analysis of colon using ImageProPlus 4.5 software package (Media Cybernetics, Inc. Silver Spring, MD). In brief, digital images of longitudinal sections (1-2 cm in length) of H/E stained colons were produced. Using the ImageProPlus 4.5 software the length of ulcerated mucosal lining was divided by the total length of the colonic mucosal surface and the value was expressed as a percentage of colon length with mucosal ulceration.

2.2.9 Detection and quantification of eosinophils by immunohistochemistry

The colon segment of the GI tract was immunostained with antiserum against mouse MBP as previously described (Mishra, Hogan et al. 1999). Briefly, 5μm sections were quenched with H2O2, blocked with normal goat serum and stained with a rabbit anti-murine eosinophil major basic protein (MBP) antiserum [kind gift from Nancy and James Lee, (Mayo Clinic, Scottsdale, AZ)] as previously described (Mishra, Hogan et al. 1999). The slides were then washed and incubated with biotinylated goat anti-rabbit antibody and avidin-peroxidase complex (Vectastain ABC Peroxidase Elite kit; Vector Laboratories). The slides were developed by nickel diaminobenzidine, enhanced cobalt chloride to form a black precipitate, and counterstained with nuclear fast red. Quantification of eosinophils was performed by counting the number of immunoreactive cells from 15-25 fields of view (magnification x 40) from at least 4-5 random sections/mouse. Values were expressed as eosinophils per mm² tissue.
2.2.10 Eosinophil peroxidase (EPO) activity assay

Mice were killed on Day 8, and the colon was excised and flushed with 1 ml PBS solution. The faecal material was vortexed vigorously for 5 min at 4°C and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and placed in a sterile Eppendorf tube and stored at -70°C until analysis. EPO activity was measured in the supernatant of cell-free colon flushes as described previously (Schneider and Issekutz 1996). This assay is based on the oxidation of o-phenylenediamine (OPD) by EPO in the presence of H₂O₂. The EPO substrate solution consisted of 12 mM OPD (Sigma Aldrich, St. Louis, MO), 0.005% H₂O₂, 10 mM HEPES, and 0.22% cetyltrimethylammonium bromide (CTAB). Substrate solution (75 µl) was added to cell-free supernatants, which were derived from colon flushes (75 µl) in a 96-well microplate and incubated at room temperature for 15 min before stopping the reaction with 50 µl cold 8 N sulfuric acid. Absorbance was measured at 490 nm. Standard EPO activity, 100 U/ml, was determined, as EPO activity produced by 1x10⁶-purified eosinophils/µl supernatants. Eosinophils were purified from the spleen of CD2-IL-5Tg mice as described previously in section 2.2.2.

2.2.11 In vitro stimulation of eosinophils with eotaxin-1

Eosinophils were purified from the spleen of CD2-IL-5 transgenic mice as previously described (Shapiro 2003). Eosinophils were consistently purified to 95-99% purity. Purified eosinophils (2 x 10⁶ cells/ml) were incubated in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% FCS (Life Technologies), 50 µM 2-ME (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Sigma), and penicillin-streptomycin (Life Technologies) [complete RPMI 1640] with mIL-5 (120ng/ml) at 5% CO₂, 37°C for 24 hours. Eosinophil chemotaxis activity was measured. In some experiments following the 24-hour incubation, eosinophils chemotactic activity to eotaxin-1 was determined using transwell tissue culture plates (Corning Costar, Cambridge, MA). In brief, 2.5 x 10⁵ eosinophils (500µl) were resuspended in complete RPMI 1640 and were placed in the upper chamber, and eotaxin-1 (100ng/ml) (PeproTech, NJ) in complete RPMI 1640 was placed in the lower chamber. Following a 3-h incubation, the permeable polycarbonate filter was removed and fixed in methanol for 5 minutes. The filter was stained using H & E and air-dried. The cells attached to the
lower chamber of the polycarbonate filter (8-μm pores) were quantitated by counting the number of eosinophils per 20 HPF/filter.

2.2.12 Fluorescent-activated cell sorter (FACS) analysis on colonic eosinophils

Experimental colitis was induced with 2.5% DSS as described above. On day 8, the mice were exsanguinated and the colon segment of the GI tract removed and flushed with 20ml of PBS. The colon tissue was cut into 1-cm segments and incubated in digestion buffer containing 48mg/ml Collagenase A, 24 U/ml Dispase II, 2.5mg/ml DNAse in RPMI 1640 and incubated for 60 minutes at 30°C. The tissue was vigorously vortexed every 10 minutes for 15 seconds. Following the 60-minute incubation the cell aggregates were dissociated by pipetting and centrifuged at 1200 rpm for 10 minutes at 4°C. The supernatant was decanted and the cell pellet resuspended in RPMI 1640 + 10% FCS and filtered through 70μm filter. The single cell suspension was prepared by Ficoll density gradient centrifugation and quantitated by trypan blue exclusion analysis. 1x10^6 colonic cells were plated in a 96 well round bottom plate and flow cytometry analysis staining for CCR3 and β7-integrin were performed as described above in section 2.2.2.

2.2.13 Statistical analysis

The significance of differences between the means of experimental groups were analysed using Student’s unpaired t-test. Values were reported as the mean ± standard error of the mean (SEM). Differences in mean values were considered significant if p < 0.05.
2.3 Results

2.3.1 Role of $\beta_7$-integrin in eosinophil recruitment into the colon and experimental colitis.

We have recently provided direct evidence of a central role for eosinophils and eosinophil-derived eosinophil peroxidase (EPO) in GI dysfunction and the pathophysiology of experimental colitis (Forbes, Murase et al. 2004). Furthermore, we demonstrate a critical role for eotaxin-1 in the recruitment of eosinophils to the large intestine during inflammation. We were next interested in elucidating the adherence pathways involved in eosinophil transmigration into the colon during experimental colitis. Initially, we examined the role of $\beta_7$ integrin, as this molecule has been shown to be critically involved in leukocyte adherence and recruitment into the GI tract (Butcher, Williams et al. 1999). To elucidate the role of $\beta_7$ integrin in eosinophil recruitment into the large intestine we employed $\beta_7$ integrin-deficient mice and the DSS-model of experimental colitis. DSS treatment of WT mice induced experimental colitis including diarrhoea, rectal bleeding, and colon shortening (Figure 2.1. a - d). Histological examination of mouse colons at day 8 showed that all DSS-treated mice developed extensive ulceration of the epithelial layer, submucosa oedema, crypt damage, fibrotic thickening of the muscularis mucosa and dense infiltration of the superficial layers of the mucosa with granulocytes and some mononuclear cells (Figure 2.1. g.). To identify tissue eosinophils we performed immunohistochemistry using a polyclonal antiserum against eosinophil-derived MBP. Eosinophils were observed throughout the mucosa and submucosa in all DSS-treated WT mice (Figure 2.1. g.). Quantification of eosinophil numbers revealed eosinophil levels to be significantly increased in the colon of DSS-treated mice as compared to control-treated mice (Figure 2.1. e.). Consistent with this observation the level of eosinophil derived EPO in the lumen of the colon of DSS-treated mice was 100-fold higher than that observed in control-treated animals (Figure 2.1. f). DSS-induced experimental colitis in $\beta_7$-integrin$^{+}$ mice was comparable to that observed in strain matched WT mice (Figure 2.1. a. – g.). We observed no significant difference in diarrhoea, rectal bleeding, colon shortening and histopathology of the colon between DSS-treated WT and $\beta_7$-integrin$^{+}$ mice (Figure 2.1. a. – d.). We next examined eosinophil numbers and EPO activity in $\beta_7$-integrin$^{+}$ mice revealed a
Figure 2.1. DSS-induced experimental colitis occurs via a β7-integrin independent mechanism.

(a) DAI, (b.) weight change, (c.) diarrhoea/rectal bleeding [0-2] score and (d.) colon length during the course of DSS treatment in β7⁻⁻⁻⁻integrin and WT mice. (e.) eosinophil numbers per high powered field (HPF) and (f.) EPO activity in lumen of the colon of control and DSS-treated β7-integrin⁻⁻⁻⁻ and WT mice. (g) Representative photomicrographs of H&E and anti-MBP-stained colon from control and DSS-treated C57BL/6 WT and β7-integrin⁻⁻⁻⁻ mice. Data represent the mean ± SEM of 4-5 random sections per mouse for 4-5 mice per group. Statistical significance of differences (p<0.05) was determined using Student’s unpaired t-test. Significant differences (*p<0.05) between groups. *p < 0.05 as compared to matched vehicle. (g) Insert depicts MBP-positive eosinophils. Magnification (g) x 40.
significant and near identical increase in these parameters in the colon of DSS-treated \( \beta_\gamma \)-integrin\(^+\) mice as compared to DSS-treated WT mice (Figure 2.1. e., f. and g.)

Previous investigations have demonstrated that VLA-4 (\( \alpha_4\beta_1 \)) is important in eosinophil recruitment during inflammatory responses (Weg, Williams et al. 1993; Abraham, Sielczak et al. 1994; Nakajima, Sano et al. 1994; Pretolani, Ruffie et al. 1994; Gonzalo, Lloyd et al. 1996). To examine the role of VLA-4/VCAM-1 pathway in eosinophil recruitment into the colon during experimental colitis, \( \beta_\gamma \)-integrin\(^+\) mice were administered either anti-\( \alpha_4 \) integrin mAb or control-Ig and exposed to 2.5% DSS in drinking water or drinking water alone for 8 days and eosinophil levels in the colon were quantitated. DSS treatment of \( \beta_\gamma \)-integrin\(^+\) mice administered control-Ig significantly increased colonic eosinophil numbers and EPO activity as compared to control treated \( \beta_\gamma \)-integrin\(^+\) mice administered control-Ig [eosinophils/HPF; 0.4 ± 0.2 vs. 24.1 ± 0.4; EPO activity (U/ml); 1.7 ± 1.4 vs. 87.1 ± 1.6; vehicle- vs. DSS-treatment of \( \beta_\gamma \)-integrin\(^+\) mice administered control-Ig; mean ± SEM (n=4-5) (p < 0.05)]. Notably, the level of eosinophils and EPO activity in the colon of DSS treated \( \beta_\gamma \)-integrin\(^+\) mice administered with anti-\( \alpha_4 \) integrin mAb was equivalent to that observed in DSS treated \( \beta_\gamma \)-integrin\(^+\) mice administered control-Ig mAb [eosinophils/HPF; 24.1 ± 0.4 vs. 22.0 ± 0.5; EPO activity (U/ml); 87.1 ± 1.6 vs. 82.1 ± 1.50; mean ± SEM (n=4-5) (p < 0.05)]. Collectively, these studies suggest that both the \( \alpha_4\)- and \( \beta_\gamma \)-integrin pathways do not play a major role in eosinophil trafficking into colon under inflammatory conditions related to experimental colitis.

2.3.2 Expression of \( \alpha \) and \( \beta \)-integrins on splenic eosinophils from CD2-IL-5Tg and CD2-IL-5Tg \( \beta_\gamma \)-integrin\(^+\) mice.

Following the demonstration that eosinophil recruitment to the colon could occur independently of \( \alpha_4 \) and \( \beta_\gamma \)-integrins, we next wanted to characterise the surface expression of integrins (\( \alpha_4\beta_7 \), \( \alpha_4 \), \( \alpha_4 \), \( \alpha_M \) and \( \beta_7 \)) on eosinophils in attempt to identify the integrins involved in eosinophil recruitment to the colon. To do this we examined splenic eosinophils from CD2-IL-5Tg and CD2-IL-5Tg \( \beta_\gamma \)-integrin\(^+\) mice. Eosinophils were analyzed by flow cytometry based on FSC-H vs. SSC-H (Figure 2.2. a. and b.). To confirm that the identified cell population was eosinophils, the gated cells were sorted by flow cytometry, cytocentrifuged and stained with chromotrope 2R. 95-99% of the
sorted cells were identified as eosinophils by histological analysis (Figure 2.2. c.). Splenic eosinophils from CD2-IL-5Tg mice expressed \( \alpha_4, \alpha_L, \alpha_M \) and \( \beta_2 \) integrins (Figure 2.2. d., f., h. and j.). Similar results were obtained with eosinophils from CD2-IL-5Tg \( \beta_2 \)-integrin\(^+ \) mice (Figure 2.2. e., g., i., and k.). The level of expression of these integrins on \( \beta_2 \)-integrin\(^+ \) eosinophils were comparable to that observed on WT eosinophils (Figure 2.2.).

2.3.3 The effect of eotaxin-1 and IL-5 on \( \alpha- \) and \( \beta \)-integrin expression on eosinophils.

Recently we have demonstrated a central role for eotaxin-1 in eosinophil recruitment into the colon during experimental colitis (Forbes, Murase et al. 2004). To identify the effect of eotaxin-1 on integrin expression on eosinophils we cultured eosinophils purified from CD2-IL-5Tg mice in the presence of eotaxin-1 and/or IL-5 and examined the surface expression of various integrins including \( \alpha_4 \beta_7, \alpha_4, \alpha_L, \alpha_M \) and \( \beta_2 \). Incubation of purified eosinophils in the presence of IL-5 for 24 hours upregulated the surface expression of \( \beta_2 \) integrin as compared to eosinophils cultured in media alone (Figure 2.3. h.). Similarly, exposure to eotaxin-1 also stimulated an increase in \( \beta_2 \) expression of eosinophils. Interestingly, addition of eotaxin-1 also promoted a modest increase in the expression of both \( \alpha_L \) and \( \alpha_M \) integrins (Figure 2.3. f and g). We observed no change in expression of \( \alpha_4 \beta_2 \) or \( \alpha_4 \) integrins on eosinophils following stimulation with either IL-5 and/or eotaxin-1 as compared to media alone (Figure 2.3. d. and e.). Collectively, these data show that eotaxin-1 selectively promotes the surface expression of ICAM-1 binding ligands, LFA-1 (\( \alpha_L \beta_2 \)) and MAC-1 (\( \alpha_M \beta_2 \)) on eosinophils.

2.3.4 The effect of blockade of \( \alpha_L \), \( \alpha_M \) and \( \beta_2 \) integrins on eosinophil chemotaxis.

To examine the role \( \alpha_L^- \), \( \alpha_M^- \) and \( \beta_2^- \)-integrins play in eotaxin-1 mediated eosinophil chemotaxis we performed in vitro eosinophil chemotaxis assays. Purified eosinophils were incubated with anti-\( \alpha_L \) integrin, anti-\( \alpha_M \) integrin, anti-\( \beta_2 \) integrin mAb or control anti-mouse Ig and eosinophil chemotaxis in response to eotaxin-1 was examined. Eosinophil chemotaxis was inhibited by anti-\( \alpha_L \), anti-\( \alpha_M \) and anti-\( \beta_2 \) mAb treatment as compared to the control-Ig treatment (Figure 2.4.). Eosinophil chemotaxis was inhibited by \( \sim 45-60\% \). However, as the assay contained purified eosinophils alone, and
Figure 2.2. Characterisation of surface expression of LFA-1 and MAC-1 integrin chains on eosinophils.

Representative histograms of $\beta_2$, $\alpha_4$, $\alpha_{L^-}$ and $\alpha_{M^-}$-integrin chain expression on splenic eosinophils from $\beta_7$-integrin $^+$ CD2-IL-5Tg and CD2-IL-5Tg mice. (a. and b.) Representative FSC-H vs. SSC-H dot blot of splenocytes from $\beta_7$-integrin $^+$ CD2-IL-5Tg mice and CD2-IL-5Tg mice. Representative histograms of (d. and e.) $\beta_2$, (f. and g.) $\alpha_4$, (h. and i.) $\alpha_{M^-}$ and (j. and k.) $\alpha_{L^-}$-integrin chain expression on splenic eosinophils from $\beta_7$-integrin $^+$ CD2-IL-5Tg mice and CD2-IL-5Tg mice respectively. Filled histogram depicts cells alone, dotted line (streptavidin-PE) and closed line anti-integrin antibody + (streptavidin-PE). (k.) Photomicrograph of cytocentrifuged cells sorted by FSC-H vs. SSC-H and polarised light criteria [depicted in box in (a.)] and stained by H&E. The purity of eosinophils was >95% on re-analysis.
Figure 2.3. Characterisation of surface expression of α4β7, LFA-1 and MAC-1 integrin chains on eosinophils following IL-5 and/or eotaxin-1 stimulation.

(a. – c.) FSC-H vs. SSC-H dot blot and autofluorescence of splenic eosinophils purified from CD2-IL5Tg mice and cultured in presence of mIL-5 (120ng/ml) and/or eotaxin-1. Representative histograms of (d.) α4β7-, (e.) α4-, (f.) αL- (g.) αM- and (h.) β2-integrin chain expression on splenic eosinophils following stimulation with mIL-5 and/or eotaxin-1. The purity of eosinophils was >95% on re-analysis. (d. – h.) Filled histogram depicts cells alone; (-----) histogram depicts unstimulated eosinophils, (- ___ -) histogram depicts IL-5 stimulated eosinophils and closed black line depicts IL-5 and eotaxin-1 stimulated eosinophils.
Chapter Two: The role of β2-integrin during inflammation of the large intestine

as such, virtually no epithelial cells, it is possible this effect is due to non-specific interactions via to surface bound antibody on transwells blocking recruitment. We have attempted to control for this phenomenon by employing control-Ig treatment, but this cannot rule out the possibility of non-specific interactions with the other mAb treatments.

2.3.5 Characterisation of the β2 integrin/ICAM-1 pathway expression in the colon.

β2 integrins, LFA-1 (αLβ2) and MAC-1 (αMβ2) both interact with ICAM-1. To delineate the role for β2-integrin/ICAM-1 pathway in eosinophil transmigration into the colon in vivo we initially examined expression of β2 integrin on colonic lamina propria cells in the colon of vehicle- and DSS-treated WT mice. By FACS analysis we show that β2-integrin lamina propria cells were significantly increased in DSS-treated mice as compared to control-treated animals (Figure 2.5. a). To identify whether CCR3+ eosinophils from the colon of DSS injury-induced WT mice express αL- and αM-integrins, we examined αL- and αM-expression on β2-integrin CCR3+ eosinophils. We demonstrate that all β2-integrin CCR3+ cells expressed αL- and αM-integrins (Figure 2.5. c. and d.). To examine the involvement of the ICAM-1 pathway, we performed anti-ICAM-1 immunofluorescence staining of colon from vehicle-and DSS-treated WT mice. Anti-ICAM-1 immunofluorescence staining of colon from vehicle-treated WT mice detected very few ICAM-1+ cells (Figure 2.5.d.). In contrast, after DSS-treatment of WT mice, a large number of ICAM-1+ inflammatory cells were detected (Figure 2.5. e.). Notably, the ICAM-1+ cells localised to the region similar to that previously shown for eosinophils (Forbes, Murase et al. 2004) (and figure 1.g.). Collectively, these studies suggest that the β2-integrin/ICAM-1 are expressed in the colon during experimental colitis and may play a role for eosinophil infiltration into the colon.
Figure 2.4. Blockade of LFA-1 and MAC-1 / ICAM-1 pathways inhibit eosinophil chemotaxis in vitro.

Eotaxin-1 mediated eosinophil chemotaxis following anti-αL-αM- and β2-integrin chain treatment. Eosinophil numbers per HPF. Data represents the mean ± SEM of triplicate eosinophil cultures. Statistical significance of differences was determined using Student's unpaired t-test. Significant differences (*p<0.05) between groups. *p<0.05 as compared to appropriate control.
Chapter Two: The role of $\beta_3$-integrin during inflammation of the large intestine

![Graph showing eosinophils/HPF](#)
Figure 2.5. $\beta_2$-integrin$^+$ CCR3$^+$ and ICAM-1$^+$ cells in the colon during DSS-induced colonic inflammation.

(a.) Percentage of $\beta_2$-integrin positive cells in colon of control- and DSS-treated WT mice. (b.). Representative histogram of isotype match control Ig (FITC) and (Alexa-Fluor-647) on $\beta_2$-integrin$^+$ gated lamina propria cells from DSS-treated WT mice. Representative histogram of (c.) $\alpha_1$-integrin- and (d.) $\alpha_M$-integrin-FITC and CCR3-Alexa-Fluor-647 on $\beta_2$-integrin$^+$ gated colonic cells from DSS-treated WT mice. Representative photomicrograph of ICAM-1$^+$ cells in colon tissue from (d.) control- and (e.) DSS-treated C57BL/6 WT mice (a). Data represents the mean + SEM (a. – f.). n = 4-5 mice per group from duplicate experiments. (e. and f.) x 200 magnification. Statistical significance of differences was determined using Student’s unpaired t-test. Significant differences (*p<0.05) between groups. *p<0.05 as compared to control.
Chapter Two: The role of β7-integrin during inflammation of the large intestine

(a.)

\[\text{\% \(\beta_7\)-integrin positive cells} \]

\[\text{Control} \quad \text{DSS}\]

(b.)

\[\text{isotype match control (Alexa 647)}\]

(c.)

\[\text{CCR3-Alexa 647}\]

(d.)

\[\text{CCR3-Alexa 647}\]

(e.)

(f.)
2.4 Discussion

It has been previously demonstrated that eosinophils express \( \alpha_d\beta_7 \) integrin and that the \( \beta_7 \)-pathway is critical for eotaxin-1 mediated eosinophil recruitment into the small intestine (Mishra, Hogan et al. 2002). In this study we have examined the adhesion systems that regulate eosinophil recruitment into the large intestine and have demonstrated 1) eosinophil accumulation in the colon can occur via a \( \beta_7 \)-integrin-independent pathway; 2) that murine eosinophils express the ligands for MAdCAM-1 and ICAM-1 (\( \alpha_d\beta_7, \beta_2, \alpha_l \) and \( \alpha_M \)); 3) eotaxin-1 (the critical chemokine for eosinophil recruitment into the GI tract) upregulates surface expression of LFA-1 and MAC-1 integrin chains (\( \alpha_l, \alpha_M \) and \( \beta_2 \)) on eosinophils; 4) antibody blockade of \( \alpha_l, \alpha_M \) and \( \beta_2 \) integrins can inhibit eotaxin-1 mediated eosinophil transmigration \textit{in vitro} and 5) \( \beta_2 \)-integrin expression on colonic eosinophils.

Leukocyte recruitment into the GI tract and gut-associated lymphoid tissue is thought to be critically regulated by \( \beta_7 \)-integrin (Wagner, Lohler et al. 1996; Butcher, Williams et al. 1999; Artis, Humphreys et al. 2000; Gurish, Tao et al. 2001). Indeed, the expression of MAdCAM-1 and \( \alpha_d\beta_7 \) expression on leukocytes in the intestinal mucosa is significantly upregulated in GI diseases including cow’s milk allergy and food allergy (Eigenmann, Tropia et al. 1999; Veres, Helin et al. 2001). Recently, experimental studies have demonstrated that leukocyte recruitment into the large intestine can occur in a \( \beta_7 \)-integrin-dependent manner (Artis, Humphreys et al. 2000; Gurish, Tao et al. 2001; Sydora, Wagner et al. 2002). Moreover, no impairment in CD4+ T-cell and mast cell infiltration into the colon of \( \beta_7 \)-integrin" mice was observed following helminth infection (Artis, Humphreys et al. 2000). In addition, the concentration of mast cell progenitors in the large intestine of \( \beta_7 \)-integrin" mice was not reduced as compared to WT mice (Gurish, Tao et al. 2001). We demonstrate that eosinophil recruitment into colon during inflammatory conditions predominantly occurs via a \( \beta_7 \)-integrin-independent mechanism, and demonstrate upregulation of the \( \beta_2 \)-integrin/ICAM-1 pathway during colonic inflammation. The contribution of the \( \beta_2 \)-integrin pathway in leukocyte and mast cell recruitment into the colon is not yet fully elucidated.

Previous studies have demonstrated that eotaxin-1 upregulates the expression of ICAM-1 binding \( \alpha_l \) and \( \alpha_M \) expression on lymphocytes (Jinquan, Quan et al. 1999). \textit{In vitro},
we demonstrate that eotaxin-1 upregulates $\alpha_L$ and $\alpha_M$ expression on eosinophils, whilst both IL-5 and eotaxin-1 enhanced $\beta_2$ expression. Furthermore, we show that eotaxin-1 mediated eosinophil chemotaxis \textit{in vitro} can be attenuated by blockade of $\beta_2$, $\alpha_L$ and $\alpha_M$ by neutralising mAb. These studies would suggest that eotaxin-1 preferentially upregulates LFA-1 and MAC-1 integrin expression and increases eosinophil sensitivity to ICAM-1 adhesion system mediated inflammatory processes. In different disease states, elevated eosinophil numbers are often restricted to specific GI compartments. For example, eosinophilic oesophagitis is characterised by elevated level of eosinophils restricted to the oesophagus. Whereas, UC is characterised by elevated levels of eosinophils in the colon without increased numbers in other GI compartments. Paradoxically, the recruitment of eosinophils into these various GI compartments is thought to be primarily regulated by eotaxin-1. How eotaxin-1 selectively orchestrates the trafficking of eosinophils into specific GI compartments remains unclear.

Our demonstration that eotaxin-1 upregulates $\alpha_L$ and $\alpha_M$ expression on eosinophils and previous investigations showing that eotaxin-1 stimulates expression of ICAM-1 on microvascular endothelial cells (Hohki, Terada et al. 1997) suggests that chemokine-modulation of integrin expression may regulate eosinophil recruitment into the colon. However, as $\alpha_L$, $\alpha_M$, $\beta_2$, and $\alpha_4\beta_2$ integrins are expressed on unstimulated eosinophils, the differential expression of integrins cannot fully account for the eotaxin-1 mediated selectivity for the ICAM-1 pathway. Another possible explanation is that chemokines such as eotaxin-1 differentially regulate integrin/adhesion molecule avidity promoting $\beta_2$/ICAM-1-dependent adhesion pathway interaction. Weber and colleagues have demonstrated that RANTES and MCP-3, eotaxin-1 receptor (CCR3) agonists, can selectively down regulate eosinophil VLA-4/VCAM-1 adhesion and promote $\beta_2$/ICAM-1 adhesion. Furthermore, they showed that the differential regulation of adhesion occurred independently of integrin surface expression and directly involved modulation of integrin avidity (Weber, Kitayama et al. 1996). We postulate that eotaxin-1 mediated recruitment involves a complex step of events involving 1) integrin expression on leukocytes (increased $\alpha_L$, $\alpha_M$, $\beta_2$); 2) adhesion molecule expression on microvascular endothelium (ICAM-1) and 3) integrin and integrin receptor avidity (LFA-1/ICAM-1, MAC-1/ICAM-1).
In conclusion, we have shown that eotaxin-1 selectively upregulates surface expression of ICAM-1 binding integrins (\( \beta_2, \alpha_4 \), and \( \alpha_M \)) on eosinophils and that eosinophil recruitment into the colon occurs via a \( \beta_7 \)-integrin-independent pathway. These investigations highlight the importance of investigating the role of \( \beta_2 \)-integrins/ICAM-1 pathways in eosinophilic inflammation in the colon during disease.
Chapter Three

The role of ICAM-1 dependent pathways in eosinophil recruitment into the large intestine
3.1 Introduction

In chapter 2, the role of adhesion pathways in eosinophil recruitment into the large intestine during homeostasis and disease was investigated. Colonic eosinophilic recruitment during disease (experimental colitis) occurs via a \( \beta_7 \)-integrin-independent pathway. Eosinophils were shown to express both the MAdCAM-1- \((\alpha_4 \beta_7)\) and ICAM-1- [MAC-1 and LFA-1 (\( \beta_2, \alpha_l \) and \( \alpha_M \))] ligands and the expression of these molecules are upregulated by *in vitro* stimulation with eotaxin-1. Performing chemotaxis assays it was demonstrated via antibody blockade of \( \alpha_l, \alpha_M \) and \( \beta_2 \) integrins that this was able to inhibit eosinophil chemotactic responses to eotaxin-1. Employing a well-established model of experimental colitis associated with the administration of DSS, an upregulation of surface expression of LFA-1 and MAC-1 integrin chains on eosinophils was observed. These studies led to the hypothesis that ICAM-1-dependent and not \( \beta_7 \)-integrin/MAdCAM-1-dependent pathways are integral to eosinophil recruitment into the colon during GI inflammation.

The integrins LFA-1 \((\alpha_l \beta_2)\) and MAC-1 \((\alpha_M \beta_2)\) bind to ICAM-1 to allow cellular transmigration. ICAM \(^{-/-}\) mice have shown clear roles for ICAM-1 in lymphocyte migration to the lung during inflammation (Keramidaris, Merson et al. 2001). Furthermore, mice deficient in ICAM-1 have reduced susceptibility to LPS-induced septic shock and impaired delayed type hypersensitivity responses (Sligh, Ballantyne et al. 1993; Xu, Gonzalo et al. 1994; Tang, Hale et al. 1997). Moreover, leukocyte rolling velocities are significantly increased during inflammation, in the absence of ICAM-1 (Steeber, Campbell et al. 1998). Clinical investigations have demonstrated an upregulation of ICAM-1 in inflammatory bowel disease (IBD) patients, and blockade of ICAM-1 function attenuates experimental colitis (Bennett, Kornbrust et al. 1997; Hamamoto, Maemura et al. 1999; Kato, Hokari et al. 2000; Vainer and Nielsen 2000).

Recruitment of inflammatory cells into the GI tract is currently believed to occur via a \( \alpha_4 \beta_7 /\text{MAdCAM-1} \) dominant interaction (Kunkel and Butcher 2002). The \( \alpha_4 \beta_7 \) integrin receptor, MAdCAM-1 is primarily expressed on GI vascular endothelium and is generally absent on non-intestinal venules and most non-GI sites of inflammation (Butcher, Williams et al. 1999). Blockade of \( \alpha_4 \beta_7 /\text{MAdCAM-1} \) interactions by neutralising monoclonal antibodies or genetic deletion inhibits T, B cell and mast cell
recruitment into GI compartments including the small intestine, mesenteric lymph nodes and Peyer’s patches. Interestingly, recent experimental investigations have demonstrated that leukocyte recruitment into the large intestine can occur via a β7 integrin independent mechanism suggesting that leukocytes utilize different adhesion systems to infiltrate various GI compartments (Artis, Humphreys et al. 2000; Sydora, Wagner et al. 2002). Identifying the dominant adhesion complex involved in eosinophil trafficking into the large intestine is particularly important, since there are numerous diseases characterised by eosinophil accumulation in the colon (e.g. allergic colitis and IBD), yet most studies have concentrated on the upper GI tract (e.g. oesophagus and small intestine).

This and our previous findings in chapter 2 led us to examine the contribution of ICAM-1 to eosinophil recruitment into the colon during homeostasis and following DSS treatment, by blockade of ICAM-1 through neutralising antibody and also employing ICAM-1 deficient mice.
3.2 Materials and Methods

3.2.1 Mice

As described in section 2.2.1. ICAM-1$^{-/-}$ and L-selectin$^{-/-}$ mice (C57BL/6 background) were kindly provided by Thomas F. Tedder, Duke University Medical Center, NC, USA.

3.2.2 Induction of colonic injury

As described in section 2.2.3.

3.2.3 Disease activity index

As described in section 2.2.4.

3.2.4 Body weight

As described in section 2.2.5.

3.2.5 Diarrhoea

As described in section 2.2.6.

3.2.6 Rectal bleeding

As described in section 2.2.7.

3.2.7 Detection and quantification of eosinophils by immunohistochemistry

As described in section 2.2.9.
3.2.8 Eosinophil peroxidase (EPO) activity assay

As described in section 2.2.10.

3.2.9 Monoclonal Antibody treatment

Mice were i.p. injected daily with either rat anti-mouse integrin α4 chain (200μl of 1mg/ml of clone PS/2 [IgG2a] mAb in saline) or anti-mouse ICAM-1 (200μl 1mg/ml clone YN1/1.7.4 [IgG2a] mAb in saline) or rat IgG control antibody (200μl 1mg/ml βGL113mAb in saline) for 4 days. Three hours following the final i.p. injection mice were sacrificed and the jejunum and colon was excised and fixed in 4% paraformaldehyde and stained for anti-MBP and eosinophil levels quantitated as described above. In some experiments mice were injected i.p. daily throughout the 8 day DSS treatment protocol with anti-mouse ICAM-1 (200μl 1mg/ml clone YN1/1.7.4 (IgG2a) mAb in saline) or rat IgG control antibody (200μl 1mg/ml βGL113mAb in saline).

3.2.10 Myeloperoxidase (MPO) activity

MPO activity, a marker of polymorphonuclear neutrophil granules, was assessed in colonic luminal contents according to the Bradley method (Bradley, Priebat et al. 1982). Mice were killed on Day 8, and the colon was excised and flushed with 1 ml PBS solution. The fecal material was vortexed vigorously for 5 min at 4°C and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and placed in a sterile Eppendorf tube and stored at −70°C until analysis. MPO activity was measured in the supernatant of cell-free colon flushes as described previously (Bradley, Priebat et al. 1982). This assay is based on the oxidation of o-dianisidine dihydrochloride (oDd) by MPO in the presence of H₂O₂. The MPO substrate solution consisted of 0.167 mg/ml oDd (Sigma Aldrich), 0.005% H₂O₂, 10 mM HEPES, 0.22% CTAB (Sigma Aldrich) and 60mM resorcinol (Sigma Aldrich). Substrate solution (75 μl) was added to cell-free supernatants, which were derived from colon flushes (75 μl) in a 96-well microplate and incubated at room temperature for 15 min before stopping the reaction with 50 μl cold 3 M sulfuric acid. Absorbance was measured at 450 nm. Standard MPO (human leukocytes, Sigma Aldrich; 50 U/mg protein), 10 U/ml, was used to generate a standard
3.2.11 Statistical analysis

As described in section 2.2.13, unless otherwise stated.
3.3 Results

3.3.1. The effect of anti-ICAM-1 treatment on eosinophil recruitment into colon of CD2-IL-5Tg mice.

We were initially interested in determining whether β7/ICAM-1 pathway contributes to eosinophil recruitment in vivo, we firstly examined colonic eosinophil numbers in CD2-IL-5Tg and CD2-IL-5Tg/β7-integrin+ mice treated with control-Ig (rat IgG1) or anti-ICAM-1 mAb. Anti-ICAM-1 mAb treatment of CD2-IL-5Tg and CD2-IL-5Tg/β7-integrin+ mice significantly reduced eosinophil levels in the colon (Figure 3.1.). This data suggests β7-integrin plays a role in eosinophil recruitment at baseline. This is most likely due to an artefact of the transgenic system, as CD4+ T-cells are producing the overexpression of IL-5 and β7-integrin is known to be necessary for the migration of T-cells into the GI tract (Butcher, Williams et al. 1999). Notably, in the absence of the β7-integrin and ICAM-1 pathway eosinophil levels in the colon were nearly ablated (Figure 3.1.). These studies suggest that the ICAM-1 pathway is the dominant adhesion system involved in eosinophil recruitment into the colon.

3.3.2 Anti-ICAM-1 treatment blocks DSS-induced experimental colitis and eosinophil recruitment into colon.

To identify a role for the β7/ICAM-1 pathway in the migration of eosinophils into the colon during an inflammatory reaction, WT mice were treated with anti-ICAM-1 neutralising mAb or control-Ig and challenged with DSS. Administration of DSS to control-Ig treated WT mice induced experimental colitis including diarrhoea, rectal bleeding and colon shortening (Figure 3.2. a. – c.). Eosinophil levels in the colon and the level of EPO in the lumen of the colon of these mice was significantly elevated compared to vehicle-challenged anti-ICAM-1-treated mice (Figure 3.2. d. – e.). In contrast, experimental colitis and eosinophil and EPO levels in the colon of DSS-administered anti-ICAM-1 treated mice were significantly reduced as compared in DSS-challenged control-Ig-treated WT mice (Figure 3.2.).

3.3.3 DSS-induced experimental colitis and eosinophil recruitment into colon occurs via an ICAM-1 dependent mechanism.

To confirm that ICAM-1 plays a role in the transmigration of eosinophils in the colon
**Figure 3.1. Anti-ICAM-1 treatment blocks eosinophil recruitment into colon of CD2-IL-5Tg mice.**

Eosinophils/mm² in colon of β₇-integrin⁺ CD2-IL-5Tg and CD2-IL-5Tg mice treated with Ig control or anti-ICAM-1 mAb. Data represents the mean ± SEM of 4-5 random sections per mouse for 4-5 mice per group. Statistical significance of differences was determined using Student’s unpaired t-test. Significant differences (*p<0.05) between groups. *p<0.05 as compared to appropriate control.
Chapter Three: The role of ICAM-1 in eosinophil recruitment into the large intestine

![Graph showing eosinophil counts per mm² for CD2 IL-5 Tg and CD2 IL-5 Tg β7−/− mice with Ig Control and anti-ICAM-1 treatments.](image-url)
Figure 3.2. Anti-ICAM-1 treatment blocks DSS-induced experimental colitis and eosinophil recruitment into colon.

(a.) DAI, (b.) % weight change, (c.) diarrhoea/rectal bleeding [0-2] score during the course of DSS treatment in anti-ICAM-1 or control-Ig-treated WT mice. (d.) eosinophil numbers per mm2 and (e.) EPO activity in lumen of the colon of control and DSS-treated WT mice administered anti-ICAM-1 or control-Ig. Data represents the mean ± SEM of 4-5 random sections per mouse for 4-5 mice per group. Statistical significance of differences was determined using Student’s unpaired t-test. (a. – c.) *p<0.05 as compared to vehicle + anti-ICAM-1; **p<0.05 as compared to DSS + anti-ICAM-1; (d. and (e.) *p<0.05 as compared to DSS + control-Ig.
Chapter Three: The role of ICAM-1 in eosinophil recruitment into the large intestine

(a.)

Vehicle + anti-ICAM-1
DSS + Ig control
DSS + anti-ICAM-1

(b.)

Weight change (% change from 100% baseline)

(c.)

Diarrhea/rectal bleeding [0-2]

(d.)

Eosinophils/mm² colon

(e.)

EPO activity (U/ml)
3.3.4 ICAM-1 deficiency results in suppression of DSS-induced experimental colitis by specific inhibition of eosinophil recruitment.

To demonstrate that the observed ablation of eosinophil recruitment into the colon by ICAM-1 blockade is directly a result of inhibition of eosinophil transmigration and not a result of the suppression of the recruitment of other inflammatory cells, we examined neutrophil levels in DSS-treated ICAM-1$^{-}$ mice. Neutrophils and not B and T cells have been shown to be important in the inflammatory response observed in experimental colonic injury (Krieglstein, Cerwinka et al. 2002; Kruidenier, van Meeteren et al. 2003; Naito, Takagi et al. 2003). To quantitate neutrophil levels, we examined MPO activity in colon luminal washes from DSS-treated mice. To block residual EPO activity, we performed the MPO assay in the presence of an EPO-specific inhibitor resorcinol (60 µM) (Schneider and Issekutz 1996; Forbes, Murase et al. 2004). DSS administration to WT mice promoted an increase in luminal MPO activity as compared with control-treated WT mice (Figure 3.4). Similarly, administration of DSS to ICAM-1$^{-}$ mice induced a significant and comparable increase in luminal MPO activity, demonstrating no reduction in neutrophil recruitment into the colon in the absence of ICAM-1 (Figure 3.4). The level of MPO activity is DSS-treated WT, and ICAM-1$^{-}$ mice were not reduced significantly in the presence of the EPO inhibitor resorcinol (Figure 3.4). These studies suggest that the $\beta_2$-integrin/ICAM-1 pathway is directly involved in eosinophil but not neutrophil recruitment into the colon during colonic injury.
Figure 3.3. DSS-induced experimental colitis and eosinophil recruitment into colon occurs via an ICAM-1 dependent mechanism.

(a.) DAI, (b.) Percentage weight change, (c.) diarrhoea/rectal bleeding [0-2] score and (d.) colon length during the course of DSS-treatment in ICAM-1\(^{+/−}\) and WT mice. (e.) eosinophil numbers per high powered field (HPF) and (f.) EPO activity in lumen of the colon of control and DSS-treated ICAM-1\(^{+/−}\) and WT mice. (g.) Representative photomicrographs of H&E and anti-MBP-stained colon from control and DSS-treated C57BL/6 WT and ICAM-1\(^{+/−}\) mice. Data represent the mean ± SEM of 4-5 random sections per mouse for 2-5 mice per group. Statistical significance of differences was determined using Student’s unpaired t-test (*p<0.05). *p<0.05 as compared to matched vehicle. *p<0.05 as compared to ICAM-1\(^{+/−}\) DSS. (g.) black arrows MBP-positive eosinophils. Magnification (g) x 40.
Chapter Three: The role of ICAM-1 dependent pathways in eosinophil recruitment into the large intestine

(a) DAL index

(b) Weight change

(c) Diarrheal rectal bleeding

(d) Colon length

(e) Eosinophils/hpf

(f) Ulmi EPO activity

(g) H&E and α-MBP images

* * *
Figure 3.4. MPO activity in the colon of DSS-treated WT and ICAM-1⁻ mice.

MPO activity in the lumen of the colon of control- and DSS-treated ICAM-1⁻ and WT mice in the presence and absence of the EPO inhibitor resorcinol (60 µM). Data represents the mean ± SEM of four to five mice per group. *, p<0.05; **, p<0.01; ***, p<0.001, significant difference among groups as per two-way ANOVA with Bonferroni post-test.
Chapter Three: The role of ICAM-1 in eosinophil recruitment into the large intestine

Cn Control
• loss
C3DSS + 60\(\mu\)M Resorcinol

WT ICAM-r

\[\text{MPO (U/mL)}\]

- **Control**
- **DSS**
- **DSS + 60\(\mu\)M Resorcinol**

\[\text{WT} \quad \text{ICAM-1}^{++}\]
3.4 Discussion

Blockade of ICAM-1 by neutralisation monoclonal antibody attenuated eosinophil recruitment into the colon in a transgenic model. Furthermore, the investigations demonstrate that the ICAM-1 adhesion pathway is important for the development of eotaxin-1 mediated eosinophil recruitment to this compartment during inflammation. Notably, ICAM-1 can bind both LFA-1 ($\alpha_{M}\beta_{2}$) and MAC-1 ($\alpha_{M}\beta_{2}$)-integrins (Bochner and Schleimer 2001). We were unable to identify either LFA-1 or MAC-1 as the ICAM-1 ligand responsible for eosinophil transmigration into the colon. Importantly, we have demonstrated that the ICAM-1 pathway is critical for eosinophil recruitment to the colon.

We have recently provided corroborative evidence demonstrating a central role for eosinophils in GI dysfunction and the pathophysiology of experimental colitis (Forbes, Murase et al. 2004). Our findings are in contrast to a previous investigation employing the same mutant mice in a DSS-model of colonic injury and inflammation, which suggest eosinophils are not required for disease pathogenesis in experimental colitis (Stevceva, Pavli et al. 2000). While we cannot fully explain these inconsistencies, Stevceva and colleagues employed a general histological staining technique (H/E) that poorly differentiates immature and degranulating eosinophils in gastrointestinal tissues, whereas we employed an eosinophil specific stain (MBP immunohistochemistry) that allows for specific identification and quantification of eosinophils in all activation states. Previously we have demonstrated that eosinophils mediate disease pathogenesis in experimental colitis through EPO-dependent pathways (Forbes, Murase et al. 2004). EPO catalyses the oxidation of halides and pseudohalides (Cl, Br and SCN) with the products of respiratory burst (O$_2$ and H$_2$O$_2$) to generate cytotoxic oxidants. Elevated levels of H$_2$O$_2$ have been reported in mucosal tissue samples from patients with UC (Shanahan and Targan 1992; Sands 1999). Interestingly, H$_2$O$_2$ has been shown to also promote an upregulation of $\beta_2$-integrin expression on eosinophils supporting a role for $\beta_2$-integrin associated eosinophil transmigration into the colon (Nagata, Yamamoto et al. 2000). We have extended these studies and demonstrated that eosinophils transmigrate into the colon via a $\beta_2$-integrin-dependent ICAM-1 pathway. However, it remains possible that anti-ICAM-1 treatment or ICAM-1 deficiency may result in an
indirect effect on disease pathogenesis by decreasing bone marrow eosinophil levels and as such cannot be discounted at this time.

Previous studies employing a similar model of experimental colitis have demonstrated an important role of VLA-4/VCAM-1, MAdCAM-1/α₄β₇, collagen-binding integrins α₄β₁ and α₄β₂ adhesion pathways in eosinophil recruitment and the development of experimental colitis (Hemler 1990; de Fougerolles, Sprague et al. 2000; Soriano, Salas et al. 2000). It is postulated that DSS-induced experimental colitis is regulated by macrophages and CD4⁺ T-cells of a mixed phenotype expressing both Th₁-type (IFNγ) and Th₂-type (IL-4, IL-5 and -13) cytokines (Dieleman, Palmen et al. 1998). CD4⁺ T-cell derived Th₂-cytokines regulate eosinophil-sensitive pathways including eotaxin-1 expression and eosinophil infiltration and activation (Rothenberg, Mishra et al. 2001; Rothenberg, Mishra et al. 2001). It is possible that studies demonstrating a critical role for MAdCAM-1/α₄β₇, VCAM-1 and α₁β₁-dependent pathways in experimental colitis by antibody neutralisation of VCAM-1 and α₁β₁, are inhibiting CD4⁺ T-cell recruitment and thereby suppressing the expression of Th₂-cytokines as well as downstream eosinophil-associated pathways. Indeed, macrophage, T-cell and neutrophil levels are decreased in the colon of DSS-treated mice following neutralisation of these adhesion systems (Kriegstein, Cerwinka et al. 2002).

Another possible explanation for these discrepancies are differential roles for adhesion pathways in discrete components of eosinophil migration (Broide 2002). The recruitment of eosinophils into tissues is a sequential multi-step process involving eosinophil intravascular tethering, firm adhesion to endothelium, followed by eosinophil diapedesis and chemotaxis into the tissue (Bochner 2000; Broide and Sriramarao 2001). Interestingly, α₄-integrins on eosinophils have been shown to be involved in the initial tethering function, reducing eosinophil rolling and facilitating subsequent firm adhesion (Sriramarao, von Andrian et al. 1994). Whereas, β₂-integrins are unable to promote initial eosinophil tethering, however following eosinophil tethering, β₂-integrin can stimulate eosinophil firm adhesion to basally expressed ICAM-1 (Sanz, Ponath et al. 1998). Notably, α₄β₇ is thought to mediate leukocyte tethering and rolling in the GI tract. Thus, blockade of the sequential multi-step pathways may halt leukocyte and eosinophil recruitment into the GI tract at various stages of transmigration and thereby suppress inflammation and disease pathogenesis.
Clinical and experimental studies have provided corroborative evidence for a role for ICAM-1 in UC (Bennett, Kornbrust et al. 1997; Hamamoto, Maemura et al. 1999; Kato, Hokari et al. 2000; Vainer and Nielsen 2000). ICAM-1 levels are elevated in sonicated colonic tissue samples from UC patients as compared to control patients and in the colon of mice experimental colitis (Vainer and Nielsen 2000). Furthermore, blockade of ICAM-1 function by neutralising monoclonal antibody or by anti-sense oligonucleotide against ICAM-1 ameliorates experimental colitis (Bennett, Kornbrust et al. 1997; Hamamoto, Maemura et al. 1999; Kato, Hokari et al. 2000). To our knowledge this is the first demonstration of a critical role for ICAM-1 adhesion pathway in eosinophilic accumulation in the colon during experimental UC. These findings are particularly important, given that activated eosinophils and eosinophil-derived granular proteins have been linked to the pathogenesis of UC in humans and that therapeutic approaches targeting ICAM-1 such as anti-sense ICAM-1 oligonucleotide (ISIS-2302) therapy are being examined for the treatment of IBD (Yacyshyn, Bowen-Yacyshyn et al. 1998; Yacyshyn, Barish et al. 2002; Yacyshyn, Chey et al. 2002).

In conclusion, we have demonstrated that eotaxin-1 mediated eosinophil recruitment into the colon occurs via an ICAM-1 dependent pathway. Furthermore, we demonstrate a pathogenic role for eosinophils and ICAM-1-mediated adhesion pathways in the development of experimental eosinophilic colonic injury. Our investigations further confirm the importance of eosinophils in gastrointestinal diseases and suggest that antagonism of ICAM-1/eosinophil pathways may be significant therapeutic approach for the treatment of UC and allergic colitis.
Chapter Four

The role of IL-9 in the development of oral antigen-induced intestinal anaphylaxis
4.1 Introduction

GI allergic diseases represent a vast spectrum of inflammatory diseases, which are increasing in both incidence and severity (Rothenberg, Mishra et al. 2001; Kweon and Kiyono 2003; Sampson 2003). To date, the aetiology of food allergy is not fully understood. Several animal models have been developed that present with manifestations comparable to those seen in clinical settings. These involve co-administration of protein antigen with adjuvants to sensitise mice followed by gastric antigen challenge (Layton, Stanworth et al. 1986; Snider, Marshall et al. 1994; Maloy, Donachie et al. 1995; Li, Schofield et al. 1999; Kweon, Yamamoto et al. 2000; Brandt, Strait et al. 2003). Each of these experimental models highlights the importance of Th₂-driven immunopathogenesis. Correspondingly, clinical studies have shown antigen-primed T cells in patients with food allergy (Eigenmann 2002). Investigators have also examined food antigen induced cytokine production in T cells and show T helper cells from allergic patients, secret IL-4, IL-5 and IL-13 (Higgins, Lamb et al. 1995; de Jong, Spanhaak et al. 1996; Eigenmann, Huang et al. 1996; Katsuki, Shimojo et al. 1996; Eigenmann 2002; Turcanu, Maleki et al. 2003).

IL-9 is a mouse T cell derived cytokine, involved in growth of mucosal mast cells suggesting a role in the regulation of type I hypersensitivity reactions (Petit-Frere, Dugas et al. 1993). IL-9 has been shown to inhibit lymphokine production from IFN-γ producing CD4⁺ T cells and promote proliferation of CD8⁺ T cells (Soussi-Gounni, Kontolemos et al. 2001). In addition, IL-9 increases production of IgE by B cells, orchestrates chemokine and mucous secretion by bronchial epithelial cells, and leads to mast cell proliferation (Hultner, Druez et al. 1990; Petit-Frere, Dugas et al. 1993; Dong, Louahed et al. 1999; Louahed, Toda et al. 2000; Soussi-Gounni, Kontolemos et al. 2001). Moreover, linkage studies have demonstrated a role of the IL9 gene locus with features of atopic disease including elevated serum IgE and airways hyperresponsiveness (Postma, Bleecker et al. 1995; Doull, Lawrence et al. 1996; Ulbrecht, Eisenhut et al. 1997). Generation of IL-9 deficient (IL-9⁻) mice and employing a model of pulmonary granuloma formation involving infection with Schistosoma mansoni, and transgenic mice overexpressing IL-9 in the lung, show a direct role for IL-9 in the development complex Th₂ inflammatory responses, which
specifically involve the induction of mastocytosis. Taken together, the data suggests IL-9 has a strong influence on the inflammatory pathways involved in allergic disease.

With an interest in determining the specific cells and mediators responsible for clinical manifestations in GI allergic disorders, a murine model of oral allergen-induced intestinal inflammation that was accompanied by strong Th2 humoral and cellular responses was developed (Brandt, Strait et al. 2003). These responses were associated with the immunopathogenesis of allergic diarrhoea (Brandt, Strait et al. 2003). Exposure of OVA/alum sensitised mice to repeated doses of intragastric OVA induced acute intestinal anaphylaxis that was associated with diarrhoea, increased intestinal permeability, eosinophilia and mastocytosis (Brandt, Strait et al. 2003). Depleting mast cells using an anti-c-kit antibody, intestinal anaphylaxis was abolished in association with a significant decrease in intestinal permeability. Using an antibody directed against IgE and mice deficient in the IgE receptor (FceRI) they implicated that intestinal anaphylaxis was shown to be IgE mediated. Thus, these results demonstrated that oral allergen-induced diarrhoea associated with experimental Th2 intestinal inflammation is mast cell and IgE dependent (Brandt, Strait et al. 2003).

With the demonstration of the importance of Th2 inflammation, involving a mast cell dependent mechanism in oral antigen-induced intestinal anaphylaxis, we hypothesised a central role for IL-9 in the development of oral antigen-induced intestinal anaphylaxis. In this chapter we employed IL-9 deficient (IL-9−/−) mice to determine the role of the Th2 cytokine, IL-9, and its function in the development of experimental oral allergen-induced intestinal anaphylaxis.
4.2 Materials and Methods

4.2.1 Mice

All mice were maintained in a barrier facility, and animals were handled under IACUC-approved protocols. There was no serologic evidence of specific pathogens and no evidence of bacterial or other infection in sentinel mice maintained with the colony. No serological evidence of viral infection or histological evidence of bacterial infection was detected in transgenic mice at autopsy. BALB/c mice were obtained from the National Cancer Institute (Bethesda, Maryland, USA) and IL-9-deficient mice (BALB/c background) were a gift from Andrew McKenzie (Medical Research Council, Laboratory of Molecular Biology, Cambridge, England); all mice were housed according to institutional guidelines. Water was food was provided ad libitum. Experiments were performed on age- and gender-matched mice (6–8 weeks old). IL-9^−/− mice were identified using following amplification primers and reaction conditions:

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<tr>
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<tr>
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<td>95°C: 0'15&quot;</td>
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<td>60°C: 0'15&quot;</td>
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WT ~ 560bp
KO ~ 1.8kb
ladder: LH3

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<tr>
<td>IL9KOR</td>
<td>TccTcTcTcATTTgcTTgATgTg</td>
</tr>
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4.2.2 Protocol

Mice were sensitised twice, 2 weeks apart, with sterile saline or 50 μg of OVA (grade V, A-5503; Sigma-Aldrich, St. Louis, Missouri, USA) in the presence of 1 mg of aluminium potassium sulfate adjuvant [alum: AlK(SO₄)₂-12H₂O] (A-7210; Sigma-
Aldrich) by intraperitoneal injection (i.p.). Two weeks later, mice were held in the supine position three times a week (every other day) and orally administered 250 µl of sterile saline that contained 50 mg of OVA. Before each intragastric (i.g.) challenge, mice were deprived of food for 3–4 hours with the aim of limiting antigen degradation in the stomach. Challenges were performed with i.g. feeding needles (01-290-2B; Fisher Scientific Co., Pittsburgh, Pennsylvania, USA). Diarrhoea was assessed by visually monitoring mice for up to 1 hour following i.g. challenge. Mice demonstrating profuse liquid stool were recorded as diarrhoea-positive animals.

4.2.3 Ribonuclease protection assay.

Jejunal RNA was obtained using Trizol reagent (Life Technologies Inc., Grand Island, New York, USA) following the manufacturer’s protocol. The ribonuclease protection assay (RPA) was performed by making a radioactive probe from the mCk-1b multiprobe template (Riboquant multi-probe RPA system; BD Biosciences PharMingen, San Diego, California, USA). RNA from OVA- and saline-challenged BALB/c WT mice was then hybridized overnight with the radioactive probe, purified, and finally separated on an urea-acrylamide gel at 75 W as described in the Riboquant protocol from BD Biosciences PharMingen. RPA analysis was performed by Dr. Eric Brandt, Allergy and Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati OH, USA.

4.2.4 Quantitative PCR.

BALB/c WT mice were obtained and the above protocol administered. Mice were sacrificed and intestinal samples were harvested. RNA was isolated from intestinal samples and cDNA was generated by standard procedures. The RNA samples (500 ng) were subjected to reverse transcription analysis using Iscript reverse transcriptase according to manufacturer’s instructions (170-8890, Bio-Rad Laboratories, Hercules, CA, USA). GAPDH and IL-9 were quantified by real-time PCR using the LightCycler instrument and iQ SYBR Green I as a ready-to-use reaction mix (170-8880, Bio-Rad Laboratories, Hercules, CA, USA). Results were then normalised to GAPDH amplified from the same cDNA mix and expressed as fold induction compared with the saline treated controls. cDNAs were amplified using the following amplification primers and reaction conditions:
A standard curve was generated using cDNA samples expressing the target gene. Samples were amplified by PCR and gel purified on a 1% agarose gel. The 114 bp fragment was cut from the gel and RNA isolated using QIAquick gel extraction kit following manufacturer’s instructions (28704, Qiagen Sciences, Maryland, USA). Sample was designated with a concentration of the arbitrary unit 1. The sample was then diluted 1:100 and an RT-PCR standard curve was generated with serial 1:10 dilutions thereafter and as such, the standard curve begins at 1x10^{-3}. RT-PCR mRNA expression analysis was performed by Richard Ahrens in Simon Hogan’s Laboratory, Allergy and Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati OH, USA.

4.2.5 ELISA measurements.

mMCP-1 serum levels were measured by ELISA according to the manufacturer’s instructions (Moredun Scientific, Midlothian, United Kingdom). ELISA determined OVA-specific IgE. Briefly, plates were coated for 2 hours with 100 µl of anti-IgE Ab (EM-95; 10 µg/ml, BD Pharmingen), blocked with 200 µl of 10% Foetal Bovine Serum (FBS; diluted in PBS) (26140-079; Gibco, Grand Island, NY, USA) before adding serial dilutions of plasma samples (100 µl/well). After overnight incubation, plates were washed, and biotinylated OVA was added (1:1000 dilution of 2.5 mg/ml, 100 µl/well). After one hour of incubation, streptavidin-HRP (1:1000 dilution) (SNN1004; 1mg/ml, Biosource, Camarillo, CA, USA) was added. Prior to the initiation of each step, plates were washed with 0.05% Tween-20 in PBS. Finally, after one-hour incubation, 100 µl of substrate (TMB Substrate Reagent Set, 55214; BD OptEIA, San Diego, CA, USA) was added. Colourimetric reaction was stopped with 50µl of 1M H₂SO₄ and was quantified by measuring optical density with an ELISA plate reader at 450 nm. OVA-specific IgG₁ was measured after coating the wells with OVA (100 µg/ml). Blocking was done with 10% FBS in PBS, and all washes were performed with 0.05% Tween-20 in PBS. Plasma samples were diluted 1:100 for IgG₁ and serially diluted 1:10 thereafter. After 2 hours of incubation, plates were washed and HRP-conjugated anti-mouse IgG₁.
(1:1000) (X56; 0.5mg/ml, BD Biosciences-PharMingen) or biotin-conjugated rat anti-mouse IgG1 (A85-1; BD Biosciences PharMingen) followed by streptavidin, HRP conjugate (1:1000) (SNN1004; 1mg/ml, Biosource, Camarillo, CA, USA). Following a one hour incubation, plates were subsequently washed and 100 µl of substrate (TMB Substrate Reagent Set, 55214; BD OptEIA, San Diego, CA, USA) was added. Colourimetric reaction was stopped with 1M H2SO4 and was quantified by measuring optical density with an ELISA plate reader at 450 nm. Blood was collected from mice and allowed to clot and centrifuged at 5000g for 10 minutes at room temperature. Serum was aliquoted and stored at -20°C until analysis.

4.2.6 Intestinal mast cell quantification

Jejunum tissue was collected 10–12 cm distal to the stomach, while ileum and colon samples were collected 1 cm proximal or distal of the cecum, respectively. All samples were fixed in 10% formalin and processed by standard histological techniques. The 5-μm tissue sections were also stained for mucosal mast cells with chloroacetate esterase activity as described elsewhere (Brandt, Strait et al. 2003) and lightly counterstained with hematoxylin. At least four random sections per mouse were analyzed. Quantification of stained cells was performed by counting the number of chloroacetate positive cells from 25-50 fields of view (magnification 40x).

4.2.7 Activated CD4+ Th2 response

Lymphocytes from mesenteric lymph nodes were subjected to activation via 100µg/ml OVA, with αCD3/αCD28 stimulation was used as a positive control, as previously described (Hogan, Foster et al. 1998). In brief, 5x10^5 lymphocytes per milliliter were cultured with αCD3 (5 μg/mL) and αCD28 (1μg/mL) for 96 hours. IL-4, IL-5, IL-13 and interferon (IFN)-γ levels were determined in supernatants from stimulated lymphocyte homogenates by using the OptEIA Mouse IL-4, IL-5 and IFN-γ kits (Pharmingen). IL-13 levels were measured using rat anti-mouse IL-13 mAb (R&D Systems; clone 38213.11 720µg/ml, 1:180 dilution) and biotinylated rat anti-mouse IL-13 mAb (R&D Systems; 36µg/ml, 1:180 dilution). Data was normalized to cytokine levels from lymphocyte culture with no stimulus i.e. no OVA or αCD3/αCD28 stimulation.
4.2.8 Proliferation Assay

In brief, 5x10^5 lymphocytes per milliliter were cultured with 0, 1, 10 or 100 μg/mL OVA or αCD3 (5μg/mL) and αCD28 (1μg/mL) in 96-well round-bottomed plates for 96 hours. Six hours before completion of the assay, 1μCi [³H]-Thymidine was added to each well and incubated in 5% CO₂ at 37°C for a further 6 hours. After the incubation each well and supernatants were frozen at -70°C to fractionate cells and release [³H]-Thymidine. [³H]-Thymidine was obtained from MP biochemicals (Solon, OH, USA). Microscint 20 was obtained from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA).

4.2.9 Mononuclear cell preparation and MCp assessment

Mice were killed by CO₂ asphyxiation and the entire jejunum was harvested. Individual jejunum lengths from 2 mice were pooled, placed in 20 mL RPMI 1640 complete (RPMI 1640 containing 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% heat-inactivated foetal calf serum [Sigma-Aldrich no. F2442, St. Louis, MO]), and processed essentially as previously described (Gurish, Tao et al. 2001; Abonia, Austen et al. 2005). Briefly, the jejunum samples (flushed out and rinsed twice in HBSS) were finely chopped with a scalpel blade and transferred separately to 50 mL plastic tubes with 30 mL RPMI 1640 complete plus 1 mg/mL collagenase Type 4 (Worthington, Lakewood, NJ). There were 3 enzymatic digestions carried out for approximately 20 minutes each at 37°C. The undigested tissue clumps were collected after each digestion period and were subjected to another enzymatic digestion, while the liberated cells were pelleted, resuspended in 44% Percoll (Sigma-Aldrich no. P1644, St Louis, MO), overlayed on a 67% Percoll layer, and spun at 400g for 20 minutes at 4°C. The mononuclear cells (MNCs) were harvested from the interfaces of the 3 digestions of the jejunum, pooled by separate tissue source, and washed in RPMI 1640 complete. The numbers of viable cells were determined by trypan blue dye exclusion with a hemocytometer. Cells were serially diluted in RPMI 1640 complete, and 100-μL samples of the MNC dilutions were added to each well of standard 96-well flat-bottomed microtitre plates (Corning no. 3596, Corning, NY). Typically, 24 wells were plated for each cell concentration. Jejunum MNCs were plated starting at 5000 to 10 000 cells/well. Then, each well received 100 μL gamma-irradiated (30 Gy) splenic feeder cells plus cytokines.
(recombinant mouse interleukin-3 [IL-3] at 20 ng/mL and recombinant mouse stem cell factor [SCF] at 100 ng/mL). The cultures were incubated in humidified 37°C incubators with 5% CO₂ for 12 to 14 days, and positive wells containing mast cell colonies were identified and counted with an inverted microscope. The mast cell (MC) colonies were easily distinguished as large colonies of nonadherent small- to medium sized cells (Crapper and Schrader 1983; Guy-Grand, Dy et al. 1984). Mast cell progenitor (MCp) concentration expressed as the number of MCps per 10⁶ MNCs isolated from the tissue. Multiplying the concentration of MCps by the MNC yield/organ derives the number of MCps/tissue. Mast cell progenitor assay performed by H. Faith Rainey of Dr. J. Pablo Abonia’s laboratory, Allergy and Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati OH, USA.

4.2.10 Statistical Analysis

Data are expressed as mean ± standard error (SEM). Statistical significance comparing different sets of mice was determined by Student’s t-test. In experiments comparing multiple experimental groups, statistical differences between groups were analyzed using the one-way ANOVA nonparametric Bonferroni post-test. P < 0.05 was considered significant. All analyses were performed using Prism 4.0 software.
4.3 Results

4.3.1 Elevation of mRNA IL-9 expression during oral antigen-induced intestinal anaphylaxis.

To examine whether the expression of IL-9 is elevated during experimental oral antigen-induced intestinal anaphylaxis, we performed RPA analysis and quantitated IL-9 mRNA expression levels in OVA-sensitised mice receiving repeated doses of i.g. saline (SAL), or OVA. Homeostatic levels of IL-9 mRNA expression, at baseline, were undetectable (Figure 4.1. a.). In contrast, OVA-sensitised and subsequently OVA challenged mice had significantly increased levels of IL-9 as compared to control challenged, OVA-sensitised mice (Figure 4.1. b.; p < 0.01). To examine the temporal expression of IL-9 mRNA in the jejunum following oral OVA-challenge, we performed quantitative PCR analysis on jejunum mRNA from OVA-sensitised mice that had received one or three OVA-challenges. We demonstrate increased levels of IL-9 mRNA in jejunum of mice following one (~100-fold compared to Day 0 baseline) and following three oral antigen challenges (Figure 4.1. c.). These studies demonstrate that oral antigen challenge of OVA-sensitised mice induces intestinal IL-9 mRNA expression.

4.3.2 Elucidation of the role of IL-9 in oral antigen-induced intestinal anaphylaxis.

To elucidate the importance of IL-9 in oral antigen-induced intestinal anaphylaxis we employed IL-9 deficient mice (IL-9−/− BALB/c) and WT (BALB/c) mice. Repeated i.g. challenges of OVA to OVA-sensitised WT mice induced diarrhoea (Figure 4.2.). WT mice began developing diarrhoea acutely after the third intragastric challenge with greater than 75% of the mice suffering from diarrhoea following the 7th challenge. Notably, intestinal mast cell and serum mMCP-1 levels were also significantly elevated as compared to vehicle (saline) challenged OVA-sensitised WT mice (Figure 4.2. b and c.). In contrast, oral antigen-induced intestinal anaphylaxis was attenuated in IL-9−/− mice. Typically, in any one experiment, 1/6 OVA-challenged OVA-sensitised IL-9−/− mice would have evidence of diarrhoea 45-60 minutes following i.g. OVA-challenge. The number of IL-9−/− mice with diarrhoea never achieved greater than 25% following 9 i.g. OVA-challenges (Figure 4.2. a.). Consistent with the attenuation in intestinal
Figure 4.1 Intestinal expression of IL-9 is elevated during oral antigen-induced intestinal anaphylaxis.

(a) RPA analysis of IL-9 mRNA expression in the jejunum of i.p. OVA-sensitised BALB/c WT mice following nine i.g. saline or OVA challenges, (b) quantification of IL-9 mRNA expression (blot (a)) by densitometry analysis of i.p. OVA-sensitised BALB/c WT mice following nine i.g. saline or OVA challenges and (c) quantitative PCR analysis of IL-9 mRNA expression in the jejunum of i.p. OVA sensitised BALB/c WT mice with 1 or 3 i.g. OVA challenges compared to saline sensitised, saline challenged BALB/c WT control. (a) The housekeeping gene L32 was used as a control for RNA loading. (b) IL-9 mRNA was quantitated by Densitometry analysis using the NIH Image 6.1 Software and standardized to the housekeeping gene L32. The black line represents the mean value of each group. (c) IL-9 mRNA expression was normalized to GADPH expression in each individual sample. Results expressed as IL-9/GADPH ratio fold change over saline. The black line represents the mean value of each group.
anaphylaxis, intestinal mast cell and serum mMCP-1 levels in oral antigen-challenged, OVA-sensitised IL-9^{−/−} mice were significantly reduced as compared to WT mice (Figure 4.2 b, c, and d - g.).

4.3.3 Evaluation of the OVA-CD4^{+} Th2 response.

To determine whether the attenuation of diarrhoea in IL-9^{−/−} mice was due to the lack of a OVA-CD4^{+} Th2 response, we examined draining lymph node cytokine production in WT and IL-9^{−/−} mice. We show an increase in cytokine production from IL-9^{−/−} and WT mesenteric lymph node (MLN) lymphocytes cultured and re-stimulated with 100μg/ml OVA over MLN lymphocytes in media alone. This effect is further amplified in cultures with the presence of αCD3/αCD28 activation. We show the level of CD4^{+} Th2 cytokines (IL-4, IL-5 and IL-13) in IL-9^{−/−} mice was equivalent to that observed in WT mice (Table 4.1.). Furthermore, we demonstrate supernatants cultured in the presence of OVA have an attenuated IFN-γ response as compared to the positive control (Table 4.1.). This suggests these T cells in the presence of the initial antigenic stimulus involves a predominantly Th2 immune response. To further confirm the ablation of oral antigen-induced intestinal anaphylaxis in IL-9^{−/−} deficient mice is not due to defects in Th2 immunity, we assessed the levels of OVA-specific IgG1 and IgE. We demonstrate the levels of OVA-specific IgG1 and IgE in saline treated or OVA-sensitised and subsequently OVA-challenged IL-9^{−/−} mice was equivalent to that in saline treated or OVA-sensitised and subsequently OVA-challenged WT mice (Figure 4.3.).

4.3.4 Effect of IL-9 deficiency on mature mast cells.

We were next interested in examining basal intestinal mast cell levels in WT and IL-9^{−/−} mice. We show that the level of intestinal mast cells in IL-9^{−/−} mice was decreased by 2-fold as compared to WT mice (Figure 4.4.). Importantly, IL-9^{−/−} mice had very low levels of intestinal mature mast cells, leading us to examine whether the observed reduction in basal mast cells numbers in IL-9^{−/−} mice was due to decreased mast cell progenitor recruitment into the intestine or due to a defect in intestinal mast cell progenitor maturation. We quantified mast cell progenitor levels in the intestine of WT and IL-9^{−/−} mice. We show that the level of mast cell progenitors in the intestine of IL-9^{−/−} mice under basal conditions was equivalent to that of WT mice (Figure 4.5.).
Figure 4.2 Oral antigen-induced intestinal anaphylaxis is attenuated in IL-9 deficient mice.

(a) Diarrhoea occurrence, (b) mean number of mast cells per high power field (hpf), (c) serum mouse mast cell protease-1 in saline or OVA i.p. sensitised and subsequently i.g. OVA-challenged BALB/c WT and IL-9^{−/−} mice and (d - g) photomicrograph of chloroacetate esterase-stained jejunal sections from saline or OVA i.p. sensitised and subsequently i.g. OVA-challenged BALB/c WT (e and f) and IL-9^{−/−} (g and h) mice. (a) Data represented as percentage of diarrhoea occurrence over number of OVA challenges. (b and c) Data represented as mean ± SEM; 4-5 mice per group from n=3 experiments. (d - g) Photomicrograph 10x magnification; insert 40x magnification. SAL/OVA represents saline sensitised i.g. OVA challenged mice and OVA/OVA represents OVA sensitised i.g. OVA challenged mice.
Chapter Four: The role of IL-9 in the development of oral antigen-induced intestinal anaphylaxis

(a) Diarrhoea Occurrence (%) vs. Number of Allergen Challenges

(b) Mean Mast Cells/HPF

(c) mMCP-1 (ng/ml) sensitisation (i.p)

(d) SAL/OVA IL-9\(^{-/-}\)

(e) SAL/OVA WT

(f) OVA/OVA IL-9\(^{-/-}\)

(g) OVA/OVA WT

(i.g) sensitisation (i.p)
Table 4.1 CD4+ cytokine productions in IL-9 deficient mice is comparable to BALB/c WT mice.

IL-4, IL-5, IL-13 and IFN-γ protein in supernatants from cultured 1.5x10^6 mesenteric lymphocytes of i.p. OVA-sensitised and subsequently i.g. OVA-challenged BALB/c WT and IL-9+ mice. Data represented as mean ± SEM; 6 mice per group from n=3 experiments. Data normalised to lymphocytes grown in the same conditions in the absence of OVA or αCD3 / αCD28 stimulation.
Table 4.1 MLN lymphocyte cytokine production from OVA-sensitised OVA-challenged IL-9^{-/-} and BALB/c WT mice.

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<td>BALB/c WT</td>
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<td>99.07 ± 23.75</td>
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1.5x10^6 cells/ml were plated in 24 well tissue culture plates and incubated with either media alone, 100μg/ml OVA or αCD3 (5μg/ml)/αCD28 (1μg/ml) and cultured for 72 hours. ELISA measured supernatant cytokine levels. Cytokine levels were normalised by removing background levels from cell cultures with media alone. *p<0.05 compared to IFN-γ 100μg/ml OVA culture.
Figure 4.3 Th2 immune response in IL-9 deficient mice is comparable to BALB/c WT mice.

(a) Serum OVA-specific IgE and (b) IgG1 in saline or OVA i.p. sensitised and subsequently i.g. OVA-challenged BALB/c WT and IL-9−/− mice. (a and b) Data represented as mean ± SEM; 4-5 mice per group from n=3 experiments.
Chapter Four: The role of IL-9 in the development of oral antigen-induced intestinal anaphylaxis

(a) 

![Graph showing OD 450 Serum OVA-specific IgE (1:100) for IL-9^-/- and BALB/c WT mice after oral sensitisation (i.p.).](image)

(b) 

![Graph showing OD 450 Serum OVA-specific IgG1 (1:100) for IL-9^-/- and BALB/c WT mice after oral sensitisation (i.p.).](image)
Figure 4.4 IL-9 plays a role in intestinal mast cell levels.

(a) Mean number of mast cells per high power field (hpf), (b and c) photomicrograph of chloroacetate esterase-stained jejunal sections from BALB/c WT (b) and IL-9⁻/⁻ (c) mice.
Chapter Four: The role of IL-9 in the development of oral antigen-induced intestinal anaphylaxis

(a) IL-9\(^{-}\) - BALB/c WT

(b) BALB/c WT

(c) IL-9\(^{+}\)
Figure 4.5 Homeostatic maintenance of intestinal mast cell progenitor numbers occurs independently of IL-9.

Intestinal mast cell progenitor numbers in IL-9\(^{-}\) and BALB/c WT mice. Data represented as mean + SEM; 4-5 mice per group from n=4 experiments.
Chapter Four: The role of IL-9 in the development of oral antigen-induced intestinal anaphylaxis

![Graph showing MCP1 levels in jejunum comparing IL-9-/- and BALB/c WT mice.](image)
4.4 Discussion

By employing an experimental model of oral antigen-induced intestinal anaphylaxis we demonstrate a non-redundant role for IL-9 in the induction of the effector phase of disease. The observed comparable levels of antigen-specific IgG, IgE and Th2 cytokines in IL-9\(^{-/-}\) and WT mice confirms antigen sensitisation and suggests that the reduction of the intestinal anaphylaxis phenotype in IL-9\(^{-/-}\) mice is not due to a defect in Th2 immunopathogenesis. The attenuation of oral antigen-induced intestinal anaphylaxis in IL-9\(^{-/-}\) mice was associated with a reduction in intestinal mast cell levels, a critical factor in the effector phase of intestinal anaphylaxis (Brandt, Strait et al. 2003). Importantly, we show no difference in intestinal mast cell progenitor levels in IL-9\(^{-/-}\) compared to WT mice. Previous studies have demonstrated IL-9 is a potent enhancer for SCF-dependent growth of mast cell progenitors, however, IL-9 could not enhance SCF-dependent generation of progeny or survival of mature mast cells (Matsuzawa, Sakashita et al. 2003). Taken together, previous findings support our data suggesting IL-9 may promote intestinal mastocytosis via intestinal mast cell maturation that is independent of mast cell progenitor generation.

The demonstration that IL-9 is of importance in the inflammatory processes of asthma has been highlighted in several studies (Postma, Bleecker et al. 1995; Doull, Lawrence et al. 1996; Ulbrecht, Eisenhut et al. 1997). However, whilst there is significant amount of clinical and experimental data defining the roles of IL-4, -5 and -13 in the regulation of discrete aspects of the allergic intestinal immune reaction little is known about the contribution of IL-9. We demonstrate that experimental oral antigen-induced intestinal anaphylaxis is associated with increased intestinal IL-9 expression. T cells, mast cells and eosinophils have been shown to produce IL-9 (Soussi-Gounni, Kontolemos et al. 2001) and our data suggests - based on the acute kinetics and maintenance of expression (within 24 hours of oral antigen challenge and this level of expression is comparable to that seen after challenge 3) - that the increased IL-9 mRNA expression is T cell derived. This is the first demonstration of increased IL-9 in a model of allergic diarrhoea, so we were interested in elucidating the role of this cytokine further. We chose to employ IL-9 deficient animals in this model and examine their response to specific parameters (diarrhoea occurrence, mastocytosis, serum mMCP-1, antigen-specific Ig and cytokine responses).
Previous studies employing IL-9" mice have demonstrated that IL-9 is a requirement for rapid and robust generation of pulmonary mastocytosis during lung challenge with *Schistosoma mansoni* eggs (Townsend 2000). Furthermore, lung selective expression of IL-9 induced increased mast cell numbers within the airway epithelium, lung inflammation and increased airway hyperresponsiveness (Temann, Geba et al. 1998). However, it has been demonstrated in an allergic airways disease model that IL-9 is not required for a robust Th2 response to allergen in sensitised mice (McMillan, Bishop et al. 2002). We demonstrate that IL-9 deficiency was associated with inhibition of oral antigen-induced intestinal anaphylaxis, and this was associated with a significant reduction in intestinal mastocytosis and serum mMCP-1 levels. Notably, antigen-specific IgG1, IgE and Th2 cytokine responses are not impaired in IL-9" mice, and in the absence of this factor comparable levels of Ig and cytokines were observed after OVA sensitisation and subsequent OVA challenge as compared to BALB/c WT counterparts. The implicit differences seen in the experimental oral antigen-induced intestinal anaphylaxis model and experimental allergic airways disease can be explained by variances in the critical factors for development of the effector phase of immunopathogenesis of these diseases, McMillan et al show that IL-9 is not obligatory for the development of eosinophilia and airways hyperresponsiveness (McMillan, Bishop et al. 2002), a late phase Th2 reaction, whereas experimental oral antigen-induced intestinal anaphylaxis is a mast cell dependent (Brandt, Strait et al. 2003), early phase acute reaction.

IL-9 is a pleiotropic cytokine with diverse effects on cells involved in allergic inflammation including functioning as a T cell growth factor, having the ability to increase IgE production by B cells, an eosinophil survival factor and as having mast cell growth enhancing activity (Donahue, Yang et al. 1990; Hultner, Druez et al. 1990; Moeller, Hultner et al. 1990; Renauld, Goethals et al. 1990; Dugas, Renauld et al. 1993; Renauld, van der Lugt et al. 1994; Renauld, Kermouni et al. 1995; Renauld, Vink et al. 1995; Vink, Warnier et al. 1999; Hauber, Bergeron et al. 2004; Knoops and Renauld 2004). However, our studies failed to show a significant role for IL-9 in the generation of antigen-driven antibody responses.

Despite IL-9 having been shown to act as a T cell growth factor (Renauld, Goethals et al. 1990) and the development of thymic lymphomas in mice that constitutively
overexpress IL-9 (generated using a fusion gene expressing in all tissues, consisting of an IL-9 genomic fragment linked to the promoter of the murine pim-1 gene, including the TATA box and the cap site, followed by two copies of the Eμ enhancer and one copy of the moloney murine leukemia virus long terminal repeat) (Godfraind, Louahed et al. 1998) the T cell responses (IL-4, -5, 13 and IFN-γ) were unimpaired in IL-9" mice. This suggests that in the absence of IL-9 alternate pathways must function to regulate T cell development and this has also been supported by other studies employing IL-9" mice (Townsend 2000).

Previously it has been shown that mice overexpressing IL-9 have marked mast cell infiltration of intestinal tissues (Godfraind, Louahed et al. 1998). We were next interested in assessing the mast cell development and levels in the small intestine in IL-9 deficient mice under homeostatic conditions. Interestingly, basal levels of mast cells in the small intestine of mice deficient in IL-9 are decreased by two-fold in comparison to WT mice. This led us to examine the levels of mast cell progenitors in the small intestine of IL-9 deficient mice. We performed a progenitor assay assessing the numbers of mast cell precursors in the small intestine and found that the levels of mast cell progenitors in the small intestine of IL-9" mice was comparable to that of WT mice. This indicates the deficiency in mast cell numbers in the small intestine of IL-9" mice is not due to a defect in the generation of mast cell progenitor cells but instead mostly likely presents a role for IL-9 in the proliferation and/or differentiation of mast cells.

These studies support previous findings of associations of IL-9 and allergic inflammation. The attenuation in the development of oral antigen induced intestinal anaphylaxis is mediated by an ablation of mature mast cell numbers in IL-9" mice. This results in the subsequent amelioration of the biomediators released from jejunal resident mast cells during degranulation via antigen cross-linking of IgE on the surface, inhibiting immunopathogenesis. Moreover, this protection is elicited even in the presence of antigen-specific IgG1, IgE and the generation of a Th2 milieu (IL-4, -5 and -13) as compared to intestinal anaphylaxis in OVA-sensitised, OVA-challenged WT mice. Further studies will attempt to address the specific role of IL-9 in the development of antigen sensitisation in oral antigen-induced intestinal anaphylaxis and these studies provide the basis to probe the involvement of IL-9 in the
immunopathogenesis of disease.
Chapter Five

The role of IL-9 in intestinal anaphylaxis, employing a transgenic approach
5.1 Introduction

It is proposed that numerous inflammatory mediators, including Th2-cytokines, regulate the orchestration of the intestinal anaphylactic immune reaction. Recently, there has been significant attention focused on the Th2-cytokine, IL-9 and its role in allergic inflammatory processes (Hauber, Bergeron et al. 2004). In chapter 4 it was demonstrated that IL-9 expression is increased during oral antigen-induced intestinal anaphylaxis. Furthermore, it was shown that the induction of oral antigen-induced intestinal anaphylaxis is dependent on IL-9. Collectively, this led to the hypothesis that overexpression of IL-9 in the small intestine would promote oral antigen-induced intestinal anaphylaxis.

Several transgenic systems have been employed to generate mice overexpressing IL-9, including systemic, lung-specific and inducible lung-specific overexpression (Renauld, van der Lugt et al. 1994; Godfraind, Louahed et al. 1998; Temann, Geba et al. 1998; Temann, Ray et al. 2002). Overexpression of IL-9 systemically, (Tg5 mice; utilising the murine pim-1 promoter containing a Eμ enhancer and a terminal repeat of the Moloney murine leukaemia virus) (Renauld, van der Lugt et al. 1994; Godfraind, Louahed et al. 1998) induced mastocytosis in the intestinal tract and the upper airways. This was characterised by an intraepithelial localization in the small intestine and upper airways, and a mixed mast cell phenotype: sharing mucosal mast cell (MMC) and connective tissue mast cell (CTMC) properties (Godfraind, Louahed et al. 1998). Interestingly, this phenotype is similar that of mast cells cultured in vitro in the presence of stem cell factor (SCF) and IL-9 (Gurish, Ghildyal et al. 1992; Eklund, Ghildyal et al. 1993).

In an attempt to further delineate the role of IL-9 in asthma, following the implication of IL-9 in atopic pathology, investigators generated mice that specifically overexpress IL-9 in the lung. This was done by utilizing the lung specific promoter, CC10, and by a CC10-IL9-SV40 transgene (Temann, Geba et al. 1998). Constitutive expression of IL-9 in the airway epithelium of these mice resulted in airway inflammation, mast cell hyperplasia, and dramatically increased airway hyperresponsiveness (Temann, Geba et al. 1998). Generation of doxycycline-inducible, lung-specific IL-9 transgenic mice to examine the contribution of IL-9 to lung inflammation further confirmed these findings: doxycycline-treated lung-specific IL-9 transgenic mice develop pathological changes of the lungs similar to those of the constitutive IL-9 transgenic as described above.
To dissect the individual contribution of IL-9 to other discreet aspects of the pathogenesis involved in oral antigen-induced intestinal anaphylaxis, we also took a transgenic approach, utilising the intestine specific promoter of the rat fatty acid binding protein (Fabpi) gene, that has been extensively used to direct the expression of gene transcripts, specifically in the enterocytes of the small intestine (Sweetser, Birkenmeier et al. 1988; Cohn, Simon et al. 1992; Mishra, Hogan et al. 2002).
Chapter Five: The role of IL-9 in intestinal anaphylaxis, employing a transgenic approach

5.2 Materials and Methods

5.2.1 Reagents

Cromolyn Na (Sigma Aldrich, St. Louis MO, USA) was dissolved in sterile PBS and administered at 0.3mg/200µl via intraperitoneal injection. Biotin-αIL-4 mAb and biotin-αIFN-γ mAb were dissolved in PBS and administered at 10µg/200µl via intravenous injection. Evan’s blue dye (Sigma Aldrich, St. Louis MO, USA) was dissolved in PBS and administered via intravenous injection at a concentration of 0.5 mg/200µl.

5.2.2 Generation of Fabpi IL-9 Transgenic Mice

The IL-9 cDNA was a gift from Andrew McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom) and was amplified by PCR employing oligonucleotides containing BamHI sites (5’-ggatccatgttggtgacatacatccttgc and 3’-ggatcctcatggtcggcttttctgcc). The 446-bp fragment containing the entire coding region of the murine IL-9 cDNA was ligated into the pCR2.1 TOPO TA cloning vector. The IL-9 cDNA was resected from the cloning vector with BamHI, gel purified and ligated into the BamHI site of the PBSIF1178-hGHpgkNeo plasmid (Sweetser, Birkenmeier et al. 1988; Cohn, Simon et al. 1992; Mishra, Hogan et al. 2002), which contained a 3.5-kb EcoRI fragment containing nucleotides −1178 to +28 of the rat Fabpi promoter linked to nucleotides +3 to +2150 of human growth hormone (hGH) gene (except for its 5’ regulatory sequences) as represented by Fig. 4.1. a. The transgene plasmid was propagated in Escherichia coli DH5α cells, and the transgene fragment was liberated by EcoRI endonuclease digestion and purified using the QIAEX DNA extraction kit after separation from the vector by gel electrophoresis (Qiagen Inc., Chatsworth, CA). After extensive dialysis, 5µg of the linearised fragment was electroporated with 5µg of circular neomycin resistance plasmid (pMC1Neo, Stratagene, La Jolla, CA, USA) into BALB/c embryonic stem (ES) cells, a generous gift of Dr. Birgit Ledermann (University of Zurich). Positive selection was performed with G418 for 10 days and seven surviving clones were screened for integration of the transgene by PCR. Of three Tg-positive ES cells one was injected into 3.5-day-old blastocysts from C57BL/6 mice and implanted into pseudopregnant females. Chimeric mice were bred with wild-type BALB/c females and the resulting germline (white) mice genotyped to identify positive transgenic mice. Heterozygote positive Tg mice were
backcrossed to wild-type BALB/c mice. Mice were maintained in microisolator cages in a specific pathogen-free mouse breeding facility. All studies were undertaken with the approval of the Australian National University Animal Ethics Experimentation Committee under the guidelines established by the Australian National Health and Medical Research Committee. Transgenic mice were identified using following amplification primers and reaction conditions;

<table>
<thead>
<tr>
<th>SAMPLES</th>
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</tr>
<tr>
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<td>TOTAL</td>
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</tr>
</tbody>
</table>

5.2.3 Intestinal mast cell quantification

As described in section 3.2.6.

5.2.4 Mononuclear cell preparation and MCp assessment

iFABPp IL-9 Tg (BALB/c) (n=4) and WT (BALB/c) mice (n=4) were killed by CO₂ asphyxiation and the small intestine, lungs, spleen, and bone marrow (BM) were harvested. The entire organs were removed except for BM, where a single femur was taken from each mouse. Individual tissues from 2 mice were pooled, placed in 20 mL complete RPMI 1640 (RPMI 1640 containing 100 U/mL penicillin, 100 µg/mL streptomycin, 10 µg/mL gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% heat-inactivated foetal calf serum [Sigma-Aldrich no. F2442, St. Louis,
MO), and processed essentially as previously described (Gurish, Tao et al. 2001; Abonia, Austen et al. 2005). Briefly, the intestines (flushed out and rinsed twice in HBSS) and lungs were finely chopped with a scalpel blade and transferred separately to 50-mL plastic tubes with 30 mL RPMI 1640 complete plus 1 mg/mL collagenase Type 4 (Worthington, Lakewood, NJ). There were 3 enzymatic digestions carried out for approximately 20 minutes each at 37°C. The undigested tissue clumps were collected after each digestion period and were subjected to another enzymatic digestion, while the liberated cells were pelleted, resuspended in 44% Percoll (Sigma-Aldrich no. P1644, St Louis, MO), overlayed on a 67% Percoll layer, and centrifuged at 400g for 20 minutes at 4°C. The cell collection procedure for BM and spleen omitted the digestion steps. BM was extruded from one femur of each animal using a 25-gauge syringe and 5 to 10 mL RPMI 1640 complete. Spleen cells were obtained from crushed whole spleens suspended in RPMI 1640 complete. The collected cells were pelleted and resuspended in 44% Percoll before centrifugation over 67% Percoll as described. The mononuclear cells (MNCs) were harvested from the interfaces of the 3 digestions of the lung and intestine, pooled by separate tissue source, and washed in RPMI 1640 complete. The numbers of viable cells were determined by trypan blue dye exclusion with a haemocytometer. Cells were serially diluted in RPMI 1640 complete, and 100 μL samples of the MNC dilutions were added to each well of standard 96-well flat-bottomed microtiter plates (Corning no. 3596, Corning, NY). Typically, 24 wells were plated for each cell concentration. Intestinal or BM MNCs were plated starting at 5000 to 10000 cells/well, and lung or spleen MNCs starting at 20000 to 40000 cells/well. Then, each well received 100 μL gamma-irradiated (30 Gy) splenic feeder cells plus cytokines (recombinant mouse IL-3 at 20 ng/mL and recombinant mouse SCF at 100 ng/mL). The cultures were incubated in humidified 37°C incubators with 5% CO₂ for 12 to 14 days, and positive wells containing mast cell colonies were identified and counted with an inverted microscope. The MNC colonies were easily distinguished as large colonies of nonadherent small- to medium sized cells (Crapper and Schrader 1983; Guy-Grand, Dy et al. 1984). The mast cell progenitor (MCp) concentrations are expressed as the number of MCps per 1x10⁶MNCs isolated from the tissue. Multiplying the concentration of MCps by the MNC yield/organ derives the number of MCps/tissue. Mast cell progenitor assay was performed by was performed by H. Faith Rainey in J. Pablo Abonia’s Laboratory, Allergy and Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati OH, USA.
5.2.5 ELISA measurements

mMCP-1, IgG1 and IgE were measured as described in section 3.2.5. TNF-α and IL-9 was measured by ELISA according to the manufacturer’s instructions (BD Biosciences PharMingen). Clonotyping of Ig complexes were performed according to manufacturer’s instructions (Southern Biotech). Serum samples were collected, processed and stored as described in section 3.2.5. For jejunal lysate samples, whole jejunum was excised and snap frozen at -70°C. Frozen jejunal sections were mechanically disrupted and suspended in 1mL of PBS containing protease inhibitors [aprotinin, autipain, chymostatin and leupeptin 100ng/ml each, Sigma-Aldrich, St. Louis, MO, USA]. The jejunal pellet was vigorously vortexed and the suspension was centrifuged at 12,000g for 10 minutes, supernatant removed, aliquoted and stored at -20°C until analysis was performed.

Clonotyping of Ig complexes was performed by Richard Ahrens in Simon Hogan’s Laboratory, Allergy and Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati OH, USA.

5.2.6 In vivo Cincinnati Cytokine Capture Assay (IVCCCA)

iFABPp IL-9 Tg (n=4) and WT (n=4) mice were intravenously injected with biotinylated rat IgG neutralizing monoclonal antibody anti-mouse IL-4 (BVD-1D11, (10 µg/mouse) and biotinylated rat IgG neutralising monoclonal antibody anti-mouse IFN-γ (R4-6A2, (10 µg/mouse) and blood collected 24 hours later. Serum levels of IL-4 and IFN-γ were determined by IVCCCA as previously described (Finkelman and Morris 1999). IVCCCA was performed by Tatiana Orekov in Professor Fred Finkelman’s laboratory, Department of Immunobiology, University of Cincinnati College of Medicine.

5.2.7 Microarray hybridization

Following TRIzol purification, RNA was repurified with phenol-chloroform extraction and ethanol precipitation. Purified RNA from iFABPp IL-9 Tg (n=4) and WT (n=4) mice were then pooled together and processed at Cincinnati Children’s Hospital Medical Center Affymetrix Gene Chip Core facility, using the murine MOE430_2, a whole
genome expression chip encoding 45101 genes, as previously described by the manufacturer (Affymetrix, Santa Clara, California, USA) (Irizarry, Hobbs et al. 2003). Differences between WT and iFABPp IL-9 Tg mice were also determined using the GeneSpring software (Silicon Genetics, Redwood City, California, USA). Data was normalized to WT mice, and genes, present in at least one sample, were screened for a greater than two-fold change over saline. A further description of the methodology, according to MIAME (minimum information about a microarray experiment) guidelines (www.mged.org/Workgroups/MIAME/miame.html). Genome wide expression gene profile comparative analysis performed by Dr. Carine Blanchard, Allergy and Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati OH, USA.

5.2.8 Lightcycler PCR

iFABPp IL-9 Tg mice (n=6) and WT (n=6) were obtained and sacrificed. Intestinal samples were harvested. RNA was isolated from intestinal samples and cDNA was generated by standard procedures as described in section 3.2.4. GAPDH, mMCP -1, -2, -4, -5, and FceRIα were quantified by real-time RT-PCR as described in section 3.2.4. Results were then normalized to GAPDH amplified from the same cDNA mix and expressed as gene of interest/GAPDH ratio. cDNAs were amplified using the following amplification primers and reaction conditions;
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence 5'-3'</th>
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<th>cycle #</th>
<th>Product size</th>
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<td>58</td>
<td>62</td>
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</tbody>
</table>

Standard curves were generated as described in section 3.2.4. Quantitative IL-9 PCR analysis was performed by Richard Ahrens and mMCP-1, -2, -4, -5 and FceRIα PCR analysis was performed by quantitative PCR analysis was performed by Elizabeth Cohen in Simon Hogan’s Laboratory, Allergy and Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati OH, USA.

5.2.9 Fluorescent-activated cell sorter (FACS) analysis

iFABPp IL-9-Tg (n=4) and WT (n=4) mice were sacrificed and the mesenteric lymph nodes (MLN) were excised. MLN were mechanically disrupted and the single cell suspensions (1x10⁶) were plated in a 96-well round bottom plate and flow cytometry analysis staining was performed. To analyse T-cell populations the following antibodies were used: PerCP anti-mouse CD4 (L3T4) (RM4-5; BD Biosciences PharMingen); PE anti-mouse CD8a (53-6.7; BD Biosciences PharMingen); APC anti-mouse CD62L (MEL-14; BD Biosciences PharMingen); FITC anti-mouse CD44 (1M7; BD Biosciences PharMingen). The following were used as appropriate isotype controls: PerCP rat IgG2a (R35-95; BD Biosciences PharMingen), PE rat IgG2a, (53-6.7; BD Biosciences PharMingen), APC rat IgG2a (R35-95; BD Biosciences PharMingen) and FITC rat IgG2a (R35-95; BD Biosciences PharMingen). Regulatory T-cell populations were identified
using the following antibodies: BD Biosciences PharMingen), APC anti-mouse CD25 (PC61; BD Biosciences PharMingen), PE anti-mouse CD45RB (16A; BD Biosciences PharMingen) and FITC anti-mouse FoxP3 (FJK-16S; BD Biosciences PharMingen). The following were used as appropriate isotype controls: PerCP rat IgG2a, (R35-95; BD Biosciences PharMingen), APC rat IgG1, (R3-34; BD Biosciences PharMingen), PE rat IgM (R4-22; BD Biosciences PharMingen), and FITC rat IgG2a. (R35-95; BD Biosciences PharMingen), B-cell populations were identified by the following antibodies: PE anti-mouse B220 (RA3-6B2; BD Biosciences PharMingen), FITC anti-mouse CD23 (B3B4; BD Biosciences PharMingen) and PE-Cy7 anti-mouse IgM (R6-60.2; BD Biosciences PharMingen). The following were used as appropriate isotype controls: PE rat IgG2a (R35-95; BD Biosciences PharMingen), FITC rat IgG2a (R35-95; BD Biosciences PharMingen), PE-Cy7 rat IgG2a (R35-95; BD Biosciences PharMingen). DC populations were analysed with the following antibodies: APC anti-mouse CD11c (HL3; BD Biosciences PharMingen), APC-Cy7 anti-mouse Gr-1 (RB6-8C5; BD Biosciences PharMingen), PE-Cy7 anti-mouse CD11b (M1/70; BD Biosciences PharMingen). The following were used as appropriate antibody controls: Armenian Hamster IgG1 (HL3; BD Biosciences PharMingen), APC-Cy7 rat IgG2b (A95-1; BD Biosciences PharMingen), PE-Cy7 rat IgG2b (A95-1; BD Biosciences PharMingen). 7-AAD was used to identify non-viable cells (BD Biosciences PharMingen).

5.2.10 Ussing chambers

iFABPp IL-9 Tg (n=4) and WT mice (n=4) were sacrificed and jejunum was excised. 1-cm segments of mucosa were stripped of muscle and mounted in U2500 Dual Channel Ussing chambers (Warner Instruments, Hamden, CT, USA) that exposed 0.30 cm² of tissue to 10 mL of Krebs buffer. Agar-salt bridges and electrodes were used to measure the potential difference. Every 50 seconds the tissues were short-circuited at 1 V (EC 800 Epithelial Cell Voltage Clamp; Warner Instruments), and the short-circuit current was monitored continuously. In addition, every 50 seconds the clamp voltage was adjusted to 1 V for 10 seconds to allow calculation of tissue resistance using Ohm’s law. After the preparation had stabilized for 10 minutes and baseline potential difference and resistance had been established, fluorescein isothiocyanate (FITC)–dextran (2.2 mg/mL; molecular mass, 4.4 kd; Sigma-Aldrich, St Louis, Mo) was added to the mucosal reservoir. Medium (0.25 mL of 10 mL) was removed from
the serosal reservoir and replaced with fresh medium every 20 minutes over a period of 180 minutes for measurement of FITC-dextran. Krebs buffer contained 4.70mM KCl, 2.52mM CaCl₂, 118.5mM NaCl, 1.18 mM NaH₂PO₄, 1.64 mM MgSO₄ and 24.88 mM NaHCO₃ on each side. The tissues were allowed to equilibrate for 15 minutes in Krebs buffer containing 5.5 mM glucose. All reagents were obtained from Sigma-Aldrich unless stated otherwise. To determine the overall luminal-to-serosal flux of HRP across the tissues, HRP (5 x 10⁻⁵ M) was added into the luminal buffer and 500 μl of serosal buffer samples were collected at 30-min intervals for 120 min and replaced with Krebs buffer. The concentration of HRP was measured by a kinetic enzymatic assay (Berin, Kiliaan et al. 1997; Yang, Berin et al. 2000). Briefly, 120 μl of sample were added to 800 μl of phosphate buffer containing 0.003% H₂O₂ and 80 μg/ml o-dianisidine (Sigma), and the enzymatic activity was determined from the rate of increase in optical density at 460 nm during a 1.5-min period. The luminal-to-serosal flux was calculated using a standard formula and expressed as ng/ml. Intestinal permeability experiments were performed by Katherine Groschwitz in Simon Hogan’s Laboratory, Allergy and Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati OH, USA.

5.2.11 Identification of vascular permeability

iFABPp IL-9 Tg (n=5) and WT (n=5) were retro-orbitally bled and peripheral blood samples were collected in EDTA microtainer tubes (Becton Dickinson, Franklin Lakes, NJ). Automated total cell counts and differential counts were performed according to manufacturer’s instructions (Fisher Diagnostics, Middletown, VA). Evan’s blue tissue extravasation was performed as previously described (Green, Johnson et al. 1988). Briefly, mice received i.v. Evan’s Blue dye in PBS; 20 mg/kg and 3.5 hours later, mice were anesthetised with pentobarbital (20mg/kg, i.p.) and heat perfusion was performed (10 ml PBS arterial perfusion). Jejunum and colon were harvested and Evan’s blue extravasation was measured in optical density at 650 nm. Tissue protein levels were quantified using a BCA protein assay kit, following manufacturer’s instructions (Pierce, Rockford, IL).
5.2.12 Statistical Analysis

Data are expressed as mean ± standard error (SEM). Statistical significance comparing different sets of mice was determined by Student’s t-test. In experiments comparing multiple experimental groups, statistical differences between groups were analyzed using the one-way ANOVA nonparametric Bonferroni post-test. $P < 0.05$ was considered significant. All analyses were performed using Prism 4.0 software.
5.3 Results

5.3.1 Generation of Fabpi IL-9 Transgenic Mice.

Enterocytes have been demonstrated to be a chief source of select inflammatory mediators in lesions in patients with diverse gastrointestinal inflammatory disorders (MacDermott, Sanderson et al. 1998; MacDermott 1999; Papadakis and Targan 2000; Mishra, Hogan et al. 2002). The 1.2-kb 5'-flanking region of the rat Fabpi gene contains all of the necessary elements to promote specific expression of transgenes into enterocytes in the small intestine (Sweetser, Birkenmeier et al. 1988; Mishra, Hogan et al. 2002). Furthermore, this promoter has been shown to produce highest gene expression in the jejunum (Sweetser, Birkenmeier et al. 1988; Mishra, Hogan et al. 2002) the primary organ involved in the development of intestinal anaphylaxis responses (Perdue and Davison 1986; Perdue and Gall 1986; Perdue and Gall 1986).

We initially generated transgenic mice that utilized this promoter to direct expression of IL-9 in the intestine (Figure 5.1. a.). RT-PCR analysis revealed markedly increased levels of IL-9 mRNA in the small intestine of transgenic mice compared with WT mice. In WT mice, IL-9 mRNA expression was not detectable (Figure 5.1. b.). IL-9 protein in the intestinal tissue and in serum of iFABPp IL-9 Tg and WT mice was examined next. A comparable marked increase in IL-9 protein levels in the sera of iFABPp IL-9 Tg mice was shown (Figure 5.1. c.) Furthermore, a 10-fold induction of IL-9 protein levels in the small intestine of iFABPp IL-9 Tg mice is observed as compared to WT (Figure 5.1. d.). The IL-9 ELISA does detect immunoreactive protein levels in the small intestine of WT mice in contrast to no mRNA expression of IL-9 in the small intestine of WT mice. However, due to the high sensitivity of quantitative PCR (and no IL-9 mRNA detection), these differences are most likely explained by non-specific binding of jejunal supernatant products during ELISA quantification. Take together, these two independent methods of IL-9 mRNA expression and protein quantification indicate an observable increase in IL-9 levels in iFABPp IL-9 Tg mice.
Figure 5.1 Generation of iFABPp IL-9 Tg mice.

(a.) Transgenic construct (b.) Quantitative PCR analysis of IL-9 mRNA expression in the jejunum of iFABPp IL-9 Tg mice and BALB/c WT and (c. and d.) IL-9 protein levels in the sera (c.) and jejunum extracts (d.) of iFABPp IL-9 Tg mice and BALB/c WT. (b.) Data normalised to GADPH expression. Data expressed as IL-9/GADPH ratio fold change compared to BALB/c WT. The black line represents the mean value in each group. (c. and d.) Data represented as mean + SEM, 4-5 mice per group. (d.) Data normalised to mg of tissue protein.
Chapter Five: The role of IL-9 in intestinal anaphylaxis, employing a transgenic approach

(a)

(b)

(c)

(d)

Average GADPH Quantity (AU $10^{-15}$): 3.29 ± 2.44, 5.48 ± 4.96

BALB/c WT iFABPp IL-9 Tg
in comparison to WT mice, and this elevation is specific to the jejunal segment (Figure 5.2. a. and b.). Mast cells in WT mice were predominantly localised to the lamina propria.Whilst iFABPp IL-9 Tg mice also had a strong localisation of mast cells to the lamina propria, there was a significant infiltration of mast cells into intracryptic and intraepithelial regions of the small intestine (Figure 5.2. a., insert and c.). Interestingly, the architecture of the small intestine remains unchanged other than compartmentalisation of mastocytosis, under homeostatic conditions. We were next interested in elucidating whether overexpression of IL-9 in the small intestine induced mast cell activation in addition to mastocytosis. To examine mast cell activation we examined serum mMCP-1 levels, since increased serum mMCP-1 is a marker of mucosal mast cell degranulation and indicates mast cell activation (Stevens, Friend et al. 1994; Knoops, Louahed et al. 2005). Indeed, serum mMCP-1 levels in iFABPp IL-9 Tg mice were significantly elevated when compared to WT littermate controls (Figure 5.2. d.). Furthermore, we show an increase in TNF-α (p<0.05), a pro-inflammatory cytokine and one that is associated with mucosal mast cell activation (Metcalfe, Baram et al. 1997; MacDonald, Pick et al. 1998) (Figure 5.3. a. and b.).

5.3.3 Effect of IL-9 overexpression on mast cell progenitor populations.

In vitro studies have shown an effect of IL-9 on bone marrow-derived mast cell (BMMC) populations (Uyttenhove, Simpson et al. 1988; Renauld, Goethals et al. 1990; Hultner, Kolsch et al. 2000; Kweon, Yamamoto et al. 2000; Stassen, Arnold et al. 2000; Soussi-Gounni, Kontolemos et al. 2001). We have shown in IL-9 deficient mice that intestinal mast cell progenitors are not decreased in comparison to WT mice, suggesting IL-9 does not play a role in mast cell progenitor generation. However, this may be due to regulatory compensation in the absence of IL-9. To confirm this observation, employing another mouse system, we examined the levels of mast cell progenitors in the intestine, lung, spleen and bone marrow of iFABPp IL-9 Tg mice. We show overexpression of IL-9 in the small intestine does not affect the generation of mast cell progenitors locally within the intestine or globally in the lung, spleen or bone marrow (Figure 5.4.). This further confirms our findings in IL-9 deficient mice and demonstrates that mast cell progenitor generation and proliferation occurs independently of IL-9.
Figure 5.2 Intestinal expression of IL-9 induces intestinal mastocytosis and mast cell activation.

(a.) Photomicrograph of chloroacetate esterase-stained jejunal sections from BALB/c WT and iFABPp IL-9 Tg, identifying mast cells (red), (b.) mean number of mast cells per high power field (HPF) (c.) villus localisation of mast cells, (d.) serum mouse mast cell protease-1. (a.) Photomicrograph 10x magnification; insert 40x magnification. (b. - d.) Data represented as mean + SEM, 4-5 mice per group from n=4 experiments.
Chapter Five: The role of IL-9 in intestinal anaphylaxis, employing a transgenic approach

(a)

(b)

(c)

(d)
Figure 5.3 Mast cell activation is associated with the release of pro-inflammatory mediators.

TNF-α protein levels in the sera (a.) and jejunum (b.) of iFABPp IL-9 Tg and BALB/c WT mice. (a. and b.) Data represents 4-5 per group. (b.) Data normalised per mg of protein.
Chapter Five: The role of IL-9 in intestinal anaphylaxis, employing a transgenic approach

(a) BALB/c WT
\[ \text{IFABPp IL-9 Tg} \]

(b) BALB/c WT
\[ \text{IFABPp IL-9 Tg} \]

**Figure Legend:**
- (a) BALB/c WT and IFABPp IL-9 Tg.
- (b) BALB/c WT and IFABPp IL-9 Tg.

**Graphs:**
- (a) TNF-\(\alpha\) (pg/ml)
- (b) TNF-\(\alpha\) (pg/ml)/mg protein

**Statistical Significance:**
- \( p < 0.01 \)
Figure 5.4 Overexpression of IL-9 does not alter mast cell progenitor levels.

Mast cell progenitor numbers in the intestine, lung, spleen and bone marrow of BALB/c WT and iFABPp IL-9 Tg mice. Data represented as mean + SEM, 4-5 mice per group.
Chapter Five: The role of IL-9 in intestinal anaphylaxis, employing a transgenic approach

HBALB/c WT
• iFABPp-IL-9 Tg

10000
1000
100
10

MCP/\times10^6 cells

Intestine Lung Spleen Bone marrow

BALB/c WT
iFABPp-IL-9 Tg
5.3.4 **Effect of IL-9 overexpression on other intestinal immune parameters.**

To examine what effect intestinal expression of IL-9 had on other intestinal immune parameters we examined CD4⁺, CD8⁺, regulatory T cell, B (B220) cell and dendritic cell (DC) levels in the mesenteric lymph node (MLN) of iFABPp IL-9 Tg and WT mice (Table 5.1). We chose to measure the immune profile of the MLN as this is the closest proximal draining lymph node tissue to the small intestine and is most likely to be representative of the immunological parameters present. We observed no difference in the levels of CD4, CD8, B220, and DC cells between iFABPp IL-9 Tg and WT mice. Furthermore, we examined T cell activation status (CD44, CD62L, and CD69) and the development of T cell subsets such as regulatory cells (CD4⁺CD25⁺) and demonstrate no difference in CD4⁺ or CD8⁺ T cell populations between groups. We also examined MLN DC subpopulations (plasmacytoid, myeloid, and lymphoid) and DC activation status (MHC-II) and observed no difference in DC subpopulations and activation status in MLN in WT and iFABPp IL-9 Tg mice (Table 5.1.). Finally, we were interested in examining if there was any evidence of an altered Th2-profile. Serum IL-4 and IFNγ and total Ig levels in WT and iFABPp IL-9 Tg mice were comparable (Figure 5.5. a.-c.).

5.3.5 **Consequence of IL-9 overexpression and elevation of intestinal mast cells.**

To gain a further understanding of the consequence of elevated intestinal IL-9 and mast cell levels, we took an empirical approach involving genome wide expression profile analysis using Affymetrix oligonucleotide chips and probing small intestinal RNA from WT and iFABPp IL-9 Tg mice. Using a criterion of 2.0-fold change, we identified 176 genes altered in the iFABPp IL-9 Tg mice. Out of these transcripts, 126 were upregulated and 52 were downregulated. Functional classification of the altered transcripts revealed a significant predominance of mast cell associated genes including phospholipase A2, group IVC (31.4-fold), carbonic anhydrase 3 (12.7-fold), mMCP-2 (7.4-fold), mMCP-1 (6.8-fold) and Fc receptor, IgE, high affinity, alpha polypeptide, and Th2-immunity genes including RELMb (12.6-fold) and small proline-rich protein 2A (SPRA) (4.6-fold). Furthermore, we performed quantitative PCR analysis examining expression levels of mast cell genes, mMCP-1, mMCP-2, mMCP-4, mMCP-5, and FcεR1. Levels of mMCP-1 (~50-fold), mMCP-2 (~100-fold), mMCP-4 (~10-fold), mMCP-5, and FcεR1 (~100-fold) in the small intestine of iFABPp IL-9 Tg mice was
Table 5.1 IL-9 in the small intestine promotes mastocytosis and mast cell activation independently of CD4$^+$ T-cell, B-cell and DC-cell dysfunction.

CD4$^+$, CD8$^+$, B220, regulatory T cell and dendritic cell percentages of BALB/c WT and iFABPp IL-9 Tg mice mesenteric lymph node populations. Data presented as % mean ± SEM, 4–5 mice per group.
Table 5.1. IL-9 in the small intestine promotes mastocytosis and mast cell activation independent of CD4$^+$ T-cell, B-cell and DC-cell dysfunction.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>BALB/c WT</th>
<th>iFABPp IL-9 Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4$^+$ T-cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4$^+$ CD8$^-$</td>
<td>53.8±0.46</td>
<td>54.93±2.23</td>
</tr>
<tr>
<td><strong>Naïve:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4$^+$ CD44$^{low}$ CD62$^{high}$</td>
<td>13.77±1.72</td>
<td>17.03±1.46</td>
</tr>
<tr>
<td><strong>Memory:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4$^+$ CD44$^{high}$ CD62$^{low}$</td>
<td>12.13±2.41</td>
<td>13.8±0.36</td>
</tr>
<tr>
<td>T$_{reg}$:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4$^+$ CD25$^+$ CD45RB$^+$ FoxP3$^+$</td>
<td>14.95±2.33</td>
<td>15.33±0.80</td>
</tr>
<tr>
<td><strong>CD8$^+$ T-cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4$^-$ CD8$^+$</td>
<td>20.27±0.85</td>
<td>21.4±1.51</td>
</tr>
<tr>
<td><strong>Naïve:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8$^+$ CD44$^{low}$ CD62$^{high}$</td>
<td>40.73±2.76</td>
<td>42.73±2.38</td>
</tr>
<tr>
<td><strong>Memory:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting memory:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8$^+$ CD44$^{high}$</td>
<td>53.47±2.11</td>
<td>49.13±2.27</td>
</tr>
<tr>
<td>Effector:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8$^+$ CD44$^{high}$</td>
<td>3.93±1.00</td>
<td>5.6±0.6</td>
</tr>
<tr>
<td><strong>B220$^+$ cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220$^+$</td>
<td>72.17±0.57</td>
<td>20.77±2.29</td>
</tr>
<tr>
<td><strong>Immature:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220$^+$ IgM$^+$</td>
<td>12.43±0.9</td>
<td>10.63±1.07</td>
</tr>
<tr>
<td>B220$^+$ CD23$^-$</td>
<td>12.23±1.50</td>
<td>11.9±1.37</td>
</tr>
<tr>
<td><strong>DC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC</td>
<td>1.19±0.4</td>
<td>12.28±.11</td>
</tr>
<tr>
<td><strong>Lymphoid:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7AAD$^-$ CD11c$^+$ Gr-1$^-$ CD11b$^-$</td>
<td>71.32±3.13</td>
<td>64.43±3.17</td>
</tr>
<tr>
<td><strong>Plasmacytoid:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7AAD$^-$ CD11c$^+$ Gr-1$^+$ CD11b$^-$</td>
<td>6.01±1.66</td>
<td>10.05±2.38</td>
</tr>
<tr>
<td><strong>Myeloid:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7AAD$^-$ CD11c$^+$ Gr-1$^+$ CD11b$^+$</td>
<td>13.68±3.55</td>
<td>15.54±3.7</td>
</tr>
</tbody>
</table>
Figure 5.5 Serum cytokine and immunoglobulin levels are unaltered in iFABPp IL-9 Tg mice.

(a.) Serum IL-4, (b.) IFN-γ and (c.) immunoglobulin levels in BALB/c WT and FABPp IL-9 Tg mice. (a. and b.) Data represented as mean ± SEM. (c.) The black line represents the mean value in each group. (a. – c.) Experiments repeated in triplicate, 4-5 mice per group.
Chapter Five: The role of IL-9 in intestinal anaphylaxis, employing a transgenic approach

(a) BALB/c WT

(b) BALB/c WT

(c) WT

• Tg

Concentration (ug/ml)

IgA IgE IgG1 IgG2a IgG2b IgG3 IgM

IL-4 (ug/ml)

IFN-γ (ug/ml)
significantly elevated as compared to WT mice (Figure 5.6. a.). These studies demonstrate that overexpression of IL-9 predominantly leads to upregulation of mast cell gene expression in the intestine. Previously whole genome wide analysis examining gene expression profile in oral allergen-induced intestinal anaphylaxis has been performed (Brandt, Strait et al. 2003). These studies have demonstrated that the most upregulated genes associated with oral antigen-induced intestinal anaphylaxis were mast cell associated genes phospholipase A2, group IVC, carbonic anhydrase 3, mMCP-2, mMCP-1, and Fc receptor, IgE, high affinity, alpha polypeptide, and Th2-immunity genes including RELMβ and small proline-rich protein 2A (Brandt, Strait et al. 2003) (Figure 5.6. b.). Remarkably, comparison of the upregulated genes in these studies and the upregulated genes in the intestine of syngeneic iFABPp IL-9 Tg mice revealed a similar gene profile (Figure 5.6. b.).

5.3.6 Consequence of IL-9 overexpression in ion transport: intestinal permeability and intravascular leakage.

Oral antigen-induced intestinal anaphylaxis in WT mice is associated with increased intestinal permeability and diarrhoea (Brandt, Strait et al. 2003). Furthermore, several other models have implicated a role for mast cells in intestinal permeability (Perdue and Gall 1986; Perdue and Gall 1986; Crowe, Sestini et al. 1990; Perdue, Marshall et al. 1990; Berin, Kiliaan et al. 1997; Berin, Kiliaan et al. 1998). The similarities in the intestinal phenotype (mastocytosis and elevated mMCP-1) and gene profile between iFABPp IL-9 Tg mice and WT mice led us to examine these parameters in WT and iFABPp IL-9 Tg mice and assess the role of mast cells in intestinal permeability. To do this we employed a mast cell stabilizer, cromolyn sodium (Crowe and Perdue 1993; Singh, Boucher et al. 1999). i.p. administration of cromolyn sodium reduced serum mMCP-1 levels confirming stabilisation of mast cells (Figure 5.7. a.). To confirm our hypothesized altered epithelial cell barrier function in iFABPp IL-9 Tg mice, we examined intestinal permeability by analysing FITC-dextran and HRP transport in jejunum segments ex vivo. FITC-dextran is a 4kD protein known to travel via a paracellular route and HRP, a 44kD protein, travels via a transcellular pathway and the subsequent luminal-to-serosal flux of proteins across the jejunal tissue is quantitated. Compared with control WT mice, iFABP IL-9 Tg control mice had increased intestinal permeability in the jejunum to FITC-dextran and HRP (Figure 5.7. b. and c.). Notably,
Figure 5.6 Intestinal expression of IL-9 is sufficient to induce a gene profile similar to that observed in mice with oral antigen-induced intestinal anaphylaxis.

(a.) Quantitative PCR analysis of mast cell gene mRNA expression in the jejunum and (b.) Genome wide expression gene profile comparative analysis of iFABPp IL-9 Tg and BALB/c WT mice compared to OVA-sentitised and subsequent i.g. saline or OVA-challenged mice. (a.) Gene expression was normalised to GADPH expression in each individual sample. The black line represents the mean value of each group. (b.) Data represents genes found to be upregulated from profile analysis. Results expressed as gene/GADPH ratio with respect to fold change over BALB/c WT.
Chapter Five: The role of IL-9 in intestinal anaphylaxis, employing a transgenic approach

(a) (b)
we show that the reduction in mast cell activity correlated with a reduction in dextran-FITC and HRP intestinal permeability in IFABPp IL-9 Tg mice (Figure 5.7. b. and c.). Collectively, these studies demonstrate increased mast cell mediated intestinal permeability in iFABPp IL-9 Tg mice.

Increased vascular permeability, a known clinical manifestation of an anaphylactic reaction (Finkelman, Rothenberg et al. 2005), results in intravascular leakage (IVL) and this occurs via a mast cell mediated mechanism (Abelli, Nappi et al. 1992; Sampson, Mendelson et al. 1992). Initially we assessed whether multiple oral OVA-challenges induced IVL during oral antigen-induced intestinal anaphylaxis. Vascular leak causes haemoconcentration (fluid leak into extracellular space with retention of blood cells resulting in a packed erythrocyte volume and measured as increased haematocrit). Previous studies have demonstrated an increased haematocrit during systemic anaphylaxis (Mitsuhata, Takeuchi et al. 1995; Strait, Morris et al. 2003). Oral antigen challenge results in a significant increase in haematocrit following 3 and 6 OVA-challenges compared to OVA-sensitised, saline-challenged WT mice (Figure 5.7. d.). Following the demonstration iFABPp IL-9 Tg mice exhibit an intestinal anaphylaxis phenotype under basal conditions, we examined whether iFABPp IL-9 Tg mice had increased IVL. Haematocrit percentage in iFABPp IL-9 Tg mice is significantly greater than that observed in WT mice (Figure 5.7. e.). However, iFABPp IL-9 Tg mice had normal red blood cell numbers and levels of haemoglobin in the blood (haemoglobin; 12.99 ± 0.6 vs 14.2 ± 0.5 dec/L; WT vs iFABPp IL-9 Tg mice; mean + SEM; n=8 per group). We therefore employed an independent method (Evan’s blue extravasation) to test for IVL in WT and iFABPp IL-9 Tg mice. We demonstrate that the level of Evan’s blue in the intestine of iFABPp IL-9 Tg mice was significantly higher than that observed in WT mice clearly indicating an increase in intestinal IVL (Figure 5.7. f.). To examine whether the IVL was associated with mast cells we examined Evan’s blue concentration in the colon of iFABPp IL-9 Tg mice which has normal mast cell levels (Figure 5.2. b.). Evan’s blue concentration in the colon was equivalent to that of WT mice, suggesting that IVL was associated with increased mast cell numbers (Figure 5.7. f.).
Figure 5.7 Overexpression of IL-9 in the small intestine induces features of intestinal anaphylaxis phenotype including mast cell dependent intestinal permeability and intravascular leakage.

(a.) Serum mouse mast cell protease-1 and mean number of mast cells per high power field (HPF) and intestinal permeability measured by dextran-FITC (b.) and HRP (c.) transport in jejunal segments ex vivo for iFABPp IL-9 Tg and BALB/c WT mice treated with control or the mast cell stabilising agent, Cromolyn Na. (d.) % haematocrit before and after 3x or 6x saline or OVA-challenges of OVA-sensitised BALB/c WT mice, (e.) % haematocrit of iFABPp IL-9 Tg mice compared to BALB/c WT and (f.) Evan’s blue extravasation in the jejunum and colon of iFABPp IL-9 Tg and BALB/c WT mice. (a.) Data represented as mean ± SEM. (b.) Data represented as levels in basolateral component ± SEM. (c.) Data represented as HRP uptake over time ± SEM. (d. and e.) Data represented as mean ± SEM (f.) Data represented as Evan’s blue concentration and normalised to mg of tissue protein. Black line represents mean value in each group.
Chapter Five: The role of IL-9 in intestinal anaphylaxis, employing a transgenic approach

(a) (b) (c) ... u u 
0) V) ^ tn n -n 
V) c 
c 
2 UJ 
45 
40' 
35 
30 
25 
20 
15' 
10' 
5-
0-
-O-
o 
oo 
Jejunum Colon

(d) (e) (f)
5.4 Discussion

In this chapter we have dissected the consequences of overexpression of IL-9 in enterocytes using the well established small intestine specific promoter, Fabpi (Sweetser, Birkenmeier et al. 1988; Cohn, Simon et al. 1992; Mishra, Hogan et al. 2002). Experimental analysis of IL-9 intestinal transgenic mice has revealed that overexpression of IL-9 results in upregulated mast cell gene expression, intestinal mastocytosis and mast cell activation and this mediates intestinal permeability. Furthermore, upregulated genes in the intestine of syngeneic iFABPp IL-9 Tg mice strikingly compare to the gene profile of oral antigen-induced intestinal anaphylaxis. Taken together these findings indicate that overexpression of IL-9 in the small intestine induces an experimental intestinal anaphylaxis transcriptome and phenotype.

Notably, the development of an experimental intestinal anaphylaxis transcriptome and phenotype occurs independently from mast cell progenitor, CD4⁺/CD8⁺ T cell or B cell dysregulation. IL-9 was originally identified as a T cell growth factor and mice that systemically overexpress IL-9 have an increased susceptibility to thymic lymphomas after irradiation or mutagenesis (Uyttenhove, Simpson et al. 1988; Renauld, van der Lugt et al. 1994). Investigation of systemic IL-9 Tg mice did not show any major abnormality in the immune system and mice overexpressing IL-9 in the lung either using a constitutive tissue-specific promoter or an inducible one show that IL-9 is predominantly exerting affects on lung epithelium, where it has been targeted to express (Steenwinckel, Louahed et al. 2007; Temann, Laouar et al. 2007). IL-9 has also been shown to promote Ig production. In vitro, IL-9 was shown to synergise with suboptimal doses of IL-4 for the production of IgE and IgG\textsubscript{1} by LPS-activated murine B cells (Petit-Frere, Dugas et al. 1993). Very similar observations in humans were reported with peripheral B cells (Dugas, Renauld et al. 1993) and systemic IL-9 transgenic mice showed global increases in spontaneous Ig production in vivo (Vink, Warnier et al. 1999). Here we observe no difference in IgE or IgG\textsubscript{1} production in mice overexpressing IL-9 in the small intestine. One explanation for our findings is IL-9 transgenic animals have >1 mg/ml of circulating IL-9 in the serum, where as iFABPp IL-9 Tg mice have 125 pg/ml circulating IL-9. Conversely, it may simply be due to an altered pattern of global vs. local expression of IL-9.
Increased IL-9 expression in the small intestine induced mastocytosis and mast cell activation. This is consistent with findings in the constitutive and lung-specific overexpression murine models (Godfraind, Louahed et al. 1998; Temann, Geba et al. 1998; Temann, Ray et al. 2002). We also performed quantitative PCR analysis to examine the expression of mast cell genes and show significant upregulation patterns in iFABPp IL-9 Tg mice, specifically mMCP-1, -2 and -4, which taken together represent MMC proteases (Stevens and Austen 1989; Serafin, Reynolds et al. 1990; McNeil, Austen et al. 1991; Reynolds, Gurley et al. 1991) and this is consistent with the localisation of intestinal mast cells. Our data is supported by in vitro studies with BMMC populations in which IL-9 in the presence of SCF drives mucosal mast cell development (Eklund, Ghildyal et al. 1993).

No difference in intestinal mast cell progenitor levels in iFABPp IL-9 Tg compared to WT mice was observed. This is consistent with our findings of comparable mast cell progenitor levels in IL-9" mice (chapter 4). Previous studies have demonstrated that IL-9 is insufficient to induce growth of mast cell precursors and alone cannot support survival of mature mast cells (Matsuzawa, Sakashita et al. 2003). Interestingly, we observe an increase in the number of intraepithelial mast cells both within the epithelium of the villi and in intracryptal locations, the site where mast cell progenitor precursors are suggested to reside. Collectively, these findings suggest IL-9 may promote intestinal mastocytosis via enhancing mast cell maturation and that mechanisms independent of IL-9 critically maintain mast cell progenitor numbers.

Using Affymetrix oligonucleotide chips to probe small intestinal RNA, 176 genes are altered in the iFABPp IL-9 Tg mice and these were comparable to those genes identified via this analysis in WT mice with oral antigen-induced intestinal anaphylaxis, further supporting our investigations showing that overexpression of IL-9 in the small intestine is sufficient to induce features of intestinal anaphylaxis. Notably, this predisposition was associated with increased intestinal permeability and this was mediated by a mast cell dependent mechanism. Employing two independent techniques we have also demonstrated increased IL-9 promotes IVL. Whilst IL-9 has not been shown to directly influence erythropoiesis, it remains possible that overexpression of IL-9 in the intestine may increase the number of these cells.
Impairment of intestinal barrier function, “leaky gut”, has been implicated as a critical determinant in the predisposition to a number of GI diseases including IBD and food allergy (Takeuchi, Maiden et al. 2004; Buhner, Buning et al. 2006). Previous experimental investigations employing mast cell-stabilising agents and mast cell deficient mice have provided corroborative evidence demonstrating mast cell involvement in intestinal epithelial permeability (Crowe, Sestini et al. 1990; Yu and Perdue 2001). Currently the mechanism by which mast cells mediate the development of intestinal permeability remains unclear. It is possible to postulate mast cell mediated intestinal permeability may be induced by the multiple preformed and newly synthesised pro-inflammatory mediators that are released during activation and subsequent degranulation of mast cells. We demonstrate intestinal expression of IL-9 was associated with elevated mMCP-1, mMCP-2 and TNFα and these may contribute to increased intestinal permeability in iFABP IL-9 Tg mice; other possible mediators include histamine, PAF and serotonin (Perdue, Forstner et al. 1984; Perdue and Gall 1986; Berin, Kiliaan et al. 1997; Berin, Kiliaan et al. 1998; Yu and Perdue 2001; Brandt, Strait et al. 2003; McDermott, Bartram et al. 2003), and other members of the laboratory are currently investigating their role in induction of intestinal permeability.

Collectively, our morphological and genetic analysis of iFABPp IL-9 Tg mice suggests that intestinal expression of IL-9 is sufficient to induce a phenotype and gene profile similar to that observed in mice with oral antigen-induced intestinal anaphylaxis.
Chapter Six

The mechanisms involved in predisposition of intestinal anaphylaxis
Chapter Six: The mechanisms involved in predisposition of intestinal anaphylaxis

6.1 Introduction

Oral antigen-induced intestinal anaphylaxis has been shown to be mediated by mast cells, IgE and FcεRI-dependent pathways, and was associated with elevated levels of Th2 cytokines (Brandt, Strait et al. 2003). Based on these findings we hypothesised that Th2 cytokines orchestrate the mast cell/IgE dependent pathways required for oral antigen-induced intestinal anaphylaxis.

The Th2 cytokines IL-4 and IL-13 have been shown to play a critical role in the development of antigen-specific IgE responses (Strait, Morrist et al. 2004; Finkelman, Rothenberg et al. 2005) and these IL-4/IL-13 mediated effects occur via STAT-6 dependent pathways (Takeda, Kishimoto et al. 1997; Wang, Yang et al. 2000; Mathew, MacLean et al. 2001; Yang, Hogan et al. 2001). It has been proposed that numerous inflammatory mediators including Th2-cytokines regulate the coordination of the intestinal anaphylactic immune reaction. Indeed, peripheral blood and tissue from patients with food allergy contains elevated numbers of activated T cells and their increased numbers correlate with elevated levels of Th2-cytokines, the degree of GI inflammation and dysfunction (Eigenmann, Huang et al. 1996; Eigenmann and Frossard 2003). Furthermore, in vitro stimulation of T cells and T cell clones from food allergic patients produce Th2-cytokines (IL-4, IL-5 and IL-13) following antigen stimulation (Turcanu, Maleki et al. 2003). In addition, genetic mapping studies in both humans and mice have demonstrated linkage between the atopic phenotype and the IL-4 and IL-9 gene, identifying IL-9 as a candidate gene in atopy (Postma, Bleecker et al. 1995; Nouri-Aria, Pilette et al. 2005).

In chapter 4 the Th2 cytokine, IL-9 was shown to be central to the development of oral antigen-induced intestinal anaphylaxis, which was linked to mast cell mediated processes. In chapter 5, overexpression of IL-9, specifically in the small intestine, was shown to induce features of intestinal anaphylaxis including intestinal mastocytosis, mast cell activation, gene expression and intestinal permeability and intravascular leakage. We were next interested in elucidating the role of overexpression of IL-9 in the small intestine during oral antigen induced intestinal anaphylaxis and the consequence of Th2-signalling (IL-4Rα, IL-13 and STAT-6) in the mediation of the immunopathogenesis of intestinal anaphylaxis.
6.2 Materials and Methods

6.2.1 Mice

As described in section 4.2.2, IL-4Ra, IL-13 and STAT-6 deficient mice (BALB/c) expressing the iFABPp IL-9 transgene were generated by mating IL-4Ra, IL-13 and STAT-6 deficient mice (Foster, Webb et al. 2003; Webb, Mahalingam et al. 2003; Webb, Matthaei et al. 2004) with iFABPp IL-9 Tg mice and subsequently mating iFABPp IL-9 Tg⁺ F1 mice with IL-4Ra, IL-13 or STAT-6 deficient mice. The resulting F2 mice were screened by PCR analysis for the presence of the IL-9 transgene (section 4.2.2) and for the homozygous deficiency of IL-4Ra, IL-13 or STAT-6 gene. Transgenic mice deficient in IL-4Ra, IL-13 or STAT-6 were identified using the following amplification primers and reaction conditions:

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th># TUBES</th>
<th>MASTER</th>
<th>PROGRAM #</th>
<th>CYCLES</th>
<th>MACHINE #</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR REAGENTS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water</td>
<td>9.2</td>
<td>0</td>
<td>1</td>
<td>x 1</td>
<td>94°C: 3'</td>
</tr>
<tr>
<td>10 x ST buffer</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>x 35</td>
<td>95°C: 0'15&quot;</td>
</tr>
<tr>
<td>5mM dNTP's</td>
<td>0.8</td>
<td>0</td>
<td>3</td>
<td>x 1</td>
<td>72°C: 3'</td>
</tr>
<tr>
<td>NeoP4</td>
<td>0.25</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR1.2B</td>
<td>0.75</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4RP2</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BE10</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUBTOTAL</td>
<td>15</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA ADDED</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2O, +/+ , +/- , -/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**PCR Conditions**

- **Water**
  - 94°C: 3'
  - 95°C: 0'15"
  - 72°C: 3'

- **NeoP4**
  - 72°C: 3'00" (WT ~2300bp)

- **CR1.2B**
  - 60°C: 0'15"
  - 25°C: 5' (KO ~1400kb)

**Amplification Primers**

- NeoP4: gcgcATgcgcTTcTATgcgcTTc
- CR1.2B: cTAGgAcTccAcTcAcTccAggT
- IL4RP2: AAcTgTgggcTgAgaAcAgAcA
### 6.2.2 Protocol

As described in section 3.2.2

### 6.2.3 Intestinal mast cell quantification

As described in section 3.2.6
6.2.4 ELISA measurements

As described in section 3.2.5

6.2.5 Statistical Analysis

Data are expressed as mean ± standard error (SEM). Statistical significance comparing different sets of mice was determined by Student’s t-test. In experiments comparing multiple experimental groups, statistical differences between groups were analyzed using the one-way ANOVA nonparametric Bonferroni post-test. P < 0.05 was considered significant. All analyses were performed using Prism 4.0 software.
6.3 Results

6.3.1 Intestinal expression of IL-9 is sufficient to predispose to intestinal anaphylaxis.

The demonstration that overexpression of IL-9 in the small intestine promotes an intestinal anaphylaxis phenotype and transcript profile, led to the examination of whether overexpression of IL-9 in the small intestine increases susceptibility to oral antigen-induced intestinal anaphylaxis. We therefore performed i.g. OVA-challenge on OVA-sensitised WT and iFABPp IL-9 Tg mice and assessed for the development of features of intestinal anaphylaxis. It was shown show that OVA-challenge of WT mice induced oral antigen-induced intestinal anaphylaxis including diarrhoea, mastocytosis, serum mMCP-1 and OVA-specific IgE (Figure 6.1). Susceptibility of intestinal anaphylaxis in iFABPp IL-9 Tg mice was significantly greater as compared to WT mice. OVA-challenge of OVA-sensitised iFABPp IL-9 Tg mice induced diarrhoea in >80% of iFABPp IL-9 Tg mice by challenge 3 as compared to <25% of WT mice (Figure 6.1. a). The increased diarrhoea occurrence in iFABPp IL-9 Tg mice correlated with increased intestinal mast cells and mast cell activation, represented as serum mMCP-1 levels, as compared to WT mice (Figure 6.1. b and c and e. – h.). Notably, we observed no difference in OVA-specific IgE levels following nine OVA-challenges, (Figure 6.1. d.) suggesting that the increased susceptibility was not due to enhanced an antigen-specific response.

Extraordinarily, OVA-challenge of non-sensitised iFABPp IL-9 Tg mice induced diarrhoea (Figure 6.1. a). In contrast, non-sensitised, OVA-challenged WT mice do not develop diarrhoea (Figure 6.1. a). OVA-challenge of naïve iFABPp IL-9 Tg mice induced diarrhoea in 25% of iFABPp IL-9 Tg mice by challenge 6, and >80% of these mice following the 9th challenge (Figure 6.1. a). The increased acute diarrhoea correlated with intestinal mastocytosis and serum mMCP-1 levels (Figure 6.1. b and c). Again, we observe no change in antigen-specific response, represented here by no significant difference in end point measurement of serum OVA-specific IgE (Figure 6.1. d.).
Figure 6.1 Intestinal expression of IL-9 is sufficient to predispose to intestinal anaphylaxis.

(a.) Diarrhoea occurrence (b.) mean number of mast cells per high power field (hpf) (c.) serum mouse mast cell protease-1 (d. - g.) photomicrograph of chloroacetate esterase-stained jejunal sections from saline (SAL) or OVA-sensitised and subsequent i.g. OVA-challenged BALB/c WT and iFABPp IL-9 Tg mice, (a.) Data represented as percentage of diarrhoea occurrence over number of OVA-challenges. (b. and c.) Data represented as mean + SEM; 4-5 mice per group from n=3 experiments. (d. - g.) Photomicrograph 10x magnification; insert 40x magnification. SAL/OVA represents i.p. saline-sensitised, i.g. OVA-challenged mice. OVA/OVA represents i.p. OVA-sensitised, i.g. OVA-challenged mice.
(a) OVA/OVA BALB/c WT
- SAL/OVA BALB/c WT
- OVA/OVA iFABPp IL-9 Tg
- SAL/OVA iFABPp IL-9 Tg

(b) BALB/c WT
- iFABPp IL-9 Tg

(c) BALB/c WT
- iFABPp IL-9 Tg

(d) BALB/c WT
- iFABPp IL-9 Tg

(e) SAL/OVA BALB/c WT

(f) SAL/OVA iFABPp IL-9 Tg

(g) OVA/OVA BALB/c WT

(h) OVA/OVA iFABPp IL-9 Tg
6.3.2 Spontaneous intestinal anaphylaxis in iFABPp IL-9 Tg mice is an antigen-specific acute immunological response.

It has previously been shown in OVA-sensitised, and then OVA-challenged WT mice, that the persistence of diarrhoea was antigen-specific (Brandt, Strait et al. 2003). We demonstrate that OVA-challenge of iFABPp IL-9 Tg mice was sufficient to induce oral antigen-induced intestinal anaphylaxis in the absence of systemic priming. To examine whether oral antigen-induced intestinal anaphylaxis in OVA-challenged iFABPp IL-9 Tg mice was antigen-specific, we administered consecutive OVA-challenges to iFABPp IL-9 Tg mice until they developed diarrhoea and subsequently i.g. administered a BSA bolus of the same antigenic concentration. BSA i.g challenge of iFABPp IL-9 Tg mice did not induce diarrhoea (figure 6.2). Following a subsequent i.g. challenge after the BSA bolus of OVA, diarrhoea was re-established in all iFABPp IL-9 Tg mice (figure 6.2). These results, taken together with the findings that OVA-challenged WT mice in the absence of OVA-sensitisation do not develop diarrhoea, demonstrate that the observed diarrhoea in OVA-challenged iFABPp IL-9 Tg mice was not due to osmotic loading of the GI tract but is indeed an antigen-specific acute immunological response.

6.3.3 Role of Th2 molecules in the development of oral antigen-induced intestinal anaphylaxis.

Anaphylaxis in mice has been shown to be orchestrated by two distinct pathways: IgE/mast cell or IgG/macrophage dependent mechanisms (Finkelman, Rothenberg et al. 2005). Previous investigations have demonstrated oral antigen-induced intestinal anaphylaxis to occur via mast cell/IgE/FcεRIα dependent pathways (Brandt, Strait et al. 2003). The Th2 molecules IL-13 and IL-4Rα have been implicated in isotype switching of B cell antibody production to IgE (Finkelman, Katona et al. 1988; Wynn 2003) and STAT-6 has been demonstrated to be a key pathway for signalling by these Th2 cytokines (Foster, Webb et al. 2003). Employing IL-13, STAT-6, and IL-4Rα deficient animals (IL-13−/−, STAT-6−/−, and IL-4Rα−/−) we assessed the development of diarrhoea, intestinal mast cell levels and associated serum mMCP-1. We observe an ablation in the development of diarrhoea in STAT-6−/− and IL-4Rα−/− OVA-sensitised, OVA-challenged
Figure 6.2 Spontaneous intestinal anaphylaxis in iFABPp IL-9 Tg mice is an antigen-specific acute immunological response.

Diarrhoea occurrence in OVA-challenged BALB/c WT and iFABPp IL-9 Tg mice and subsequently challenged with BSA. Data represented as percentage of diarrhoea occurrence over number of OVA-challenges and then subsequent BSA challenge.
Chapter Six: The mechanisms involved in predisposition of intestinal anaphylaxis

- BALB/c WT
- iFABPp IL-9 Tg

OVA-challenges

% Diarrhoea Occurrence

i.g. BSA challenge
mice compared to the WT OVA-sensitised, OVA-challenged mice (Table 6.1.). In concordance, we see a significant decrease in the number of intestinal mast cells and serum mMCP-1 (Table 6.1.). Antigen specific IgE is also significantly decreased in mice deficient in STAT-6 and IL-4Rα (Figure 6.3. a.). Control (saline (SAL)-sensitised, OVA-challenged) WT and STAT-6⁻/⁻ and IL-4Rα⁻/⁻ mice did not develop diarrhoea, intestinal mastocytosis, increased serum mMCP-1 or antigen-specific IgE (Table 6.1. and figure 6.3. a.). Notably, whilst diarrhoea, intestinal mastocytosis, serum mMCP-1 and OVA-specific IgE are attenuated in OVA-sensitised, OVA-challenged IL-13⁻/⁻ mice in comparison to OVA-sensitised, OVA-challenged WT mice (Table 6.1. and figure 6.3. a.), these levels are significantly increased (p<0.01 in all) in comparison to SAL-sensitised, OVA-challenged IL-13⁻/⁻ mice and OVA-sensitised OVA-challenged STAT-6⁻/⁻ and IL-4Rα⁻/⁻ mice.

To elucidate whether oral antigen-induced intestinal anaphylaxis in iFABPp IL-9 Tg mice is dependent on Th2 signalling driving IgE/mast cell mediated responses, we generated iFABPp IL-9 Tg mice deficient in IL-13, STAT-6 or IL-4Rα. We demonstrate that oral antigen-induced intestinal anaphylaxis in iFABPp IL-9 Tg is ablated in the absence of STAT-6 and IL-4Rα (Table 6.2). Notably, the absence of intestinal anaphylaxis in these mice was associated with the absence of intestinal mastocytosis, serum mMCP-1 and OVA-specific IgE (Table 6.2 and figure 6.3. b.). In the presence of overexpression of IL-9 in the small intestine but in the absence of IL-13, we see a downregulation in the parameters of oral antigen-induced intestinal anaphylaxis [diarrhoea occurrence, mastocytosis in the jejunum, serum mMCP-1 and OVA-specific IgE] (Table 6.2. and figure 6.3. b.).

These studies suggest oral antigen-induced intestinal anaphylaxis is mediated via STAT-6, IL-4Rα and partially IL-13 dependent pathways, via mast cells and IgE.
Table 6.1 Role of Th$_2$ molecules in the development of oral antigen-induced intestinal anaphylaxis.

Diarrhoea, mean number of mast cells per high power field and serum mMCP-1 for BALB/c WT mice and BALB/c mice deficient in IL-13, STAT-6 or IL-4R$\alpha$, following i.p. sensitisation with OVA or saline and subsequently nine i.g. OVA-challenges.
Table 6.1 Role of Th2 molecules in the development of oral antigen-induced intestinal anaphylaxis

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Treatment</th>
<th>% Diarrhoea Occurrence (on day 9)</th>
<th>Mast cells/HPF (mean + SEM)</th>
<th>Serum mMCP-1 (ng/ml) (mean + SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c WT</td>
<td>Vehicle</td>
<td>OVA 0/12</td>
<td>0.68 ± 0.07</td>
<td>32.28 ± 1.954</td>
</tr>
<tr>
<td>BALB/c WT</td>
<td>OVA</td>
<td>OVA 14/14</td>
<td>0.13 ± 0.01</td>
<td>23.91 ± 3.64</td>
</tr>
<tr>
<td>IL-13^--;</td>
<td>Vehicle</td>
<td>OVA 0/8</td>
<td>0.44 ± 0.06</td>
<td>37.90 ± 1.60</td>
</tr>
<tr>
<td>IL-13^--;</td>
<td>OVA</td>
<td>OVA 5/9</td>
<td>0.23 ± 0.017</td>
<td>39.6 ± 5.61</td>
</tr>
<tr>
<td>STAT-6^--;</td>
<td>Vehicle</td>
<td>OVA 0/9</td>
<td>0.26 ± 0.04</td>
<td>26.20 ± 0.91</td>
</tr>
<tr>
<td>STAT-6^--;</td>
<td>OVA</td>
<td>OVA 0/10</td>
<td>0.10 ± 0.03</td>
<td>10.70 ± 0.24</td>
</tr>
<tr>
<td>IL-4Ra^--;</td>
<td>Vehicle</td>
<td>OVA 0/8</td>
<td>0.33 ± 0.04</td>
<td>27.46 ± 2.87</td>
</tr>
<tr>
<td>IL-4Ra^--;</td>
<td>OVA</td>
<td>OVA 0/11</td>
<td>0.11 ± 0.03</td>
<td>19.6 ± 2.37</td>
</tr>
</tbody>
</table>

Chapter Six: The mechanisms involved in predisposition of intestinal anaphylaxis
Table 6.2 Role of Th2 molecules in the development of oral antigen-induced intestinal anaphylaxis in iFABPp IL-9 Tg mice.

Diarrhoea, mean number of mast cells per high power field and serum mMCP-1 for iFABPp IL-9 Tg mice and iFABPp IL-9 Tg mice deficient in IL-13, STAT-6 or IL-4Ra, following i.p. sensitisation with OVA or saline and subsequently nine i.g. OVA-challenges
### Table 6.2 Role of Th2 molecules in the development of oral antigen-induced intestinal anaphylaxis in IFABPp IL-9 Tg mice.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Treatment</th>
<th>% Diarrhoea Occurrence (on day 9)</th>
<th>Mast cells/HPF (mean + SEM)</th>
<th>Serum mMCP-1 (ng/ml) (mean + SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iFABPp IL-9 Tg</td>
<td>Vehicle</td>
<td>15/15</td>
<td>7.20 ± 0.33</td>
<td>152.5 ± 0.95</td>
</tr>
<tr>
<td>iFABPp IL-9 Tg</td>
<td>OVA</td>
<td>12/16</td>
<td>97.52 ± 2.19</td>
<td>83399.48 ± 7880.93</td>
</tr>
<tr>
<td>iFABPp IL-9 Tg x IL-13&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Vehicle</td>
<td>9/10</td>
<td>16.45 ± 0.78</td>
<td>2206.2 + 117.52</td>
</tr>
<tr>
<td>iFABPp IL-9 Tg x IL-13&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>OVA</td>
<td>8/9</td>
<td>105.7 ± 1.57</td>
<td>51757.6 + 10337.43</td>
</tr>
<tr>
<td>iFABPp IL-9 Tg x STAT-6&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Vehicle</td>
<td>0/9</td>
<td>2.93 ± 0.21</td>
<td>87.30 ± 19.92</td>
</tr>
<tr>
<td>iFABPp IL-9 Tg x STAT-6&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>OVA</td>
<td>1/8</td>
<td>11.91 ± 0.66</td>
<td>1168.17 ± 268.46</td>
</tr>
<tr>
<td>iFABPp IL-9 Tg x IL-4Ra&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Vehicle</td>
<td>0/10</td>
<td>3.04 ± 0.14</td>
<td>100.26 ± 15.01</td>
</tr>
<tr>
<td>iFABPp IL-9 Tg x IL-4Ra&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>OVA</td>
<td>0/9</td>
<td>8.05 ± 0.19</td>
<td>808.07 ± 154.09</td>
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</tbody>
</table>
Figure 6.3 Oral allergen-induced intestinal anaphylaxis is mediated by an IgE-dependent pathway.

(a.) OVA-specific serum IgE in BALB/c WT and STAT-6-, IL-4Rα- and IL-13 deficient mice after OVA or SAL-sensitisation and subsequent OVA-challenge and (b.) OVA-specific serum IgE in iFABPp IL-9 Tg and iFABPp IL-9 Tg x STAT-6-, iFABPp IL-9 Tg x IL-4Rα- and iFABPp IL-9 Tg x IL-13 deficient mice after OVA or SAL i.p. sensitisation and subsequent i.g. OVA-challenge. (a. and b.) Data represented as mean ± SEM, n = 3 experiments, 4-5 mice per group.
Chapter Six: The mechanisms involved in predisposition of intestinal anaphylaxis

(a) HBALB/C WT
- STAT-6
- IL-13

(b) iFABp IL-9 Tg
- STAT-6
- IL-4Ra
- IL-13

(i.g) OVA sensitisation (i.p) SAL OVA

OD450: Serum OVA-specific IgE (1:100)

SAL OVA OVA OVA OVA OVA OVA OVA

(p<0.001; p<0.001)
(p<0.01; p<0.001)
(p<0.05; p<0.01)
(p<0.001; p<0.001)
(p<0.01; p<0.01)
(p<0.01; p<0.01)
6.4 Discussion

A model of oral antigen induced intestinal anaphylaxis has been employed to assess the role of IL-9 and mast cells in intestinal hypersensitivity and oral sensitisation. We show that overexpression of IL-9 in the small intestine results in a more severe oral antigen-induced intestinal anaphylactic reaction, and was sufficient to predispose to oral antigen-induced intestinal anaphylaxis, that is antigen-specific, in the absence of systemic priming. This is a novel finding in experimental models of allergic diarrhoea and importantly more closely mimics the development of food allergic responses in humans. These results demonstrate a central role for IL-9 in the oral-antigen intestinal sensitisation phase of mast cell-mediated intestinal anaphylaxis.

It has been previously demonstrated that oral antigen-induced intestinal anaphylaxis is associated with a CD4^+ Th2-cytokine production (Brandt, Strait et al. 2003). The demonstration of IL-4Rα-, STAT-6-dependent and partial IL-13-dependent intestinal anaphylaxis in iFABPp IL-9 Tg mice suggests that IL-9 may drive the generation of an antigen-specific CD4^+ Th2-cell response. This was confirmed ex vivo by detection of OVA-specific Ig in iFABPp IL-9 Tg mice following 9 i.g. OVA-challenges. Recent investigations employing a Leishmania major infection model have demonstrated a role for IL-9 in the promotion of a detrimental Th2/type 2 intestinal response (Arendse, Van Snick et al. 2005). These studies demonstrated that IL-9 neutralisation led to the development of a protective Th1-immune response. The ability of IL-9 to influence T-helper cell-dichotomy has been associated with alteration in macrophage phenotype from alternative to classical macrophage activation (Arendse, Van Snick et al. 2005). The contribution of macrophages to the predisposition to oral antigen induced intestinal anaphylaxis in the unprimed iFABPp IL-9 Tg mice requires further investigation.

Interestingly, we demonstrate that IL-9 overexpression in the small intestine, resulting in intestinal mastocytosis, can occur in the absence of IL-13, STAT-6 and IL-4Rα. Moreover, intestinal mast cell levels in iFABPp IL-9 Tg mice backcrossed onto the IL-13, STAT-6 or IL-4Rα backgrounds were significantly higher than those observed in IL-13, STAT-6 and IL-4Rα deficient mice. Consistent with this observation, previous studies have demonstrated that GI nematode-induced intestinal mastocytosis in the combined IL-4-, IL-5-, and IL-13-deficient mice was dependent on IL-9 (Fallon, Jolin
et al. 2002). Importantly, whilst mast cell levels in iFABPp IL-9 Tg/IL-13-deficient, STAT-6 deficient or IL-4Rα deficient mice were elevated compared to the respective factor deficient mice, these levels were reduced by ~50% as compared to iFABPp IL-9 Tg IL-13, STAT-6 and IL-4Rα sufficient mice. These studies suggest that optimal intestinal mastocytosis requires factors dependent on IL-13, IL-4Rα and STAT-6 signalling. Antibody neutralisation of IL-3 and IL-4 has been shown to block GI nematode-induced intestinal mastocytosis by ~85% (Madden, Urban et al. 1991). We speculate that IL-9 is a potent inducer of intestinal mastocytosis, however, it may act in synergy with other factors, most likely IL-3 and IL-4, for maximal effect.

Employing a passive model of systemic anaphylaxis, investigators have recently demonstrated susceptibility to fatal systemic anaphylaxis in IL-9 Tg mice (eIL-9Tg mice) (murine pim-1 promoter containing an Eμ enhancer and moloney murine leukemia virus long terminal repeat (Renauld, van der Lugt et al. 1994)) (Godfraind, Louahed et al. 1998) and mice administered recombinant IL-9 suggesting that IL-9 promotes systemic anaphylaxis. However, these investigations demonstrated that IL-9R deficient mice were also susceptible to both passive and cutaneous anaphylaxis. This suggests that IL-9 promotes systemic anaphylaxis however it is not essential for systemic anaphylaxis response (Knoops, Louahed et al. 2005). Employing IL-9+/- mice we demonstrate that IL-9 is critical for oral allergen-induced intestinal anaphylaxis (chapter 4). A possible explanation for these contrasting findings is the pathways involved in systemic and intestinal anaphylaxis. Experimental studies employing gene-deficient mice have demonstrated that intestinal anaphylaxis is mediated via IL-4/IL-4Rα-, mast cell-, FcεRI- and IgE-dependent pathways (Strait, Morris et al. 2002). In contrast, murine systemic anaphylaxis can occur via an IgE-independent pathway (Oettgen, Martin et al. 1994; Miyajima, Dombrowicz et al. 1997; Strait, Morris et al. 2002). This pathway of systemic anaphylaxis can occur in the absence of mast cells, FcεRI, and IgE. This second pathway is complement independent but requires IgG antibody, macrophages, FcγRIII and PAF (but not histamine, serotonin, or leukotriene) (Strait, Morris et al. 2002). Notably, most studies in which mice are primed by immunization with antigen and challenged weeks later with the same antigen (as was performed in the Knoops et al., study), the response appears to activate both IgE-dependent and IgE-independent anaphylaxis pathways. Therefore, pathways independent of IL-9, mast cells and IgE may contribute to the systemic anaphylaxis,
whereas mast cell/IgE dependent pathways mediate intestinal anaphylaxis. Understanding the differences between systemic anaphylaxis and intestinal anaphylaxis is an important question because patients with food allergy often possess GI symptoms without systemic manifestations.
Chapter Seven

The role of CD4+ Th2 cells in oral sensitisation and intestinal anaphylaxis
7.1 Introduction

The gastrointestinal (GI) tract is the largest immunologic organ in the body. It has constant exposure to a myriad of dietary proteins. However, despite the significant dietary antigenic exposure only a small percentage of this is sampled by the intestinal immune system due to restrictive sites of antigen sampling in the gut and maintenance of barrier integrity. Of the antigens that are sampled, the host should be able to develop an active non-response to antigenic exposure, known as oral tolerance. Disturbances at differing steps in the path to oral tolerance induction have been described in food hypersensitivity resulting in the establishment of an aberrant immunological response. It is postulated that a breakdown in oral tolerance mechanisms or the failure to induce oral tolerance results in hypersensitivity (Chehade and Mayer 2005). Recent investigations using animal models have shown antigen processing in the lumen and sites of antigen sampling are most likely the causes of a breakdown in oral tolerance mechanisms (Berin, Kiliaan et al. 1998; Barone, Reilly et al. 2000; Hogan, Mishra et al. 2000; Rescigno, Urbano et al. 2001; Spahn, Fontana et al. 2001; Brandt, Strait et al. 2003; McDermott, Bartram et al. 2003; Untersmayr, Scholl et al. 2003; Kraus, Toy et al. 2004; Man, Bertelli et al. 2004).

In chapter 5, it is demonstrated that increased IL-9 expression in the small intestine results in intestinal permeability. Mast cells have previously been shown to disrupt epithelial barrier function (Berin, Kiliaan et al. 1998; Brandt, Strait et al. 2003; McDermott, Bartram et al. 2003). Clinically, investigators have shown intestinal permeability to predispose to GI disease. Children presenting with cow’s milk sensitivity exhibited a three-fold rise during a provocation intestinal permeability test (Dupont, Barau et al. 1989) and this has subsequently been supported in adults with adverse reactions to food (Ventura, Polimeno et al. 2006). It has also been reported that patients with inflammatory bowel disease (IBD) fail to induce oral tolerance to soluble protein. This may reflect a defect in mucosal suppression, however, may alternatively reflect a defect in mucosal barrier function (Kraus, Toy et al. 2004), a well documented clinical manifestation in IBD patients (Fiocchi 1998; Blumberg, Saubermann et al. 1999; Hendrickson, Gokhale et al. 2002).

In chapter 6, mice overexpressing IL-9 in the small intestine are susceptible to oral
antigen-induced intestinal anaphylaxis in the absence of systemic sensitisation. Our investigations demonstrate that intestinal anaphylaxis is antigen specific and IL-9 susceptibility is mediated via IL-13/STAT-6/IL-4Rα mechanisms, most likely working in synergy. Taken together, we hypothesise mast cell mediated intestinal permeability predisposes to oral antigen sensitisation and intestinal anaphylaxis.
7.2 Materials and Methods

7.2.1 Reagents

Mouse IgEαTNP (IGEL 2a) (Rudolph, Burrows et al. 1981) from ATCC were prepared as described (Finkelman, Kessler et al. 1981; Strait, Morris et al. 2002). TNP-BSA was produced by mixture of 50 mg of BSA in 5 ml of bicarbonate buffer with serial 4-fold dilutions of TNP-succinyl-Osu (starting concentration, 25 mg/ml) in DMSO. TNP-BSA-NIP was produced by a mixture of NIP-succinyl-Osu (Biosearch Technologies Inc.) with TNP0.4-BSA at a 1:2 weight ratio in DMSO and dialyzing as above. TNP-BSA was biotinylated with E-Z Link sulfo-NHSbiotin (Pierce) at a 10:1 weight ratio in DMSO.

7.2.2 Mice

As described in section 4.2.2.

7.2.3 Protocol

As described in section 3.2.2

7.2.4 ELISA measurements.

As described in section 3.2.5 and section 4.2.5.

7.2.5 Activated CD4+ Th2 response

As described in section 3.2.7.

7.2.6 Proliferation Assay

As described in section 3.2.8.

7.2.7 Mononuclear cell isolation from jejunal tissue

Approximately 5 cm of jejunum was excised and flushed with 1ml of calcium and magnesium free Hank's buffered salt solution (CMF-HBSS). The jejunum was
dissected longitudinally and placed in 5 ml CMF-HBSS and shaken vigorously for 30 seconds at room temperature to remove luminal debris. Tissue was then incubated in CMF-HBSS containing 10% foetal calf serum (FBS), 25 mM HEPES and 5 mM EDTA for 10 minutes at 37°C and shaken in 5 minute intervals to remove epithelia and intraepithelial lymphocytes. The tissue was then washed and incubated in CMF-HBSS to block any remaining EDTA activity. The remaining tissue was cut into small pieces and incubated with incomplete RPMI-1640 supplemented with Collagenase A (2.4 mg/ml) for 30 minutes at 37°C. The cell suspension was filtered using sterile gauze, washed in incomplete RPMI-1640, centrifuged and the remaining pellet was resuspended in RPMI-1640 + 10% FCS. Mononuclear cell suspension was used in in vitro stimulation assays.

7.2.8 In vitro stimulation of jejunum mononuclear cells

Mononuclear cells were plated at 5x10^5 cells/well in a 96 well plate. Plated cells were cultured for 6 hours in the presence of IL-2 (10ng/ml, BD Pharmingen) and αCD3/αCD28 (5μg/ml and 1μg/ml respectively, BD Pharmingen). Intracellular protein transport was prevented with monensin (1000x, 1:1000 dilution, ebioscience) during the 6-hour culture.

7.2.9 Fluorescent-activated cell sorter (FACS) analysis

CD4^+ IL-4^+ cells were identified with PE anti-mouse CD4 (L3T4; BD Biosciences PharMingen) and PE-Cy7 anti-mouse IL-4 (BVD6-24G2; ebioscience). The following were used as appropriate isotype controls: PE Rat IgG2b (A95-1; BD Biosciences PharMingen) and PE-Cy7 Rat IgG1 (R3-34; BD Biosciences PharMingen).

7.2.10 Lightcycler PCR

iFABPp IL-9 Tg mice (n=6) and WT (n=6) were obtained and i.g. OVA challenged as per protocol, section 3.2.2. Intestinal samples were harvested. RNA was isolated from intestinal samples and cDNA was generated by standard procedures as described in section 3.2.4. HRPT and mIL-4 were quantified by real-time PCR as described in section 3.2.4. Results were then normalized to HPRT amplified from the same cDNA mix and expressed as fold induction compared with the controls. cDNAs were amplified using the commercially available primers following the manufacturer’s instructions.
Quantitative IL-4 PCR analysis was performed by Richard Ahrens in Simon Hogan’s Laboratory, Allergy and Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati OH, USA.

7.2.11 Passive anaphylaxis model

Mice were i.v. primed with 10µg of IgEαTNP then challenged i.v. 24 hours later with 100 ng TNP-BSA.

7.2.12 Anaphylaxis

The severity of the anaphylactic shock was assessed by change in rectal temperature (Physitemp model BAT-12), as previously described (Strait, Morris et al. 2002; Strait, Morris et al. 2003)

7.2.13 Statistical Analysis

Data are expressed as mean ± standard error (SEM). Statistical significance comparing different sets of mice was determined by Student’s t-test. In experiments comparing multiple experimental groups, statistical differences between groups were analyzed using the one-way ANOVA nonparametric Bonferroni post-test. P < 0.05 was considered significant. All analyses were performed using Prism 4.0 software.
Chapter Seven: The role of CD4+ Th2 cells in oral sensitisation and intestinal anaphylaxis

7.3 Results

7.3.1 Intestinal expression of IL-9 predisposes to oral antigen sensitisation.

The demonstration of Th2 dependent susceptibility to oral antigen-induced intestinal anaphylaxis in unsensitised iFABPp IL-9 Tg mice led us to hypothesise that oral antigen challenge of these transgenic mice was by promoting the development of oral antigen sensitisation. To test this hypothesis we challenged iFABPp IL-9 Tg and WT mice orally with OVA and examined intestinal IL-4 and IFN-γ protein levels. We show OVA-challenges of WT mice do not induce significant changes in intestinal IL-4 or IFN-γ (Figure 7.1. a. and b.). Furthermore, intestinal OVA-specific IgG1 and total IgE remains comparable to naive WT mice (Figure 7.1. c. and d.). In contrast, OVA-challenged iFABPp IL-9 Tg mice had a significant increase in intestinal IL-4 levels. (Figure 7.1. b.) The increases in IL-4 were associated with increased OVA-specific IgG1 and total IgE in the intestine of OVA-challenged iFABPp IL-9 Tg mice (Figure 7.1. c. and d.). This data collectively demonstrates intestinal IL-9 expression results in oral antigen-induced type-2 sensitisation.

7.3.2 Role of CD4+ T cells in the regulation of oral antigen sensitisation.

The demonstration that OVA-challenges to iFABPp IL-9 Tg mice results in antigen induced, Th2 mediated, oral sensitisation led us to examine the CD4+ cytokine response in the draining mesenteric lymph nodes (MLN), to assess the local response and the spleen, for a global immune characterisation, of OVA-challenged WT and iFABPp IL-9 Tg mice. We assessed production of IL-4, IL-5, IL-9, IL-13 and IFN-γ from T-cells stimulated in vitro with either 100μg/ml of OVA or aCD3/aCD28 (5μg/ml and 1μg/ml respectively) and normalised with concurrent identical cultures with no stimulus. We examined cytokine production after one and five i.g. OVA-challenges. We chose these time points as diarrhoea in unprimed, OVA-challenged iFABPp IL-9 Tg mice begins to develop after the 6th i.g. OVA challenge and as such, priming of lymphocytes to an aberrant Th2 immune response, in theory, should have occurred after five i.g. challenges, and could have been possible even after one i.g. challenge. Surprisingly, we did not find any differences in cytokine production from lymphocytes cultures from the spleen or MLN of iFABPp IL-9 Tg mice and WT mice after one or five i.g. OVA-
Figure 7.1 Overexpression of IL-9 in the intestine increases local Th2 responses after OVA i.g. challenge.

(a.) IL-4 and (b.) IFN-γ protein levels in jejunal lysates from BALB/c WT and iFABPp IL-9 Tg mice after 5 i.g. OVA-challenges and (c.) antigen-specific IgG, and (d.) antigen-specific IgE protein levels in jejunal lysates from BALB/c WT and iFABPp IL-9 Tg mice under basal conditions and after 5 i.g. OVA-challenges. (a. and b.) Data expressed as protein level in pg/ml. The black line represents the mean value in each group. (c. and d.) Data represented as mean ± SEM, 4-5 mice per group from at least n = 2 experiments.
Chapter Seven: The role of CD4+ Th2 cells in oral sensitisation and intestinal anaphylaxis

(a) 
- BALB/c WT
- iFABPp IL-9 Tg

(b) 
- BALB/c WT
- iFABPp IL-9 Tg

(c) 
IL-4 μg/ml per mg of protein

(d) 
IFN-γ (μg/ml) per mg of protein

- naive
- 5x i.g. OVA

- BALB/c WT
- iFABPp IL-9 Tg

- naive
- 5x i.g. OVA

OD450: OVA-Specific IgG1

OD450: Total IgE

ND

naive 5x i.g. OVA

p<0.05

p<0.01

p<0.05

p<0.01
We were next interested in investigating intestinal CD4\(^+\) T cells in the orchestration of oral antigen sensitisation. As shown in Figure 7.1. a., we observe a significant increase in intestinal protein IL-4 levels in iFABPp IL-9 Tg mice after 5 i.g. OVA-challenges. We further confirmed this with increased IL-4 mRNA expression in the intestine of OVA-challenged iFABPp IL-9 Tg compared to OVA-challenged WT mice (Figure 7.2. a.). To assess the role of intestinal CD4\(^+\) T cells, we isolated jejunum lamina propria mononuclear cells and then cultured these in the presence of IL-2 and \(\alpha\)CD3/\(\alpha\)CD28 to activate T cells. The *in vitro* stimulation was conducted in the presence of monensin, an inhibitor of intracellular protein transport. Incubation of cells in culture with monensin leads to blockade of protein transport to the Golgi complex (GC) and accumulation of proteins in the endoplasmic reticulum (ER). Addition of monensin during *in vitro* activation of cells results in enhanced detection of intracellular cytokines. We show that the percentage of CD4\(^+\) IL-4\(^+\) T cells from the lamina propria of OVA-challenged iFABPp IL-9 Tg mice following *in vitro* stimulation was approximately 3 fold greater than that in repeated oral antigen challenged WT mice (Figure 7.2. b.). These studies suggest that intestinal expression of IL-9 predisposes to oral antigen CD4\(^+\) Th\(_2\)-type sensitisation.

7.3.3 *Intestinal IL-9/mast cell mediated intestinal permeability predisposes to oral antigen sensitisation in iFABPp IL-9 Tg mice.*

Our next question to address was to determine the involvement of mast cell mediated intestinal permeability in oral antigen sensitisation – was this predisposition to intestinal anaphylaxis in iFABPp IL-9 Tg mice mast cell mediated?

In chapter 5 we have demonstrated that iFABPp IL-9 Tg mice exhibit intestinal mastocytosis and intestinal permeability. Furthermore, we have demonstrated the intestinal permeability to be mast cell dependent. Previous investigations have shown intestinal permeability to predispose to GI disease, and as such, we were interested in assessing the role mast cells play in oral antigen sensitisation. To investigate our hypothesis that mast cell mediated intestinal permeability was driving the development of oral antigen sensitisation, we treated iFABPp IL-9 Tg and WT mice with cromolyn
Table 7.1 OVA-challenge of iFABPp IL-9 Tg mice does not predispose to a Th$_2$ response in the draining mesenteric lymph nodes or the spleen.

IL-4, IL-5, IL-9, IL-13 and IFN-$\gamma$ protein levels (normalised to baseline lymphocyte cultures; no stimulation) from in vitro stimulated lymphocytes of the spleen and mesenteric lymph nodes of BALB/c WT and iFABPp IL-9 Tg mice. Data represents mean ± SEM, conducted in triplicate n=4-5 mice per group.
Table 7.1 OVA-challenge of iFABPp IL-9 Tg mice does not predispose to a Th2 response in the draining mesenteric lymph nodes or the spleen.

<table>
<thead>
<tr>
<th></th>
<th>BALB/c WT [100µg/ml OVA]</th>
<th>iFABPp IL-9 Tg [100µg/ml OVA]</th>
<th>BALB/c WT [αCD3/αCD28]</th>
<th>iFABPp IL-9 Tg [αCD3/αCD28]</th>
</tr>
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<tr>
<td><strong>1x i.g. OVA challenge Spleenocytes</strong></td>
<td></td>
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<tr>
<td>IL-4 pg/ml</td>
<td>235.4 ± 62.84</td>
<td>131.0 ± 19.34</td>
<td>222.9 ± 34.82</td>
<td>155.6 ± 17.80</td>
</tr>
<tr>
<td>IL-5 pg/ml</td>
<td>2050 ±100.0</td>
<td>1175 ±172.2</td>
<td>1193 ±186.0</td>
<td>2475 ± 207.2</td>
</tr>
<tr>
<td>IL-9 ng/ml</td>
<td>56.09 ±10.02</td>
<td>53.92 ±13.42</td>
<td>74.04 ±21.13</td>
<td>67.60 ± 18.46</td>
</tr>
<tr>
<td>IL-13 pg/ml</td>
<td>204.3 ±21.97</td>
<td>270.7 ±10.22</td>
<td>279.4 ±23.70</td>
<td>383.8 ± 21.16</td>
</tr>
<tr>
<td>IFN-γ pg/ml</td>
<td>255.3 ± 48.43</td>
<td>265.3 ± 23.90</td>
<td>909.9 ±124.2</td>
<td>870.6 ± 90.19</td>
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<tr>
<td><strong>MLN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4 pg/ml</td>
<td>187.0 ± 20.78</td>
<td>126.6 ± 235.9</td>
<td>312.0 ± 17.10</td>
<td>246.3 ± 17.36</td>
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<td>IL-5 pg/ml</td>
<td>2070 ±104.9</td>
<td>3302 ±175.7</td>
<td>2637 ±122.5</td>
<td>3131 ±259.1</td>
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<td>IL-9 ng/ml</td>
<td>17.61 ±1.174</td>
<td>70.97 ±11.37</td>
<td>50.27 ±6.924</td>
<td>75.36 ± 9.573</td>
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<tr>
<td>IL-13 pg/ml</td>
<td>343.4 ±53.29</td>
<td>620 ±21.10</td>
<td>504.2 ±41.15</td>
<td>706 ± 45.54</td>
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<tr>
<td>IFN-γ pg/ml</td>
<td>109.0 ± 23.00</td>
<td>63.82 ± 17.19</td>
<td>651.4 ± 63.88</td>
<td>801.2 ± 103.6</td>
</tr>
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<td><strong>5x i.g. OVA challenge Spleenocytes</strong></td>
<td></td>
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<td></td>
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<tr>
<td>IL-4 pg/ml</td>
<td>277.5 ± 10.11</td>
<td>218.5 ± 29.44</td>
<td>141.6 ± 493.6</td>
<td>152.5 ± 42.44</td>
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<tr>
<td>IL-5 pg/ml</td>
<td>2788 ±218.4</td>
<td>1172 ±217.2</td>
<td>1251 ± 335.1</td>
<td>992.6 ± 135.9</td>
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<td>IL-9 ng/ml</td>
<td>58.14 ±13.15</td>
<td>87.2 ±27.77</td>
<td>56.57 ±9.837</td>
<td>94.70 ± 22.68</td>
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<tr>
<td>IL-13 pg/ml</td>
<td>2008 ±678.2</td>
<td>1723 ±395.6</td>
<td>2206 ±607.1</td>
<td>2029 ± 133.5</td>
</tr>
<tr>
<td>IFN-γ pg/ml</td>
<td>69.66 ± 11.90</td>
<td>93.61 ± 30.79</td>
<td>818.2 ± 29.86</td>
<td>376.8 ± 56.03</td>
</tr>
<tr>
<td><strong>MLN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4 pg/ml</td>
<td>136.4 ± 34.69</td>
<td>174.5 ± 18.71</td>
<td>134.5 ± 31.11</td>
<td>203.1 ± 16.80</td>
</tr>
<tr>
<td>IL-5 pg/ml</td>
<td>1315 ±92.78</td>
<td>1402 ±15.24</td>
<td>1994 ±20.72</td>
<td>1393 ± 31.29</td>
</tr>
<tr>
<td>IL-9 ng/ml</td>
<td>11.97 ±20.95</td>
<td>70.33 ±14.88</td>
<td>69.4 ± 7.710</td>
<td>80.58 ± 11.89</td>
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<tr>
<td>IL-13 pg/ml</td>
<td>1378 ± 30.84</td>
<td>822.4 ± 30.52</td>
<td>1280 ±34.28</td>
<td>1491 ± 60.93</td>
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<td>IFN-γ pg/ml</td>
<td>126.20 ± 12.39</td>
<td>130.33 ± 9.828</td>
<td>395.1 ± 26.83</td>
<td>451.2 ± 26.02</td>
</tr>
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</table>
Figure 7.2 IL-9 predisposes to oral antigen CD4⁺ Th2-type sensitisation.

(a.) Fold increase in IL-4 mRNA expression in iFABPp IL-9 Tg compared to BALB/c WT at baseline and after 3x i.g. OVA challenges and (b.) percentage of CD4⁺ intracellular IL-4⁺ T cells in the intestine of iFABPp IL-9 Tg and BALB/c WT mice after OVA challenge and subsequent isolation and in vitro stimulation. (a.) Data normalised to HRPT expression. Data expressed as IL-4/HRPT ratio fold change compared to BALB/c WT. (b.) Data represented as percentage of CD4⁺/IL-4⁺ T cells. (a. and b.) Data represents mean ± SEM, conducted in triplicate, n=4-5 mice per group.
Chapter Seven: The role of CD4⁺ Th₂ cells in oral sensitisation and intestinal anaphylaxis

(a) BALB/c WT

(b) BALB/c WT

Figure captions:
(a) Graph showing the fold increase in IL-4/HPRT ratio in naïve and 3 x i.g. OVA-treated animals. The y-axis represents the fold increase, with error bars indicating variability. The x-axis has two groups: naïve and 3 x i.g. OVA.

(b) Graph showing the percentage of CD4⁺ IL-4⁺ cells. The y-axis represents the percentage of CD4⁺ IL-4⁺ cells, with error bars indicating variability. The x-axis has two groups: naïve and 3 x i.g. OVA. The p-value is given as p<0.05.
Na or vehicle to block basal mast cell activation and permeability, as shown in chapter 5. We then began i.g. OVA-challenge of these mice whilst continuing to stabilise mast cells with cromolyn Na, over the next 6 days (3x i.g. OVA-challenges). (Figure 7.3. a.). Consistent with our previous findings, i.g OVA- challenged, vehicle-treated iFABPp IL-9 Tg mice induced diarrhoea (Figure 7.3. b.). OVA-challenged cromolyn Na treated iFABPp IL-9 Tg mice did not develop diarrhoea (Figure 7.3. b.) We examined serum IgE levels and demonstrate elevated serum total IgE in OVA-challenged, vehicle-treated iFABPp IL-9 Tg compared to OVA-challenged, cromolyn Na-treated iFABPp IL-9 Tg mice (Figure 7.3. c.).

To confirm the inhibition of diarrhoea was not due to complete ablation of mast cell activity during the effector phase by cromolyn Na mediated suppression, we examined serum mMCP-1 levels. We show that serum mMCP-1 levels in iFABPp IL-9 Tg mice treated with cromolyn Na were comparable to vehicle treated iFABPp IL-9 Tg mice after OVA-challenge (Figure 7.3. d.) To confirm that administration of cromolyn Na did not block mast cell function we performed passive sensitisation in control- and cromolyn Na-treated iFABp IL-9 Tg mice. Following the 9th OVA-challenge, the mice were i.v. administered 10 μg anti-2,4,6-trinitophenyl (TNP)-IgE and subsequently challenged i.v. with TNP-BSA. Body temperature was measured over the next 60 minutes following i.v. challenge. We show TNP-BSA challenge of naïve iFABPp IL-9 Tg mice alone did not affect body temperature (maximum temperature decrease, presented at 20 minutes, Figure 7.3. e). In contrast, TNP-BSA i.v. challenge of iFABPp IL-9 Tg mice i.v. administered IgEαTNP induced a significant decrease in body temperature, suggestive of systemic anaphylaxis. Similarly, iFABPp IL-9 Tg mice that had been administered cromolyn Na or vehicle and OVA-challenged before TNP-BSA and i.v. challenge with IgEαTNP induced an equivalent decrease in body temperature, demonstrating mast cell effector activity (Figure 7.3. e.). Notably, the decrease in body temperature in OVA-challenged, vehicle treated TNP-BSA i.v. administered subsequent to IgEαTNP i.v. challenged iFABPp IL-9 Tg mice had a significantly greater body temperature decrease that naïve or cromolyn Na treated and OVA-challenged, TNP-BSA i.v. administered subsequent to IgEαTNP i.v. challenged iFABPp IL-9 Tg mice (Figure 7.3. e.). Collectively these studies demonstrate a link between mast cell mediated intestinal permeability promoting oral sensitisation and subsequent predisposition to intestinal anaphylaxis.
Figure 7.3 Treatment with mast cell stabilising agent Cromolyn sodium protects against antigen sensitisation.

(a.) Experimental protocol, (b.) diarrhoea occurrence, (c.) antigen-specific IgE (d.) serum mouse mast cell protease-1 for iFABPp IL-9 Tg and BALB/c WT mice treated with control or the mast cell stabilising agent, Cromolyn sodium and subsequently OVA-challenged, (e.) maximal temperature decrease for iFABPp IL-9 Tg and BALB/c WT mice treated with control or the mast cell stabilising agent, Cromolyn sodium, subsequently i.g. OVA-challenged or control and treated with passive anaphylaxis mediators. (b.) Data represented as percentage of diarrhoea occurrence over number of OVA-challenges. (c. and d.) Data represented as mean ± SEM, 4-5 mice per group. (e) Data represented as temperature drop over time in minutes, ± SEM, 4-5 mice per group
Chapter Seven: The role of CD4+ Th2 cells in oral sensitisation and intestinal anaphylaxis

(b) 

- BALB/c WT i.p. PBS/i.g. OVA
- iFABPp IL-9 Tg i.p. PBS/i.g. OVA
- iFABPp IL-9 Tg i.p. Cromolyn Na/i.g. OVA

I.g. OVA challenge (50mg/250µL)

Days -3 -2 -1 0 1 2 3 4 7 9 11 14 16 18 19

I.p. Cromolyn Na (300µg/200µL) or PBS control (200µL) every 12 hours

(c) 

- BALB/c WT
- iFABPp IL-9 Tg

Serum IgE (µg/mL)

p<0.05

I.p. PBS + + -
I.p. cromolyn Na - - +
I.g. OVA + + +

(e) 

A. OVA + + + +
IgE TNP - - + +
Cromolyn Na - - - +
7.4 Discussion

These investigations demonstrate that IL-9/mast cells can predispose to oral sensitisation and subsequent development of intestinal anaphylaxis. Notably, the predisposition to oral antigen sensitisation in iFABPp IL-9 Tg mice was associated with increased intestinal permeability. Impairment of intestinal barrier function “leaky gut” as been implicated as a critical determinant in the predisposition to a number of GI diseases including IBD and food allergy (Dupont, Barau et al. 1989; Kraus, Toy et al. 2004; Takeuchi, Maiden et al. 2004; Buhner, Buning et al. 2006; Ventura, Polimeno et al. 2006). We (chapter 5) and others have shown employing mast cell-stabilising agents and mast cell deficient mice have provided corroborative evidence demonstrating mast cell involvement in intestinal epithelial permeability (Perdue and Gall 1986; Crowe, Sestini et al. 1990; Yu and Perdue 2001). Consistent with these observations was that the increased intestinal permeability and predisposition to oral antigen sensitisation was associated with intestinal mastocytosis.

Currently we do not fully understand the underlying molecular basis of increased intestinal permeability in iFABPp IL-9 Tg mice; however our data suggests that this is a mast cell-dependent pathway. We demonstrate IL-9 mediated intestinal permeability is mast cell dependent, as mast cell stabilisation resulting in attenuation of mast cell activity prevents oral sensitisation, suggesting a defect in barrier integrity can predispose to oral sensitisation and the development of allergic food reactions. It has been previously demonstrated that there is a role for PAF and serotonin-dependent pathways in the regulation of intestinal permeability during the effector phase of oral-allergen-induced intestinal anaphylaxis (Kubes, Arfors et al. 1991; Kanwar, Wallace et al. 1994; Tan, Chang et al. 2000; Brandt, Strait et al. 2003). Furthermore, a number of cytokines secreted by mast cells including IL-4 and TNFα have also been shown to directly regulate permeability (Metcalf, Baram et al. 1997; MacDonald, Pick et al. 1998). We have demonstrated elevated levels of serum mMCP-1 and TNFα in iFABPp IL-9 Tg mice (chapter 5). Future studies are needed to dissecting out the contribution of TNFα, mMCP-1 and mast cells in the increased intestinal permeability in iFABPp IL-9 Tg mice.

We demonstrate here that IL-9 overexpression was sufficient to predispose to intestinal anaphylaxis in the absence of sensitisation with adjuvant. Furthermore, we show this
response is regulated via CD4\(^+\) Th\(_2\) signalling. Consistent with this observation is the demonstration of a role for IL-9 in the promotion of a Th\(_2\) intestinal response during experimental *Leishmania major* infection (Arendse, Van Snick et al. 2005). In addition, several studies have shown antigen-primed T cells in patients with food allergy (Eigenmann 2002). These and others have also examined food antigen induced cytokine production in T cells and show allergic patient T helper cells with a Th\(_2\) profile, secreting IL-4, IL-5 and IL-13 (Higgins, Lamb et al. 1995; de Jong, Spanhaak et al. 1996; Eigenmann, Huang et al. 1996; Katsuki, Shimojo et al. 1996; Eigenmann 2002; Turcanu, Maleki et al. 2003).

Our studies and previous investigations linking IL-9 with atopy identify IL-9/mast cell-regulated pathways as a critical determinant in the induction of food allergic reactions and these findings highlight the critical demand for assessment of IL-9 expression and its function in patients presenting with food allergic reactions.
Chapter Eight

General Discussion
8.1 Chemokines, cytokines, cells and their receptors: The roles they play in the induction and exacerbations of gastrointestinal inflammation.

The central aims of this thesis were to investigate 1) the role of β7-integrin during inflammation and to identify candidate integrins and their receptors in the recruitment of eosinophils to the large intestine; 2) the role of ICAM-1 in eosinophil recruitment into the colon during homeostasis and disease; 3) the role of the Th2 cytokine, IL-9, in the development of experimental oral antigen-induced intestinal anaphylaxis; 4) the individual contribution of IL-9 to other discreet aspects of disease pathogenesis of oral antigen-induced intestinal anaphylaxis employing a transgenic approach; 5) the effect of overexpression of IL-9 in the small intestine during oral antigen-induced intestinal anaphylaxis and the consequence of Th2-signalling (IL-4Rα, IL-13 and STAT-6) in the mediation of the immunopathogenesis of intestinal anaphylaxis, and 6) the requirement of mast cell mediated intestinal permeability in the occurrence of oral antigen sensitisation and subsequent development of intestinal anaphylaxis. This chapter intends to summarise the major findings described within this thesis and to discuss the possible implications of these data to understanding the cellular and molecular mechanisms underlying IBD and intestinal anaphylaxis.

8.2 The role of chemokine signalling, adhesion molecules and integrin receptors in transmigration of eosinophils into the colon during injury and inflammation.

In an attempt to elucidate the cellular and molecular mechanisms involved in the immunopathogenesis of UC, a mouse model of colonic injury was developed that mimicks certain pathophysiologic characteristics observed in human UC (Blumberg, Saubermann et al. 1999; Strober, Fuss et al. 2002). Oral administration of DSS in drinking water results in acute inflammation of the colon, pronounced weight loss and bloody diarrhoea (Blumberg, Saubermann et al. 1999; Strober, Fuss et al. 2002). Furthermore, eosinophils were observed throughout the mucosa and submucosa in DSS-treated mice (Forbes, Murase et al. 2004). In particular, eosinophil numbers were significantly increased in the colon of DSS-treated mice as compared with control-treated mice (Forbes, Murase et al. 2004). The colonic eosinophilia was associated with eosinophil-derived EPO in the lumen of the colon of DSS-treated mice (Forbes, Murase et al. 2004).
8.2.1 Eosinophil recruitment in the intestine is compartmentalised.

We hypothesised that eosinophil accumulation into the colon was regulated by the $\beta_7$-integrin. This rationale was based upon this molecule has been shown to be critically involved in leukocyte adherence and recruitment into the GI tract and in eosinophil recruitment into the small intestine (Butcher, Williams et al. 1999; Mishra, Hogan et al. 2002). We have shown that eotaxin-1 selectively upregulates surface expression of ICAM-1 binding integrins ($\beta_2$, $\alpha_L$ and $\alpha_M$) on eosinophils and that eotaxin-1 mediated eosinophil recruitment into the colon occurs via a $\beta_7$-integrin-independent pathway. We demonstrate that blockade of ICAM-1 by using a neutralising monoclonal antibody attenuated eosinophil recruitment into the colon in a transgenic model and that the ICAM-1 adhesion pathway is important for the development of eotaxin-1 mediated eosinophil recruitment into the colon during experimental colonic eosinophilic inflammation.

Current therapeutics designed against ICAM-1 are being examined for the treatment of IBD (Yacyshyn, Bowen-Yacyshyn et al. 1998; Yacyshyn, Barish et al. 2002; Yacyshyn, Chey et al. 2002). Usage of these anti-sense oligonucleotides against ICAM-1 or small molecule antagonists to block ICAM-1 signalling in clinical trials, to assess if this blockade will result in attenuation of eosinophil recruitment in IBD may be a significant therapeutic approach, particularly for UC. We have shown that ICAM-1 deficient mice were protected against the development of experimental colonic eosinophilic inflammation, further implicating a role for eosinophils in disease pathogenesis. This is consistent with our previous studies employing eotaxin-1 and also EPO deficient mice, demonstrating that eosinophils and EPO are important for disease pathogenesis.

There are significant implications of eosinophil compartmentalisation in different adhesion pathways, chemotactic signals and eosinophil recruitment, which will require selective antagonisms for the treatment of specific EGID. For example, anti-eotaxin-3 therapy will possibly be very effective in the treatment of eosinophilic oesophagitis, however the experimental data would suggest it is not going to be effective for treatment of small intestine/large intestine eosinophilic diseases, as they have been shown to be eotaxin-1 mediated (Hogan, Mishra et al. 2000; Forbes, Murase et al. 2004; Forbes, Smart et al. 2004; Hogan and Rothenberg 2006; Rothenberg and Hogan 2006).
Yet, usage of the eotaxin receptor, CCR3 antagonists would be very effective in blocking both pathways, as eotaxin-1 and eotaxin-3 signal through CCR3. As previously mentioned, ICAM-1 antagonism for the treatment of large intestinal eosinophilic inflammation is promising, however experimental data suggests that this may not be effective for the treatment of small bowel eosinophilic inflammation because that pathway seems to be $\alpha_4\beta_7$/MAdCAM-1 dependent. These findings indicate the critical importance of assessing therapeutic approaches to EGID, encompassing a wide range of compartmentalised disorders in which eosinophil recruitment can occur via differing and complex integrin/receptor recruitment pathways (figure 8.1).

8.3 IL-9 is a critical factor regulating experimental oral antigen-induced intestinal anaphylaxis.

Employing IL-9 factor deficient mice and mice overexpressing IL-9 in the small intestine we demonstrate a non-redundant role for IL-9 in the development of intestinal anaphylaxis. Our investigations show IL-9 is required for the development of experimental oral antigen-induced intestinal anaphylaxis and iFABPp IL-9 Tg mice are more susceptible to the induction of intestinal anaphylaxis. Furthermore, we demonstrate that IL-9 overexpression in the small intestine is sufficient to induce an experimental intestinal anaphylaxis transcriptome and phenotype and predispose to experimental oral antigen-induced intestinal anaphylaxis, remarkably, in the absence of systemic priming.

8.3.1 IL-9 regulates intestinal mastocytosis.

We have shown that IL-9 regulates intestinal mastocytosis via a mechanism that is independent of mast cell progenitor levels in the small intestine. These findings are consistent with the in vitro data indicating that IL-9 cannot support the survival of mature mast cells nor enhance SCF-dependent generation of progeny (Matsuzawa, Sakashita et al. 2003). IL-9 does however have a potent effect of enhancing SCF-1 induction of mast cell maturation (Matsuzawa, Sakashita et al. 2003). We demonstrate that optimal intestinal mastocytosis requires factors dependent on IL-13, IL-4R$\alpha$ and STAT-6 signalling. Antibody neutralisation of IL-3 and IL-4 has been shown to block GI nematode-induced intestinal mastocytosis by $\sim$85% (Madden, Urban et al. 1991).
Figure 8.1 Transmigration of eosinophils to the small and large intestine.

The transmigration of eosinophils to the GI tract involves a series of complex and specific events from chemokines signalling to initiate tethering, rolling and subsequent diapadesis of the eosinophil into the tissue. Depicted here is a schematic of the required molecules for transmigration of eosinophils into the small and large intestine respectively. We have demonstrated in contrast to the small intestine, transmigration of eosinophils into the colon occurs via a β7-integrin-independent mechanism. Figure created by Dr. S.P. Hogan.
8.3.2 IL-9/mast cells and ion transport.

The major manifestations of food anaphylaxis include respiratory and cardiovascular complications. It is possible that this pulmonary response reflects an increase in pulmonary interstitial fluid caused by an increase in pulmonary vascular permeability (Finkelman, Rothenberg et al. 2005). The increased vascular permeability results in intravascular leakage (IVL) and this occurs via a mast cell mediated mechanism (Abelli, Nappi et al. 1992; Sampson, Mendelson et al. 1992). Vascular leakage results in haemoconcentration and subsequently a high haematocrit. In previous studies, employing two independent methods we have demonstrated increased haematocrit during systemic anaphylaxis, as well as increased IVL in iFABPp IL-9 Tg that is associated specifically with mastocytosis of the jejunum induced via IL-9. We speculate that IL-9 is a potent inducer of intestinal mastocytosis, however, it may also act in synergy with other factors, most likely IL-3 and IL-4, for maximal effect.

Previous investigations have established a role for mast cells in intestinal permeability (Perdue and Gall 1986; Perdue and Gall 1986; Crowe, Sestini et al. 1990; Perdue, Marshall et al. 1990; Berin, Kiliaan et al. 1997; Berin, Kiliaan et al. 1998). Moreover, a compelling link between IL-9 and mast cell mediated epithelial barrier disruption during enteric nematode infection (McDermott, Bartram et al. 2003), led us to examine the role of IL-9/mast cells in intestinal permeability under homeostatic conditions. We employed a mast cell stabiliser, cromolyn sodium and examined permeability. We demonstrate a stabilisation of mast cells results in an ablation of intestinal permeability in iFABPp IL-9 Tg mice treated with cromolyn sodium. The mechanism by which mast cells mediate permeability remains unclear. Our gene chip analysis of iFABPp IL-9 Tg mice indicates no loss or decrease in mRNA expression of tight junction proteins. However, it remains possible that the significant increase in mast cell protease (mMCP-1, -2 and -4) mRNA expression (and at least increased mMCP-1 protein expression) may degrade junction proteins. As such, the cause of increased permeability present in the IL-9 intestinal transgenic mice could be due to the degradation of tight junction proteins by these proteases, with physiological relevance demonstrated here with significant increase in paracellular ion transport (Dextran-FITC) in iFABPp IL-9 Tg mice. In vitro experiments employing a cell line such as the Caco-2 BBc, an intestinal epithelial cell line known to form a polarised monolayer complete with tight junctions (Ma, Boivin et al. 2005), and recombinant mMCP-1, -2 and -4 could be proposed to
address this hypothesis. At this time, we also cannot rule out that IL-9 may promote mast cell independent mechanisms involved in increasing intestinal permeability. IL-9 has been shown to act on other cells; B cells remain a possible candidate target via an indirect mechanism. An alternate explanation is that an upregulation or increased activation of CaCl\textsubscript{2} channels, in particular, gob5, a putative calcium-activated chloride channel involved in the regulation of mucous production and/or secretion, increasing intestinal permeability. However, based on our findings, we postulate that the intestinal permeability is indeed mast cell mediated and this is most likely through the pro-inflammatory mediators released upon degranulation. We have shown increased mMCP-1 and TNF\textsubscript{a} protein levels in iFABP\textsuperscript{p} IL-9 Tg mice and other possible mediators such as histamine, PAF and serotonin have been identified as targets for investigation to elucidate the mechanism of action (Perdue, Forstner et al. 1984; Perdue and Gall 1986; Berin, Kiliaan et al. 1997; Berin, Kiliaan et al. 1998; Yu and Perdue 2001; Brandt, Strait et al. 2003).

8.3.3 IL-9 is sufficient to predispose to oral antigen-induced intestinal anaphylaxis.

Induction of an intestinal allergic response, including oral antigen-induced intestinal anaphylaxis, is dependent on antigen sensitisation with adjuvant and subsequent multiple antigen challenges (Layton, Stanworth et al. 1986; Snider, Marshall et al. 1994; Maloy, Donachie et al. 1995; Ohtsuka, Naito et al. 1999; Kweon, Yamamoto et al. 2000; Liu, Moriyama et al. 2002; Brandt, Strait et al. 2003). We demonstrate IL-9 is sufficient to predispose mice to intestinal anaphylaxis in the absence of systemic sensitisation. Notably, we show that the development of intestinal anaphylaxis in the absence of systemic sensitisation was associated with the development of a CD4\textsuperscript{+} Th\textsubscript{2}-type response and was dependent on these signalling pathways (IL-4R\alpha and STAT-6). These studies suggest that intestinal overexpression of IL-9 promotes the generation of antigen-specific CD4\textsuperscript{+} Th\textsubscript{2} responses. We demonstrate that IL-9 promotion of an oral antigen-specific CD4\textsuperscript{+} Th\textsubscript{2} response is primarily via mast cell mediated intestinal permeability.

Clinical and experimental studies have previously demonstrated a role for mast cells in the end stage effector mechanisms of food allergy (Li, Schofield et al. 1999; Brandt, Strait et al. 2003). We provide corroborative data supporting a role for mast cells in
exacerbation of the intestinal anaphylactic reaction. Importantly, we have identified a role for mast cells in oral antigen sensitisation. Interestingly, patients with systemic mastocytosis often present with intestinal manifestations and some have impaired intestinal absorption (Cherner, Jensen et al. 1988). Clinical studies have demonstrated that cromolyn is a successful treatment for intestinal symptoms associated with systemic mastocytosis (Horan, Sheffer et al. 1990). A major drawback of cromolyn sodium is its poor absorption properties, thus it is not unreasonable to speculate that the ability of cromolyn to successfully treat the intestinal symptoms in systemic mastocytosis may be at least, in part, due to increased intestinal permeability (Horan and Austen 1991). We demonstrate a central role for IL-9 in the regulation of oral antigen-induced intestinal anaphylaxis and identify a previously unappreciated role for mast cell induced intestinal permeability in oral antigen sensitisation and predisposition to intestinal anaphylaxis. These studies demonstrate the importance of intestinal barrier function in oral antigen sensitisation and identify a role for IL-9-driven mast cells in this process (figure 8.2).

8.4 Scope for further investigation.

These two very complex and fascinating projects have exciting prospects for future investigations.

The role of eotaxin-1 in eosinophil chemotaxis has been unequivocally proven (Mishra, Hogan et al. 1999; Rothenberg 1999; Rothenberg, Zimmermann et al. 1999; Hogan, Mishra et al. 2000; Hogan, Mishra et al. 2001; Rothenberg, Mishra et al. 2001; Rothenberg, Mishra et al. 2001; Rothenberg, Mishra et al. 2001; Forbes, Murase et al. 2004; Hogan and Rothenberg 2004; Hogan, Rothenberg et al. 2004; Rothenberg 2004; Forbes, Hulett et al. 2006; Rothenberg and Hogan 2006). In chapters 2 and 3, we have demonstrated eosinophils in response to eotaxin signalling upregulate $\beta_2$ integrins on their surface to allow for transmigration to the large intestine. The specific cells involved in the generation of eotaxin-1 in the large intestine remains to be elucidated. In situ hybridization of eotaxin-1 in colonic samples at baseline and during colonic injury has been performed. Colleagues have identified a mononuclear population positive for eotaxin-1 subsequent to DSS induced injury (S.P. Hogan., unpublished data). Alternatively activated macrophages have been shown to regulate eosinophil recruitment in vivo (Voehringer,
Figure 8.2 IL-9 promotes oral sensitisation and is a critical factor in the development of experimental oral antigen induced intestinal anaphylaxis.

A schematic overview of the role IL-9 plays in orchestrating oral sensitisation and the development of intestinal anaphylaxis. IL-9 activates mast cells resulting in increased intestinal permeability allowing for oral sensitisation that results in a subsequent Th2-driven immune response and intestinal anaphylaxis. Figure created by Dr. S.P. Hogan
End Stage Effector Function
food induced anaphylaxis

IL-9

Oral Sensitisation

Intestinal anaphylaxis (diarrhoea)

IL-9/IL-9R

OVA sensitisation

"leaky gut" increased permeability
van Rooijen et al. (2007) and as such, gave rise to the hypothesis that a possible candidate for the cell responsible for the upregulation of eotaxin-1 in the colon is the alternatively activated macrophage (AAM). Furthermore, mice infected with *Shistosoma mansoni* had marked infiltration of macrophages into the colonic lamina propria and protection from DSS-induced colitis which was shown to be macrophage dependent (Smith, Mangan et al. 2007). Smith *et al.* propose a new mechanism of suppression of DSS-induced colitis via colonic macrophage population via parasitic infection. Hogan and colleagues are currently investigating the hypothesis that macrophages are involved in eotaxin-1 production during DSS-induced colonic injury, through isolation of macrophage populations from DSS-treated and WT mice and assessing the eotaxin-1 expression. This will have profound effects on delivery a therapeutic approach to patients as AAM can be selectively targeted within the large intestine.

The underlying molecular basis of increased intestinal permeability in iFABPp IL-9 Tg mice is currently undetermined; however our data suggests that this is a mast cell-dependent pathway. In chapters 4 and 6 we demonstrate a critical role for IL-9 in the development of experimental oral antigen induced intestinal anaphylaxis and furthermore identify that IL-9 can promote oral sensitisation. In chapter 5 we identify IL-9 mediated intestinal permeability is mast cell dependent, based on data showing attenuation of mast cell activity prevents oral sensitisation. Taken together, our investigations suggest that defective barrier integrity can predispose to oral sensitisation and the development of allergic food reactions. Numerous mast cell specific pro-inflammatory mediators have been suggested to regulate intestinal permeability, and we have identified several of these mediators to be upregulated in both mice with experimental oral antigen induced intestinal anaphylaxis and iFABPp IL-9 Tg mice. Furthermore, employing models of hypersensitivity and stress, investigators have provided evidence that changes in mucosal function are most likely due to either direct action of mast cell mediators on epithelial receptors and/or indirect action via nerves/neurotransmitters (Yu and Perdue 2001). It is possible the actions of cromolyn sodium may be via nerves or neurotransmitters as its method of action is currently unknown. Current data employing anti-c-kit antibody demonstrated that mast depletion abrogated intestinal permeability in iFABPp-IL-9 Tg mice further supports our findings (K.R. Groschwitz and S.P. Hogan *et al.* data unpublished). Taken together, these findings have led to the exploration of the molecular basis of mast cell mediated
intestinal permeability, and are a continuing focus in Dr. Hogan’s laboratory. Specifically utilizing an established model of mouse mast cell deficiency, such as the mutant W/Wv or B6.Cg-kitW-sh strains, will be useful for investigating the implications of oral antigen-induced intestinal anaphylaxis.

Overexpression of IL-9 in the small intestine is sufficient to predispose to intestinal anaphylaxis, and our data suggests this response is regulated by CD4\(^+\) Th\(_2\) signalling. This is consistent with clinical findings of CD4\(^+\) T-cells from food allergic patients with a Th\(_2\) profile and the role of IL-9 in Th\(_2\) driven responses during *Leishmania major* infection in mice, as described here in chapter 7. The mechanism by which IL-9 overexpression results in end stage effector function of the CD4\(^+\) Th\(_2\) driven immune reaction during intestinal anaphylaxis remains to be elucidated. Further investigations into the immunopathogenesis of disease may be vital in the discovery of a therapeutic approach for the treatment of food-induced anaphylaxis.
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References


Appendix

Peer Reviewed Journal Articles


Immunopathogenesis of Experimental Ulcerative Colitis Is Mediated by Eosinophil Peroxidase

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Nancy A. Lee,† Paul S. Foster,* and Simon P. Hogan‡*‡

The precise role that individual inflammatory cells and mediators play in the development of gastrointestinal (GI) dysfunction and extraintestinal clinical manifestations of ulcerative colitis (UC) is unknown. In this study, we have used a mouse model of UC to establish a central role for eotaxin and, in turn, eosinophils in the development of the immunopathogenesis of this disease. In this model the administration of dextran sodium sulfate (DSS) induces a prominent colonic eosinophilic inflammation and GI dysfunction (diarrhea with blood and shortening of the colon) that resembles UC in patients. GI dysfunction was associated with evidence of eosinophil cytolytic degranulation and the release of eosinophil peroxidase (EPO) into the colon lumen. By using IL-5 or eotaxin-deficient mice, we show an important role for eotaxin in eosinophil recruitment into the colon during experimental UC. Furthermore, using EPO-deficient mice and an EPO inhibitor resorcinol we demonstrate that eosinophil-derived peroxidase is critical in the development of GI dysfunction in experimental UC. These findings provide direct evidence of a central role for eosinophils and EPO in GI dysfunction and potentially the immunopathogenesis of UC. The Journal of Immunology, 2004, 172: 5664–5675.

Inflammatory bowel diseases (IBD),† Crohn’s disease, and ulcerative colitis (UC) are chronic, relapsing, remitting gastrointestinal (GI) diseases characterized by chronic inflammation of the intestine (1–3). UC and Crohn’s disease are associated with intestinal and extraintestinal clinical manifestations of disease, which include weight loss, diarrhea accompanied by blood and/or mucus, fever, gastric dysmotility, and shortening of the colon (4, 5). UC is a condition that primarily affects the superficial layer of the colon mucosa, and histological analysis showed ulceration of the mucosa, blunting and loss of crypts, and an inflammatory infiltrate (1). The cellular composition of the inflammatory infiltrate in the colon is characterized by increased numbers of CD4⁺ T lymphocytes, mast cells, neutrophils, and eosinophils (1, 2).

Recently, there has been increasing interest in the involvement of eosinophils in the pathogenesis of UC (6). Elevated levels of eosinophils have been observed in colonic biopsy samples from UC patients and increased numbers of this cell have been shown to correlate with morphological changes to the GI tract, disease severity, and gastrointestinal dysfunction (7–10). A number of inflammatory mediators (platelet-activating factor, IL-5, and chemokines (RANTES, macrophage chemoattractant protein, macrophage inhibitor protein, eotaxin-1 (eotaxin), -2, and 3) have chemotactic activity for human eosinophils and are candidates for the control of colonic eosinophils in UC (11, 12). In particular, IL-5 regulates eosinophil growth, differentiation, and activation and the chemokine eotaxin is primarily involved in the regulation of eosinophil chemotaxis and effector functions including respiratory burst (13).

Eosinophils are multifunctional leukocytes possessing the capacity to initiate or potentiate inflammatory reactions through the release of a range of inflammatory cytokines, chemokines, and lipid mediators (14–16). In addition, eosinophils may induce GI dysfunction through the release of lipid mediators (platelet-activating factor and leukotriene C₄) and eosinophil granular proteins (major basic protein (MBP), eosinophil peroxidase (EPO), and eosinophil-associated ribonucleases, i.e., eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN)). Clinical investigations of bowel biopsy specimens from UC patients have demonstrated a correlation between the eosinophil numbers in the mucosa, the levels of MBP, EPO, ECP, and EDN in perfusion fluid samples, and disease severity (6, 8–10). However, the pathological role of the eosinophil and these factors in UC is not understood.

Several experimental models of UC have been developed in mice to dissect out the key cellular and molecular mechanisms predisposing to disease (1, 2, 17). These models mimic certain pathophysiological features of human UC including weight loss, diarrhea with blood and/or mucus, shortening of the colon, crypt abnormalities, and infiltration of inflammatory cells, particularly eosinophils, into the GI mucosa (1, 2, 17). However, these investigations have provided conflicting evidence concerning the contribution of eosinophils in the development of GI dysfunction in experimental UC (1, 2, 17).

In this study we have dissected out the potential contribution of eosinophils to the pathogenesis of UC by using a model of disease that is induced by dextran sulfate sodium (DSS). We show in this model that the administration of DSS induces a prominent colonic eosinophilic inflammation and GI dysfunction (diarrhea with blood and shortening of the colon) which resembles UC in patients. In
addition, studies with IL-5 and eotaxin-deficient mice demonstrate a central role for eosinophils to the development of disease pathogenesis. Significantly, our data demonstrate that GI dysfunction is associated with evidence of eosinophilic cytotytic degranulation and identifies EPO as a key mediator of disease in experimental UC.

Materials and Methods

Animals

We used IL-5-deficient (IL-5−/−) mice on the C57BL/6 background (18) and mice deficient in eotaxin (eotaxin−/−) (19) that were backcrossed 10 generations into the C57BL/6 and their appropriate C57BL/6 wild-type (WT) controls. We also used B6/129 X Hsd X 129/SvJ background, and strain and aged-matched WT control mice. All mice were obtained from specific pathogen-free facilities at the Australian National University and housed in approved containment facilities. Mice were treated according to the Australian National University animal welfare guidelines and age- and sex-matched animals were used throughout these studies.

Induction of experimental UC

DSS was used for the induction of experimental UC (ICN Biomedical, Costa Mesa, CA) was supplied as the sodium salt with an average M, of 41. Draining water was supplemented with DSS 2.5% (w/v) for 8 days.

Disease activity index (DAI)

DAI was derived by scoring three major clinical signs (weight loss, diarrhea, and rectal bleeding) (22). The clinical features were scored separately and then correlated with a histological score: DAI = (body weight loss) + (diarrhea score) + (rectal bleeding score).

Body weight

Changes in body weight were calculated as the difference between the expected and actual weight on a particular day. The formula for predicted body weight was derived by simple regression using the body weight data for the control group. The following formula was used: \( Y = a + kx \), where \( Y \) = body weight change (loss or gain), \( k \) = daily increase in body weight, \( x \) = day, and \( a \) = starting body weight.

Diarrhea

The appearance of diarrhea was defined as mucus/fecal material adherent to anal fur. The presence or absence of diarrhea was scored as either 1 or 0, respectively. The presence or absence of diarrhea was confirmed by examining the colon following completion of the experiment (22). Mice were sacrificed and the colon excised from the animal. Diarrhea was defined by the absence of fecal pellet formation in the colon and the presence of continuous fluid fecal material in the colon.

Rectal bleeding

The appearance of rectal bleeding was defined as diarrhea containing visible blood and/or mucus or gross rectal bleeding and scored as described for diarrhea.

Assessment of Inflammation

Assessment of body weight, evaluation of stool consistency (diarrhea), and rectal bleeding were performed on a daily basis. Body weight was expressed as percent body weight change from baseline. Diarrhea and rectal bleeding were defined as described above. The presence/absence of diarrhea and rectal bleeding was given a score of 0 or 1 and the diarrhea/rectal bleeding score (0–2) is the accumulation of these two values.

Histopathological examination

Animals were sacrificed on day 8 and the colon was excised. The length of the colon was measured using digital calipers (Mitutoyo, Kawasaki, Japan). Tissue specimens were then fixed in 4% paraformaldehyde and stained with H&E and Masson’s trichrome using standard histological techniques. Percent colon length with mucosal ulceration was determined by performing morphometric analysis of the colon using an ImageProPlus 4.5 software package (Media Cybernetics, Silver Spring, MD). In brief, digital images of longitudinal sections (1–2 cm in length) of H&E-stained colons were produced. Using the ImageProPlus 4.5 software, the length of ulcerated mucosal lining was divided by the total length of the colonic mucosal surface and the value was expressed as a percentage of colon length with mucosal ulceration.

Detection and quantification of eosinophils by immunohistochemistry

The colon segment of the GI tract was immunostained with antiserum against mouse MBP as previously described (23). Briefly, 5-μm sections were quenched with H2O2, blocked with normal goat serum, and stained with a rabbit anti-murine eosinophil MBP anti-serum as described earlier (23). The slides were then washed and incubated with biotinylated goat anti-rabbit Ab and avidin-peroxidase complex (Vectastain ABC Peroxidase Elite kit; Vector Laboratories, Burlingame, CA). The slides were developed with nickel diaminobenidine, enhanced catalase chloride to form a black precipitate, and counterstained with nuclear fast red. Quantification of eosinophils was performed by counting the number of immunoreactive cells from 15 to 25 fields of view (magnification, ×40) from at least four to five random sections per mouse. Values were expressed as eosinophils per high-powered field.

Lamina propria cell isolation and flow cytometry

Experimental UC was induced as described above. On day 6, control- and DSS-treated mice were sacrificed and the colon was surgically removed. Colon lamina propria cells were isolated as described in detail in Current Protocols of Immunology (24). In brief, 5-cm colon strips were digested in RPMI 1640 supplemented with 10% FCS, 2 mg/ml collagenase (Roche, Basel, Switzerland), 1.2 U/ml Streptokinase (Roche), and 5 U/ml DNase 1 (Pharmacia LKB Biotechnology, Uppsala, Sweden) and incubated at 37°C for 60 min. The mononuclear cells were separated from the epithelial cells by centrifugation at 4°C for 30 min at 1600 × g in Ficoll-Plaque Plus (Amersham Biosciences, Uppsala, Sweden). The cell suspension was washed and filtered through 40-μm mesh. To examine the level of CD4 and intracellular IL-4 and IFN-γ expression, lamina propria cells were stained with FITC-conjugated anti-CD4, PE-conjugated anti-mouse IFN-γ, and Alexa647-conjugated rat anti-mouse IL-4 as described by the manufacturer (BD PharMingen, San Diego, CA). In brief, the cell suspension was incubated at 10^7 cells/ml with 1 μl of GolgiPlug at 4°C for 20 min. The cells were washed with PBS/1% FCS and then incubated with 50 μl of 1% FCS/PBS containing FITC-conjugated anti-CD4 (clone GK1.5 BD, 1 μg/10^6 cells; BD PharMingen). Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences). The ratio of IL-4^+CD4^+ T cells to IFN-γ^+CD4^+ T cells was calculated from cells events acquired by using gates set by forward and side scatter and CD4-FITC and IFN-γ-PE double-positive cells. CD4-FITC and IL-4-Alexa647 double-positive cells and CD4-FTC and IFN-γ-PE double-positive cells. Negative control samples were incubated with irrelevant isotype-matched Abs in parallel with all experimental samples.

EPO activity assay

Mice were sacrificed on day 8 and the colon was excised and flushed with 1 ml of PBS solution. The fecal material was vortexed vigorously for 5 min at 4°C and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was collected and placed in sterile Eppendorf tubes and stored at −70°C until analysis. EPO activity was measured in the supernatant of cell-free colon flushes as previously described (25). This assay is based on the oxidation of 0-phenylenediamine (OPD) by EPO in the presence of hydrogen peroxide (H₂O₂). The EPO substrate solution consisted of 12 mM OPD (Sigma-Aldrich, St. Louis, MO), 0.005% H₂O₂, 10 mM HEPES, and 0.22% cysteine-hydrochloride (GIBA). Substrate solution (75 μl) was added to cell-free supernatants that were derived from colon flushes (75 μl) in a 96-well microplate and incubated at room temperature for 15 min before stopping the reaction with 50 μl of cold 8 N sulfuric acid. Absorbance was measured at 490 nm. Standard EPO activity of 100 U/ml was determined as EPO activity produced by 1 × 10^6 purified eosinophils/μl supernatant. Eosinophils were purified from the spleen of CD2-IL-5-transgenic mice as previously described (26).
Inhibition of EPO activity by resorcinol

Resorcinol (Sigma-Aldrich; concentration range: 10⁻⁷–10⁻¹³ M) was titrated (1/10 serial dilutions) into supernatants isolated from 2 × 10⁵ purified eosinophils in a 96-well flat-bottom microplate. Samples (75 µl) were added to EPO substrate solution (75 µl) and incubated at room temperature for 15 min before stopping the reaction with 50 µl of cold 8 N sulfuric acid. Absorbance was measured at 490 nm. EPO activity in the presence of 10⁻⁵–10⁻¹³ M resorcinol was compared with negative control (no resorcinol-diluent) and expressed as percent inhibition of EPO activity.

Resorcinol treatment in vivo

Mice were provided with drinking water supplemented with 2.5% (w/v) DSS to induce experimental UC and were i.p. injected with 200 µl of resorcinol (1.25 mg/kg) or vehicle (PBS/200 µl) daily for 8 days. On day 8, mice were sacrificed and disease parameters were analyzed. In some experiments, mice were provided with drinking water supplemented with 2.5% (w/v) DSS for 8 days to induce experimental UC and on days 6 and 7 i.p. injected with 200 µl of resorcinol (1.25 mg/kg). The disease score for the latter experiments were derived by the following score: 1 = healthy plus normal stool (formed pellets); 2 = macroscopic bleeding (occult anal bleeding) plus normal stool (formed pellets); 3 = pilar eructi, decreased activity and body mass, macroscopic bleeding (observable anal bleeding), and diarrhea (semiformed stools that do not stick to the anus); and 5 = pilar eructi, abdominal distention, shrunken eyes, dehydration, hunched posture, decreased activity and body mass, excreted perianal mucus, gross macroscopic bleeding (blood around the anus or in the cage), and diarrhea (liquid stools that stick to the anus). Since weight variation does not occur within 24–48 h following administration of resorcinol, weight change was not included as a disease parameter.

Results

DSS-induced experimental UC was associated with eosinophilic inflammation and GI dysfunction

Administration of DSS to C57BL/6 mice induces an acute inflammation of the colon, pronounced weight loss, and bloody diarrhea (Fig. 1a). The first characteristics of pathologies are apparent after 6-day exposure to DSS and weight loss is the most predominant feature of the DAI at this time. Six to 8 days following exposure, mice develop diarrhea, rectal bleeding, and shortening of the colon (Fig. 1a–f). Histological examination of mouse colons at day 8 showed that DSS-treated mice developed extensive ulceration of the epithelial layer, bowel wall edema, crypt damage, and fibrotic thickening of the muscularis mucosa and dense infiltration of the superficial layers of the mucosa with granulocytes and mononuclear cells (Fig. 2a–f). We performed immunohistochemistry using a polyclonal antiserum against eosinophil-derived MBP to elucidate whether the
granulocytes were eosinophils. Eosinophils were observed throughout the mucosa and submucosa in DSS-treated mice. In particular, eosinophil numbers were significantly increased in the colon of DSS-treated mice as compared to control-treated mice (eosinophils/HPF, 1.5 ± 0.05 vs 25.9 ± 1.05, mean ± SE, n = 4−5 mice/group, control vs DSS-treated mice, respectively, p < 0.005). To characterize the T cell inflammatory response we performed intracellular cytokine staining on colonic lamina propria CD4+ T cells from control-treated and DSS-treated mice. The ratio of IL-4-positive CD4+ T cells to IFN-γ+CD4+ T cells in DSS-treated mice was significantly higher than that observed in control mice, suggesting that the T cell inflammatory infiltrate was predominantly Th2 type (ratio of IL-4+CD4+ T cells to IFN-γ+CD4+ T cells; 0.94 ± 0.22 vs 2.42 ± 0.53, mean ± SE, n = 3 mice/group, control vs DSS-treated mice, respectively, p < 0.05).

Masson’s trichrome stains of colonic sections to evaluate the presence and distribution of collagen showed blue stained, thickly packed collagen fibers, in the mucosa beneath the muscularis mucosa in DSS-treated mice but not in control-treated mice (Fig. 2, a, b, and f). The thick collagen layer present in DSS-treated mice contained large numbers of infiltrating eosinophils (Fig. 2, e and f). The histopathology of the colon is similar to that observed in UC patients and suggests that a similar pathological mechanism may contribute to tissue damage in both types of inflammation.

**FIGURE 2.** Histopathology in the colon of DSS-treated mice. a−f. Representative photomicrographs of colon from control (b and d) and DSS-treated (a, c, e, and f) C57BL/6 WT mice. a and b. Photomicrographs of Masson’s trichrome stain sections of colon. c−e. Immunohistochemically stained sections of colon from control- and DSS-treated mice using the eosinophil-specific anti-MBP Ab. f. Masson’s trichrome-stained sections of colon from DSS-treated mice. a. Arrows depict ulceration of the epithelial cell layer; a−f, white filled arrowheads depict fibrotic thickening of the muscularis mucosa; and c−e, black filled arrowheads depict MBP-positive eosinophils. Magnification: a and b, ×100; c and d, ×500; and e and f, ×50.

Eosinophilic inflammation and GI dysfunction in DSS-induced experimental UC is regulated by eotaxin

The regulation of eosinophil recruitment during allergic airways disease and trafficking into the upper GI tract (small bowel) at baseline as well as following allergen challenge appears to be regulated by IL-5 and eotaxin (16, 27, 28). We used IL-5- or eotaxin-deficient mice to examine the contribution of IL-5 and eotaxin in eosinophil recruitment into the colon during experimental UC. Neither of the untreated IL-5−/− or eotaxin−/− mice exhibited any of the pathological symptoms (diarrhea, rectal bleeding, or cachexia) or gross morphological changes to the GI tract normally associated with spontaneous intestinal inflammation. Administration of DSS to IL-5−/− mice induced experimental UC, including diarrhea and rectal bleeding, and colon shortening similar to that observed in DSS-treated WT mice (Fig. 3, a−d). Drinking volume was similar in all groups (data not shown). Histological examination of mouse colons showed extensive tissue ulceration, massive bowel wall edema, fibrosis of the muscularis mucosa, and a dense cellular infiltration characterized by eosinophils (results not shown). Quantification of eosinophil numbers revealed a significant and near identical increase in the colon of DSS-treated IL-5−/− mice as compared with DSS-treated WT mice (Fig. 3e). In contrast to WT and IL-5−/− mice, DSS-induced experimental UC
FIGURE 3. Critical role for eotaxin in DSS-induced experimental UC. DAI (a), weight change (b), and diarrhea/rectal bleeding score (c) during the course of DSS treatment in IL-5+/−, eotaxin−/−, and strain-matched C57BL/6 WT mice. Colon lengths (d) and eosinophil numbers (e) per HPF in the colon of control and DSS-treated IL-5+/−, eotaxin−/−, and WT mice. Eosinophils were quantitated by counting 20 similar HPF (magnification, ×40) for each group. Data represent the mean ± SEM of four to five random sections per mouse for four to five mice per group. Statistical significance of differences (p < 0.05) was determined using Student’s unpaired t test. Significant differences (*, p < 0.05) as compared with matched controls; #, p < 0.05 as compared with eotaxin−/− DSS. b, *, p < 0.05 as compared with matched controls. c, *, p < 0.05 as compared with matched control; #, p < 0.05 as compared with eotaxin−/− DSS. d, **, p < 0.01 as compared with matched control. e, #, p < 0.05 as compared with eotaxin−/− DSS and **, p < 0.01 as compared with matched control.

in eotaxin−/− mice was significantly attenuated (Fig. 3, a–d). The attenuation of experimental UC in eotaxin−/− mice was associated with a reduction (~45% compared with DSS-treated WT and IL-5+/− mice) in the number of eosinophils infiltrating into the colon (Fig. 3, a–e).

DSS-induced experimental UC is associated with eosinophil cytolytic degranulation

Clinical investigations have provided ultrastructural evidence of eosinophil degranulation in patients with IBD (29–31). In this study, we show administration of DSS induced extensive eosinophil degranulation. In control-treated mice, colonic eosinophils were primarily located in the lamina propria and possessed features including intact plasma membranes, heterochromatic-segmented nuclei, and abundant granules with characteristic electron-dense cores and matrices (Fig. 4, a and b). In comparison, eosinophils in DSS-treated mice appeared to be undergoing cytolytic eosinophilic degranulation as evidenced by nuclear chromatolysis, disruption of plasma membrane, and the presence of free eosinophilic granules in the extracellular spaces adjacent to these eosinophils (Fig. 4, c and d).

The demonstration of extensive eosinophilic degranulation suggested that eosinophil granule proteins may contribute to the pathogenesis of experimental UC. We examined the level of EPO in the lumen of the colon of control- and DSS-treated mice and showed that EPO activity in DSS-treated mice was ~1,000-fold higher than that observed in control-treated animals (Fig. 4e). We also examined luminal EPO levels in control- and DSS-treated IL-5+/− and eotaxin−/− mice. In both IL-5+/− or eotaxin−/− mice luminal EPO levels were elevated in comparison to the control-treated mice (~500- and ~100-fold, respectively). However, consistent with our observation of attenuated experimental UC in eotaxin−/− mice, EPO levels and eosinophil intestinal numbers were attenuated when compared with WT and IL-5+/− DSS-treated mice (Figs. 3e and 4e).
EPO plays an important role in the pathophysiology of DSS-induced experimental UC

We next challenged MBP−/−, EPO−/−, and strain-matched WT mice with DSS to examine the contribution of EPO and MBP in DSS-mediated experimental UC. DSS treatment of strain-matched WT mice induced experimental UC similar to that previously described for WT C57BL/6 mice (Fig. 5, a–d). Experimental UC was associated with increased DAI, diarrhea, rectal bleeding, eosinophilic inflammation, and colon shortening and elevated levels of colon EPO activity (Fig. 5). Similarly DSS-induced experimental UC in MBP−/− mice was comparable to that observed in strain-matched WT mice (Fig. 5, a–d). However, in EPO−/− mice, DSS-induced colitis was significantly attenuated as compared with either strain-matched WT or MBP−/− mice (Fig. 5, a–d). This attenuation was associated with the loss of EPO activity in DSS-treated EPO−/− mice (Fig. 5f) and occurred despite no reduction in eosinophil intestinal accumulation (Fig. 5e). Histological analysis showed the eosinophils to be dispersed throughout the lamina propria and beneath the muscularis mucosa in both DSS-treated WT and EPO−/− mice. However, only in the DSS-treated WT mice was the characteristic colonic mucosal ulceration and crypt damage observed (Fig. 5, g and h). We observed no significant difference in DAI or gross morphological changes to the Gl tract between strain-matched WT control-treated and MBP−/− and EPO−/− control-treated mice (results not shown). Thus, these studies demonstrate that eosinophil-derived EPO plays an important role in the immunopathogenesis of experimental UC.

EPO inhibitor resorcinol can attenuate DSS-induced experimental UC

The demonstration that EPO plays an important role in the manifestations observed in experimental UC suggests that an inhibitor of EPO activity would attenuate the development of disease. We tested ex vivo the suppressive effects of a peroxidase inhibitor resorcinol on EPO activity and found that resorcinol inhibited EPO activity at concentrations as low as 100 nM with an IC50 of 3 pM...
FIGURE 5. Critical role for EPO in DSS-induced experimental UC. a, DAI on day 8 and weight change (b) and diarrhea/rectal bleeding score (c) during the course of DSS treatment in strain-matched WT, MBP<sup>−/−</sup>, and EPO<sup>−/−</sup> mice. d, Colon lengths and eosinophil numbers per HPF (e) in the colon of control and DSS-treated strain-matched WT, MBP<sup>−/−</sup>, and EPO<sup>−/−</sup> mice. f, Colonic luminal EPO activity in control and DSS-treated strain-matched WT, MBP<sup>−/−</sup>, and EPO<sup>−/−</sup> mice. No significant difference in the DAI between strain-matched WT control-treated and MBP<sup>−/−</sup> and EPO<sup>−/−</sup> (Figure legend continues)
Resorcinol (M) log

% Inhibition EPO Activity

0  25  50  75  100

-16 -14 -12 -10 -8 -6

Eosinophil supernatants (U/ml)

FIGURE 6. Inhibition of EPO activity by resorcinol. Resorcinol (concentration range: $10^{-5}$–$10^{-12}$ M) was added with purified eosinophil extracts ($2 \times 10^6$ purified eosinophils/20 U/ml) in a 96-well flat-bottom microplate. Peroxidase substrate solution (12 mM OPD, pH 5.0, 0.005% H$_2$O$_2$, 10 mM HEPES, and 0.2% cetyltrimethylammonium bromide) was added to the extracts and incubated at room temperature for 15 min before stopping the reaction with 50 µl of cold 8 N sulfuric acid. Absorbance was measured at 490 nm. Eosinophils were purified from the spleen of CD2-IL-5-transgenic mice as previously described (26). Eosinophils were >99.5% pure. (results not shown). Insert, Standard EPO activity assay: EPO activity (100 U/ml) was determined as the amount of EPO activity produced by $1 \times 10^6$ purified eosinophils/µl extracts. Eosinophil extracts (0.5–100 U/ml) were added to the peroxidase substrate solution and incubated at room temperature for 15 min before stopping the reaction with 50 µl of cold 8 N sulfuric acid. Absorbance was measured at 490 nm. Data represent the mean ± SEM of triplicate wells.

and maximal inhibition occurred at 10 nM (Fig. 6). Next, we examined the effects of resorcinol on inhibiting EPO activity in vivo. Mice were provided with drinking water supplemented with 2.5% (w/v) DSS and were i.p. injected with 200 µl of resorcinol (1.25 mg/kg) or vehicle (PBS/200 µl) daily for 8 days. Experimental UC in mice treated with resorcinol was significantly attenuated as compared with DSS-treated vehicle-injected mice (Fig. 7, a–c). Furthermore, the degree of colon shortening was also reduced in mice treated with DSS and i.p. injected with resorcinol as compared with DSS-treated vehicle-injected mice (Fig. 8a). Luminal EPO levels in DSS-treated resorcinol-injected mice were significantly reduced as compared with DSS-treated vehicle-injected mice, although eosinophil levels were equivalent in both groups (Fig. 8, b and c). Notably, a reduction in pathology following resorcinol treatment was associated with decreased mucosal ulcration of the colon (Fig. 8d). To examine whether resorcinol could also suppress established experimental UC, mice received an i.p. injection of vehicle or resorcinol on days 6 and 7 of the 8-day experimental regime (Fig. 9a). Experimental UC in mice receiving DSS and administratively resorcinol on days 6 and 7 (but not vehicle alone) was significantly attenuated (Fig. 9a). Symptoms of experimental UC (rectal bleeding and diarrhea) but not of weight loss (results not shown) or colon shortening (colon length: 39.945 ± 1.95 mm vs 44.30 ± 2.56 mm, mean ± SE, n = 4–5 mice/group, vehicle vs resorcinol-treated DSS-challenged mice 24 h following treatment, respectively) were reduced within 24 h and were ablated within 48 h (day 8) of resorcinol treatment (Fig. 9a). Notably, the reduction in pathology in DSS-treated resorcinol-injected mice as compared with DSS-treated vehicle-injected mice was not associated with eosinophil recruitment but in fact associated with a reduction in luminal EPO activity. (Fig. 9, b and c). Collectively, control-treated mice (results not shown). g and h, Representative photomicrographs of immunohistochemically stained sections of colon from DSS-treated WT (g) and EPO$^{-/-}$ (h) mice. White filled arrowheads depict mucosal ulceration and black filled arrowheads depict MBP-positive eosinophils. Magnification: g and h, ×100. Data represent the mean ± SEM of four to five mice (a–e) per group and four to eight (f) superfusion samples per group. Statistical significance of differences (p < 0.05) was determined using Student’s unpaired t test. a, *, p < 0.05 as compared with WT control and #, p < 0.05 as compared with EPO$^{-/-}$ DSS, b and c, *, p < 0.05 as compared with WT control and #, p < 0.05 as compared with EPO$^{-/-}$ DSS. D, #, p < 0.05 as compared with control. e, *, p < 0.05 as compared with WT control. f, *, p < 0.005 as compared with matched control and #, p < 0.01 as compared with EPO$^{-/-}$ DSS.
The model that possesses the immunopathological features of UC, in experimental UC.

That blockade of EPO activity can attenuate the development of EPO is an important mediator in the development of the colitis and releasing protein-laden granules; and 4) that eosinophil-derived infiltration of eosinophils degranulates by a process of cytolysis recruitment of eosinophils into the colon during experimental UC; in light of our studies and previous investigations, it is tempting to speculate that IL-13 derived from NK T cells promotes chemokine production and subsequent eosinophil infiltration which leads ultimately to disease. Notably, we also demonstrate that eosinophil plays an important role in the expression of the pathophysiological features of experimental UC. Using eosinophil-/- mice, we show that while eosinophil levels are reduced by 2-fold, disease pathology (diarrhea, rectal bleeding, and colon shortening) was markedly attenuated. These data suggest that eosinophil recruitment may occur via an eosinxin-independent process; however, eosinxin is critical for the development of the disease pathology. Recently, experimental investigations have demonstrated that eosinophil can induce eosinophil activation and respiratory burst (13). It is possible that eosinophil plays multiple roles in eosinophil function, regulating both trafficking and also eosinophil activation and degranulation and the

These studies confirm the important role of eosinophil-derived EPO in the pathophysiology of experimental UC and the potential for disease treatment through inhibition of peroxidase activity.

Discussion

UC is a chronic relapsing inflammatory disease that is primarily driven by an underlying inflammatory response leading to the pathophysiological manifestations of the disease. DSS treatment of mice promotes a chronic experimental UC, which possesses certain pathophysiological features of UC. These features include extensive ulceration of the epithelial layer, massive bowel wall edema, fibrotic thickening of the mucosa, and a dense cellular infiltrate characterized by eosinophils. The similarities between DSS-induced experimental UC and UC suggest that both diseases could be explained by common underlying pathological mechanisms. In this study, we define these mechanisms which include 1) a direct link between eosinophilic inflammation and the pathogenesis of experimental UC; 2) an important role for eosinxin in the recruitment of eosinophils into the colon during experimental UC; 3) that infiltrating eosinophils degranulate by a process of cytolysis releasing protein-laden granules; and 4) that eosinophil-derived EPO is an important mediator in the development of the colitis and that blockade of EPO activity can attenuate the development of experimental UC.

Studies using an oxazolone colitis model, another Th2 colitis model that possesses the immunopathological features of UC, including a colonic eosinophilic infiltrate, have demonstrated a critical role for IL-13-producing NK T cells and IL-13 in the pathogenesis of experimental UC (32). During inflammatory responses, IL-13 has been shown to regulate an array of eosinophil-sensitive functions including eosinophil production and eosinophil recruitment and survival (33–36). Elevated levels of eosinxin have also been observed in serum and biopsy samples from patients with UC consistent with a role for eosinxin in eosinophil accumulation into the GI tract during UC (6, 11, 37, 38). Using eosinxin-/- mice, we demonstrate an important role for eosinxin in the recruitment of eosinophils into the colon during experimental UC. In light of our studies and previous investigations, it is tempting to speculate that IL-13 derived from NK T cells promotes chemokine production and subsequent eosinophil infiltration which leads ultimately to disease. Notably, we also demonstrate that eosinxin plays an important role in the expression of the pathophysiological features of experimental UC. Using eosinxin-/- mice, we show that while eosinophil levels are reduced by 2-fold, disease pathology (diarrhea, rectal bleeding, and colon shortening) was markedly attenuated. These data suggest that eosinophil recruitment may occur via an eosinxin-independent process; however, eosinxin is critical for the development of the disease pathology. Recently, experimental investigations have demonstrated that eosinxin can induce eosinophil activation and respiratory burst (13). It is possible that eosinxin plays multiple roles in eosinophil function, regulating both trafficking and also eosinophil activation and degranulation and the

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subsequent development of pathological symptoms. The demonstration of a central role for eotaxin and eosinophils in experimental UC in the present study is in contrast to a previous investigation using IL-5−/− mice in a DSS model of colonic injury and inflammation (22). Although we cannot fully explain these inconsistencies, Stevceva et al. (22) used a general histological staining technique (H&E) that poorly differentiates immature and degranulating eosinophils in GI tissues, whereas we used an eosinophil-specific stain (MBP immuno histochemistry) that allows for specific identification and quantification of eosinophils in all activation states.

Previous clinical investigations have demonstrated collagen deposition in the intestinal biopsy samples from UC patients with elevated levels of MBP, EPO, and eosinophil-associated ribonucleases that are capable of inducing tissue injury, including epithelial cell damage (14, 43). Eosinophils possess an array of cytotoxic granule proteins (MBP, EPO, and eosinophil-associated ribonucleases) that are capable of inducing tissue injury, including epithelial cell damage (14, 43). Eosinophils produce both granular proteins and other inflammatory mediators (cytokines and lipid mediators) by a number of mechanisms (granule extrusion (exocytosis), piecemeal degranulation, and cytolysis) (44–47). In the present study, we show that eosinophils undergo degranulation, releasing protein laden eosinophil granules into the surrounding tissue. Previous experimental and clinical investigations have demonstrated eosinophil degranulation, and cytolysis) (44–47). In the present study, we show that eosinophils undergo degranulation, releasing protein laden eosinophil granules into the surrounding tissue. Previous experimental and clinical investigations have demonstrated eosinophil degranulation, and cytolysis) (44–47).

Clinical investigations of bowel biopsy specimens and perfusion fluid samples from UC patients have demonstrated elevated levels of MBP, EPO, and eosinophil-associated ribonucleases that are capable of inducing tissue injury, including epithelial cell damage (14, 43). Eosinophils possess an array of cytotoxic granule proteins (MBP, EPO, and eosinophil-associated ribonucleases) that are capable of inducing tissue injury, including epithelial cell damage (14, 43). Eosinophils produce both granular proteins and other inflammatory mediators (cytokines and lipid mediators) by a number of mechanisms (granule extrusion (exocytosis), piecemeal degranulation, and cytolysis) (44–47). In the present study, we show that eosinophils undergo degranulation, releasing protein laden eosinophil granules into the surrounding tissue. Previous experimental and clinical investigations have demonstrated eosinophil degranulation, and cytolysis) (44–47). In the present study, we show that eosinophils undergo degranulation, releasing protein laden eosinophil granules into the surrounding tissue. Previous experimental and clinical investigations have demonstrated eosinophil degranulation, and cytolysis) (44–47). In the present study, we show that eosinophils undergo degranulation, releasing protein laden eosinophil granules into the surrounding tissue. Previous experimental and clinical investigations have demonstrated eosinophil degranulation, and cytolysis) (44–47). In the present study, we show that eosinophils undergo degranulation, releasing protein laden eosinophil granules into the surrounding tissue. Previous experimental and clinical investigations have demonstrated eosinophil degranulation, and cytolysis) (44–47).
neutrophil derived MPO and/or other peroxides, primarily contributes to the luminal peroxidase activity and pathology associated with DSS-induced colitis.

EPO catalyzes the oxidation of halides and pseudohalides (Cl\textsuperscript{-}, Br\textsuperscript{-}, and SCN\textsuperscript{-}) with the products of respiratory burst (O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2}) to generate cytoxic oxidants (3-bromotyrosine, 3-chlorotyrosine, and hypochloroiccytate). These cytoxic oxidants induce tissue damage and cell death (55). Recently, EPO has also been shown to preferentially catalyze the oxidation of nitrite (NO\textsubscript{2} \textsuperscript{-}), generating the highly toxic reactive nitrogen species (RNS) 3-nitrotyrosine and peroxynitrite (56, 57). Clinical and experimental studies have demonstrated elevated levels of RNS in bronchial aspirates from asthma patients, and that EPO directly contributes to the generation of these reactive nitrogen species (nitrotyrosine and peroxynitrite) (57). UC has also been shown to be associated with increased inducible NO synthase activity as well as NO and RNS production (58). Furthermore, recent clinical studies have demonstrated an imbalance in secondary mucosal antioxidant pathways and production of reactive oxygen metabolites (ROM) including H\textsubscript{2}O\textsubscript{2} and hypochlorous acid as well as RNS in IBD (59). It is possible that the release of EPO in the lumen during experimental UC leads to the generation of RNS and ROM and the subsequent development of the pathophysiological features of the disease. We are further investigating the role of EPO in the generation of ROM and RNS in experimental UC.

In conclusion, we have shown that during experimental UC, eosinophils transmigrate into the colonic lumen where they degranulate, releasing EPO, and induce a progressive colitis resembling UC in patients. Furthermore, blockade of EPO activity by genetic manipulation or by drug treatment suppressed the development of experimental UC and, importantly, administration of an EPO inhibitor reversed established disease. These studies suggest that antagonism of EPO activity may be a therapeutic approach for the treatment of UC.

References


ICAM-1-dependent pathways regulate colonic eosinophilic inflammation

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Abstract: Eosinophilic inflammation is a common feature of numerous eosinophil-associated gastrointestinal (EGID) diseases. Central to eosinophil migration into the gastrointestinal tract are the integrin-mediated interactions with adhesion molecules. Although the mechanisms regulating eosinophil homing into the small intestine have begun to be elucidated, the adhesion pathways responsible for eosinophil trafficking into the large intestine are unknown. We investigated the role of adhesion pathways in eosinophil recruitment into the large intestine during homeostasis and disease. First, using a hapten-induced colonic injury model, we demonstrate that in contrast to the small intestine, eosinophil recruitment into the colon is regulated by a β2-integrin addressin cell adhesion molecule-1-independent pathway. Characterization of integrin expression on colonic eosinophils by flow cytometry analysis revealed that colonic CC chemokine receptor 3+ eosinophils express the intercellular adhesion molecule-1 (ICAM-1) counter-receptor integrins α4, αM, and β2. Using ICAM-1-deficient mice and anti-ICAM-1 neutralizing antibodies, we show that hapten-induced colonic eosinophilic inflammation is critically dependent on ICAM-1. These studies demonstrate that β2-integrin/ICAM-1-dependent pathways are integral to eosinophil recruitment into the colon during GI inflammation associated with colonic injury. J. Leukoc. Biol. 80: 330–341; 2006.

Key Words: eosinophils • adhesion molecules • gastrointestinal tract

INTRODUCTION

Eosinophil accumulation in the gastrointestinal (GI) tract is a common feature of numerous eosinophil-associated GI disease (EGID) disorders including food allergy, eosinophilic esophagitis (EE), eosinophilic gastroenteritis, allergic colitis, and inflammatory bowel disease (IBD; ulcerative colitis (UC) and Crohn’s disease) [1, 2]. Although the underlying causes of EGID are not fully understood, clinical investigations suggest an important role for eosinophils in the etiology of disease. Indeed, a strong correlation has been demonstrated among clinical symptoms, disease severity, and increased numbers of this cell type in the GI tract [1–3]. Furthermore, recent experimental investigations have provided corroborative evidence supporting a role for eosinophils in the pathogenesis of EGID [4].

Numerous inflammatory mediators have been implicated in regulating eosinophil accumulation, including interleukin (IL)-1, -3, -4, -5, and -13 and granulocyte macrophage-colony stimulating factor (GM-CSF) and the chemokines regulated on activation, normal T expressed and secreted (RANTES), monocyte chemoattractant protein (MCP)-3, macrophage inflammatory protein-1α, and eotaxin-1, -2, and -3 [5, 6]. Of the mediators implicated in modulating eosinophil accumulation, eotaxin-1 appears to be the most important molecule in the regulation of eosinophil trafficking into the GI tract [7]. Eotaxin-1 is ubiquitously expressed in all segments of the GI tract [8]. Furthermore, eosinophil levels in the GI tract are reduced significantly in eotaxin-1-deficient mice as compared with wild-type (WT) mice, and overexpression of eotaxin-1 in the GI tract promotes a pronounced eosinophilia in the small intestine [9, 10]. Clinical studies have demonstrated increased expression of eotaxin-1 in EGID, including cows milk-associated reflux esophagitis and UC [11–15].

The transmigration of leukocytes, such as eosinophils, across the vascular epithelium into mucosal tissues, is regulated by coordinated interaction among networks involving...
chemokine and cytokine signaling, eosinophil adhesion molecules (e.g., selectins and integrins), and integrin receptors [e.g., vascular cell adhesion molecule-1 (VCAM-1), mucosal addressin cell adhesion molecule-1 (MAdCAM-1), and intercellular adhesion molecule 1 (ICAM-1)] expressed on vascular endothelial cells [16, 17]. Integrins are heterodimeric surface molecules consisting of an α- and β-chain, and eosinophils express members of the β1 (α5β1, α6β1), β2 (αIL2, αCM2, αCM2 and α5β2), and β3 (αβ2)-integrin families [18-22]. These various integrin molecules interact selectively with adhesion receptors (VCAM-1, MAdCAM-1, ICAM-1, -2, and -3, and fibrinogen) expressed on the vascular endothelium. αβ1 selectively binds to VCAM-1 and fibrinectin, and αβ3 is the primary ligand for the extracellular matrix protein laminin. The αβ1-integrin selectively binds to MAdCAM-1, and lymphocyte function-associated antigen-1 (LFA-1; αβ2) and membrane-activated complex-1 (MAC-1; αβ2β2) bind to ICAM-1. In addition, LFA-1 (αβ2) can bind ICAM-2 and -3, and αβ2 has been shown to bind VCAM-1 and ICAM-3. Leukocyte integrin/intercellular adhesion molecule interactions, particularly LFA-1/ICAM-1 and very late antigen (VLA)-4/VCAM-1, are regulated by cytokines and chemokines such as IL-1 (α and β), tumor necrosis factor (TNF; α and β), IL-4, and IL-13 [23]. IL-1 and TNF stimulate both VCAM-1 and ICAM-1 expression on a variety of cell types. By contrast, IL-4 and IL-13 selectively enhance the expression of VCAM-1. Chemokines also alter the activation state and selectivity of adhesion molecules [24, 25]. For example, treatment of eosinophils with MCP-3, RANTES, or eotaxin-2 switches eosinophils from a β2-integrin/VCAM-1-dominant to a β2-integrin/VCAM-1-dominant, interacting cell [24].

The specific interaction of cell surface integrins with adhesion receptors (VCAM-1, MAdCAM-1, ICAM-1, ICAM-2, ICAM-3, and fibrinogen) facilitates eosinophil migration into various tissue compartments during inflammation. For example, eosinophil recruitment to the site of allergic inflammation in the lung and skin is regulated by a VLA-4 (αβ2-integrin)/VCAM-1-dependent processes [26-30]. Pretreatment of mice with neutralizing monoclonal antibodies (mAb) against αβ2 or β2-integrin or genetic deletion of VCAM-1 attenuates eosinophil accumulation in the lung during allergic airways disease [26-30]. In contrast, eotaxin-1-dependent eosinophil recruitment to the small intestine of the GI tract is MAdCAM-1/αβ2-integrin-dependent [10].

Recruitment of inflammatory cells into the GI tract is currently believed to occur via an αβ2/MAdCAM-1-dependent interaction [16]. The αβ2-integrin receptor MAdCAM-1 is expressed primarily on GI vascular endothelium and is generally absent on nonintestinal venules and most non-GI sites of inflammation [31]. Blockade of αβ2/MAdCAM-1 interactions by neutralizing mAb or genetic deletion inhibits T and B cell and mast cell recruitment into GI compartments including the small intestine, mesenteric lymph nodes, and Peyer’s patches. It is interesting that recent experimental investigations have demonstrated that leukocyte recruitment into the large intestine can occur via a β2-integrin-independent mechanism, suggesting that leukocytes use different adhesion systems to infiltrate various GI compartments [32, 33]. We were therefore interested in identifying the dominant adhesion complex involved in eosinophil trafficking into the large intestine. This is particularly important, as there are numerous diseases characterized by eosinophil accumulation in the colon (e.g., allergic colitis and IBD), yet most studies have concentrated on the upper GI tract (e.g., esophagus and small intestine).

In the present study, we demonstrate that colonic eosinophils express the ICAM-1 ligands [MAC-1 and LFA-1 (β2, α4, and α4)]. Furthermore, using in vivo models of colonic eosinophil inflammation, we demonstrate that eosinophil accumulation in the colon is regulated by a β2-integrin pathway (ICAM-1) and can occur independently of α4- and β2-integrin-independent pathways. This observation has significant implications for the treatment of disease states characterized by colonic eosinophilic inflammation.

MATERIALS AND METHODS

Mice

BALB/c and C57BL/6 mice (6-8 weeks of age) used in our experiments were obtained from the Specific Pathogen Free Facility or the Gene Targeting Facility of the John Curtin School of Medical Research (Australian National University, Canberra). ICAM-1- and L-selectin-/- mice (C57BL6 background) were kindly provided by Thomas F. Tedder (Duke University Medical Center, Durham, NC). CD2-IL-5 transgenic (Tg) β2-integrin mice were generated by crossing CD2-IL-5Tg (BALB/c) mice into the β2-integrin-/- mice (C57BL6 background) (kindly provided by Prof. N. Wagner, City Hospital of Dortmund, Germany). The Tg-positive offspring (N1) were subsequently back-crossed with WT C57BL6 mice, generating background-matched CD2-IL-5Tg β2-integrin-/- mice, which were treated according to the Australian National University Animal Welfare guidelines and were housed in an approved containment facility.

Fluorescein-activated cell sorter (FACS) analysis

CD2-IL-5Tg mice were killed and spleens were removed, and a single-cell suspension was prepared into single-cell suspensions as described previously [34]. To analyze integrin expression on splenic and colonic eosinophils, cells were incubated with phycoerythrin (PE)-conjugated anti-αβ2 (Clone DACTK32, BD Pharmingen, San Diego, CA; 1 μg/10^6 cells), biotin-conjugated anti-α5 (Clone M1/70 15.1, Chemicon Europe CBL, UK), biotin-conjugated anti-α4 (Chemicon Europe CBL), PE-conjugated anti-β2-integrin (G1/16; BD Pharmingen), Alexa Fluor 647 rat anti-mouse CC chemokine receptor 3 (CCR3: 83103; BD PharMingen), or isotype-matched control immunoglobulin (Ig; rat IgG1, Southern Biotechnology, Birmingham, AL) in phosphate-buffered saline (PBS)/1% fetal calf serum (FCS) on ice for 30 min and washed twice in PBS/1% FCS. To visualize binding of the anti-integrin antibodies, cells were incubated with streptavidin-fluorescein isothiocyanate (FITC; 1:1600 dilution) in PBS/1% FCS on ice for 30 min and washed twice in PBS/1% FCS. Cells were analyzed by flow cytometry on a FACS advantage SE buffer.
correlated with a histological score. DAI = (body weight change) + (diarrhea score) + (rectal bleeding score).

**Body weight**

Changes in body weight were calculated as the difference between the predicted body weight and the actual weight on a particular day. The formula for predicted body weight was derived by simple regression using the body weight data for the control group. The following formula was used: $Y = a + kx$, where $Y = $ body weight change (loss or gain), $k = $ daily increase in body weight, $x = $ day, and $a = $ starting body weight.

**Diarrhea**

The appearance of diarrhea was defined as mucus/fecal material adherent to anal fur. The presence or absence of diarrhea was scored as 1 or 0, respectively. The presence or absence of diarrhea was confirmed by examination of the colon following completion of the experiment [36]. Mice were killed, and the colon was excised from the animal. Diarrhea was defined by the absence of fecal pellet formation in the colon and the presence of continuous fluid fecal material in the colon.

**Rectal bleeding**

The appearance of rectal bleeding was defined as diarrhea containing visible blood or gross rectal bleeding and scored as described for diarrhea.

**Histopathological examination**

Animals were killed on Day 8, and the colon was excised. The length of the colon was measured using digmatic calipers (Mitutoyo, Kawasaki, Japan). Tissue specimens were fixed in 10% formaldehyde and stained with hematoxylin and eosin (H&E) and Masson's trichrome using standard histological techniques. The percentage of colon length with mucosal ulceration was determined by performing morphometric analysis of colon using the ImagePro-Plus 4.5 software package (Media Cybernetics, Inc., Silver Spring, MD). In brief, digital images of longitudinal sections (1-2 cm in length) of H&E-stained colon sections were captured using Epix (Hoefer Scientific Instruments, San Francisco, CA). Briefly, the images were digitized using a 12-bit digitizing tablet, and the percentage of ulcerated mucosal lining was quantified using ImageJ software (National Institutes of Health, Bethesda, MD). The value was expressed as a percentage of colon length with mucosal ulceration.

**Detection and quantification of eosinophils by immunohistochemistry**

The colon segment of the GI tract was immunostained with antiserum against mouse major basic protein (MBP) as described previously [8]. Briefly, 5 μm sections were rehydrated and blocked with normal goat serum, and stained with a rabbit antimurine eosinophil MBP antisera (kind gift from Nancy and James Lee, Mayo Clinic, Scottsdale, AZ). The slides were then washed and incubated with biotinylated goat anti-rabbit antibody and avidin-biotin-peroxidase complex (Vectorstain ABC Peroxidase Elite kit, Vector Laboratories, Burlingame, CA). The slides were developed by nickel diaminobenzidine and counterstained with Nuclear Fast Red. Quantification of eosinophils was performed by counting the number of immunoreactive cells from 15-25 fields of view (magnification X40) from at least four to five random sections/mouse. Values were expressed as eosinophils per mm² tissue.

**Eosinophil peroxidase (EPO) activity assay**

Mice were killed on Day 8, and the colon was excised and flushed with 1 ml PBS solution. The fecal material was vortexed vigorously for 5 min at 4°C and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and diluted with a 1:10 PBS solution. The MPO activity produced by 1 X 10⁶-purified eosinophils/μl supernatants purified from the spleen of C57BL/6J mice was assayed as described previously.

**mAb treatment**

Mice were injected intraperitoneally (i.p.), daily, with rat anti-mouse integrin α4β1 chain [200 μl 1 mg/ml Clone PS/2 ( IgG2a) mAb in saline], anti-mouse ICAM-1 [200 μl 1 mg/ml Clone YN1/1.7.4 ( IgG2a) mAb in saline], or rat IgG control antibody (200 μl 1 mg/ml BGL113 mAb in saline) for 4 days. Three hours following the final i.p. injection, mice were killed, the jejunum and colon were excised and fixed in 4% formaldehyde and stained with anti-MBP, and eosinophil levels were quantitated as described above. In some experiments, mice were injected i.p. daily throughout the 8-day DSS treatment protocol with anti-mouse ICAM-1 [200 μl 1 mg/ml Clone YN1/1.7.4 ( IgG2a) mAb in saline] or rat IgG control antibody (200 μl 1 mg/ml BGL113 mAb in saline).

**FACS analysis on colonic eosinophils**

Colon injury was induced with 2.5% DSS as described above. On Day 8, the colon segment of the GI tract was removed and flushed with 20 ml PBS. The colon tissue was cut into 1-cm segments and incubated in digestion buffer containing 80 mg/ml Collagenase A, 24 U/ml Dispase II, and 2.5 mg/ml DNase in RPMI 1640 and incubated for 60 min at 30°C. The tissue was vortexed vigorously every 10 min for 15 s. Following the 60-min incubation, the cell aggregates were dissociated by pipetting and centrifuged at 1200 revolutions per minute for 10 min at 4°C. The supernatant was decanted, and the cell pellet was resuspended in RPMI 1640 + 10% FCS and filtered through a 70-μm filter. The single-cell suspension was prepared by Ficoll density gradient centrifugation and quantitated by trypan blue exclusion analysis. Colonic cells (1X10⁶) were plated in a 96-well round-bottom plate, and flow cytometry analysis staining for CCR3 and β2-integrin was performed as described above.

**Myeloperoxidase (MPO) activity**

MPO activity, a marker of polymorphonuclear neutrophil granules, was assessed in colonic luminal contents according to the Bradley method [38]. Mice were killed on Day 8, and the colon was excised and flushed with 1 ml PBS solution. The fecal material was vortexed vigorously for 5 min at 4°C and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and placed in a sterile Eppendorf tube and stored at −70°C until analysis. MPO activity was measured in the supernatant of cell-free colon flushes as described previously [38]. This assay is based on the oxidation of o-dianisidine dihydrochloride (ODH) by MPO in the presence of H₂O₂. The MPO substrate solution consisted of 0.16% o-dihydrochloride (Sigma Aldrich), 0.005% H₂O₂, 10 mM HEPES, and 0.022% CTAB. Substrate solution (75 μl) was added to cell-free supernatants, which were derived from colon flushes (75 μl) in a 96-well microplate and incubated at room temperature for 15 min before stopping the reaction with 50 μl cold 3 M sulfuric acid. Absorbance was measured at 450 nm. Standard MPO (human leukocytes, Sigma Aldrich; 50 U/mg protein), 10 U/ml, was used to generate a standard curve.

**Statistical analysis**

The significance of differences between the means of experimental groups was analyzed using Student's unpaired t-test. Values were reported as the mean ± SEM. Differences in mean values were considered significant if P < 0.05. In some experiments, one-way and two-way ANOVA with Bonferroni post-test were performed as indicated. All these calculations were performed using SAS, Version 9.0 (SAS Institute, Cary, NC). To test the hypotheses, that the means were equal, one-factor or two-factor ANOVA was used, and the level of significance was at 0.05. In the one-factor analyses, the comparison groups were the treated groups. If the overall F were statistically significant, Bonferroni's multiple comparison was applied to determine where the differences were. In the case of two factors, strain of mice and treatment, if the overall F were less than 0.05, Bonferroni's adjustment was made, dividing the F by the number of comparisons made to adjust the P value.
RESULTS

Role of \( \beta_7 \)-integrin in eosinophil recruitment into the colon

To delineate the adhesion systems involved in eosinophil recruitment into the colon, we used a model of hapten (DSS)-induced colonic injury. DSS treatment of WT mice for 8 days induced colonic injury and an associated cellular infiltrate of the superficial layers of the mucosa comprising of granulocytes and some mononuclear cells (Fig. 1a). To identify tissue eosinophils, we performed immunohistochemistry using a polyclonal antiserum against eosinophil-derived MBP. Eosinophils were observed throughout the mucosa and submucosa in all DSS-treated WT mice (Fig. 1a). Quantification of eosinophil numbers revealed eosinophil levels to be increased significantly in the colon of DSS-treated mice as compared with control-treated mice (Fig. 1b). Furthermore, using an EPO-specific assay [37], we demonstrate that the level of eosinophil-derived EPO in the lumen of the colon of DSS-treated mice was 100-fold higher than that observed in control-treated animals (Fig. 1c).

We hypothesized that eosinophil accumulation in the colon is regulated by the \( \beta_7 \)-integrin, as this molecule has been shown to be critically involved in leukocyte adherence and recruitment into the GI tract and in eosinophil recruitment into the small intestine [10, 31]. DSS-induced eosinophilic inflammation in \( \beta_7 \)-integrin \(^{-/-} \) mice was comparable with that observed in strain-matched WT mice (Fig. 1, a–c). Furthermore, EPO activity in the lumen of the colon of DSS-treated \( \beta_7 \)-integrin \(^{-/-} \) mice revealed a significant and near-identical increase as compared with DSS-treated WT mice (Fig. 1c). No significant difference in peripheral blood or GI eosinophil numbers was observed between WT and \( \beta_7 \)-integrin \(^{-/-} \) mice at baseline (results not shown [10]).

Previous investigations have demonstrated that VLA-4 (\( \alpha_4 \beta_1 \)) is important in eosinophil recruitment during inflammatory responses [26–30]. To examine the role of the VLA-4/VCAM-1 pathway in eosinophil recruitment into the colon, \( \beta_7 \)-integrin \(^{-/-} \) mice were administered anti-\( \alpha_4 \)-integrin mAb (PS/2 mAb) or control Ig and exposed to 2.5% DSS in drinking water or drinking water alone for 8 days, and markers of eosinophilic inflammation in the colon were examined. DSS

Fig. 1. DSS-induced colonic eosinophilic inflammation occurs via a \( \beta_7 \)-integrin-independent mechanism. (a) Representative photomicrographs of H&E and anti-MBP-stained colon from control and DSS-treated C57BL/6 WT and \( \beta_7 \)-integrin \(^{-/-} \) mice. (b) Eosinophil numbers per high-powered field (HPF) and (c) EPO activity in lumen of the colon of control and DSS-treated \( \beta_7 \)-integrin \(^{-/-} \) and WT mice. Data represent the mean ± SEM of four to five random sections per mouse for four to five mice per group. Statistical significance of differences (\( P<0.05 \)) was determined using Student’s unpaired t-test. Significant differences (\( P<0.05 \)) between groups, \( * \), \( P < 0.05 \), as compared with matched control. Inset depicts MBP-positive eosinophils. Original magnification (a), \( \times 40 \).

Forbes et al. ICAM-1-mediated colonic eosinophilia 333
Fig. 2. DSS-induced colonic eosinophilic inflammation occurs via an α4-integrin-independent mechanism. (a) Eosinophil numbers per HPF and (b) EPO activity in lumen of the colon of control- and DSS-treated β1-integrin −/− mice treated with control Ig or anti-α4-integrin mAb. Data represent the mean ± SEM of four to five random sections per mouse for three to six mice per group. Statistical significance of differences (P<0.05) was determined using Student's unpaired t-test. Significant differences (P<0.05) between groups. *, P < 0.05, as compared with matched control.

Treatment of β1-integrin −/− mice administered control Ig significantly increased colonic eosinophil numbers and EPO activity as compared with control-treated β1-integrin −/− mice administered control Ig (Fig. 2a). Similarly, DSS treatment of β1-integrin −/− mice administered with anti-α4-integrin mAb significantly increased colonic eosinophil levels, as compared with control-treated β1-integrin −/− mice administered anti-α4-integrin mAb (Fig. 2a). It is notable that the level of eosinophils and EPO activity in the colon of DSS-treated β1-integrin −/− mice administered with anti-α4-integrin mAb was equivalent to that observed in DSS-treated β1-integrin −/− mice administered control Ig mAb (Fig. 2b). Collectively, these studies suggest that the VLA-4/VCAM-1 and β1-integrin pathways do not play a major role in eosinophil trafficking into the colon under inflammatory conditions related to colonic injury.

Expression of β2-integrin subunits on splenic eosinophils from CD2-IL-5Tg mice

Following the demonstration that eosinophil recruitment to the colon could occur independently of α4- and β2-integrins, we were next interested in elucidating the involvement of the β2-integrin members (LFA-1 (αLβ2) and MAC-1 (αMβ2)). β2-Integrin members have previously been shown to be involved in eosinophil recruitment into the lung during allergic pulmonary disease [30, 39, 40]. Initially, we examined the surface expression of β2-integrin members (αL, αM, and β2) on splenic eosinophils from CD2-IL-5Tg mice. Eosinophils were analyzed by flow cytometry based on FSC-H versus SSC-H (Fig. 3a). To confirm that the identified cell population was eosinophils, the gated cells were sorted by flow cytometry, cytocentrifuged, and stained with chromotrope 2R. The sorted cells (95–99%) were identified as eosinophils by histological analysis (Fig. 3b). Splenic eosinophils from CD2-IL-5Tg mice expressed β2−, αL−, αM−, and αL-integrins (Fig. 3, c–f). Collectively, these studies demonstrate that peripheral eosinophils express the ICAM-1-binding LFA-1 (αLβ2) and MAC-1 (αMβ2) integrin subunits.

Characterization of the β2-integrin/ICAM-1 pathway expression in the colon

To identify whether the β2-integrin is involved in eosinophil transmigration into the colon during colonic injury, we examined expression of β2-integrin on colonic lamina propria cells from the colon of control- and DSS-treated WT mice by flow cytometry. We show that β2-integrin + lamina propria cells were increased significantly in DSS-treated mice as compared with control-treated animals (Fig. 1a). To determine whether β2-integrin + cells were eosinophils, we examined the CCR3 expression on β2-integrin + cells. In contrast to humans, CCR3 has been localized specifically to eosinophils in the mouse, and only one study has reported CCR3 on murine mast cells.

Fig. 3. Characterization of surface expression of LFA-1 and MAC-1 integrin chains on eosinophils. Representative histograms of β2−, αL−, αM−, and αL-integrin chain expression on splenic eosinophils from CD2-IL-5Tg mice. (a) Representative FSC-H versus SSC-H dot-plot of splenocytes from CD2-IL-5Tg mice. (b) Photomicrograph of cytocentrifuged cells sorted by FSC-H versus SSC-H and polarized light criteria (depicted in box in a) and stained by H&E. The purity of eosinophils was >95% on re-analysis. Representative histograms of (c) β2−, (d) αL−, (e) αM−, and (f) αL-integrin chain expression on splenic eosinophils from CD2-IL-5Tg mice, respectively. Filled histogram depicts cells + isotype-matched control Ig; dotted line (streptavidin-PE) and closed line, anti-integrin antibody + (streptavidin-PE).
It is notable that ~70% of β2-integrin+ cells were CCR3+, suggesting that colonic eosinophils expressed β2-integrin (results not shown). To identify whether CCR3+ eosinophils from the colon of hapten-treated WT mice express αL- and αM-integrins, we examined αL and αM expression on β2-integrin+ CCR3+ eosinophils. We demonstrate that all β2-integrin+ CCR3+ cells expressed αL- and αM-integrins (Fig. 4, c and d). To exclude the possibility that the CCR3+ cells could be mast cells, we performed chloroacetate esterase staining on the colon of control- and DSS-treated WT mice and quantified mast cell levels. We observed no significant increase in mast cell levels in the colon following DSS treatment (results not shown). Collectively, these studies demonstrate that DSS treatment promotes the recruitment of β2-integrin+ CCR3+ eosinophils into the colon. Furthermore, these studies suggest that colonic eosinophils express the ICAM-1 counter-receptors, LFA-1 (αLβ2) and MAC-1 (αMβ2) integrins.

The effect of blockade of αL-, αM-, and αz-integrins on eosinophil recruitment into the colon at baseline

We were next interested in elucidating whether blockade of the β2-integrin pathway would inhibit eosinophil recruitment into the colon. To do this, we initially took a Tg approach, using CD2-hL-5Tg mice and neutralizing mAb against the β2-integrin counter-receptor ICAM-1. Although LFA-1 (αLβ2) interacts with a number of counter-receptors including ICAM-1, ICAM-2, ICAM-3, and fibrinogen, and MAC-1 (αMβ2) can bind to ICAM-1 and the iC3b component of complement, the β2-integrin (LFA-1 and MAC-1) principal receptor is ICAM-1 [46–48]. Overexpression of IL-5 in T cells promotes a pronounced colonic eosinophilic inflammation. Treatment of CD2-hL-5Tg mice with anti-ICAM-1 neutralizing mAb significantly reduced the colonic eosinophilic infiltrate induced by the IL-5Tg [eosinophils/HPF; 176.9±3.8 vs. 101.1±9.3; CD2-hL-5Tg mice+Ig control vs. CD2-hL-5Tg mice+anti-ICAM-1 mAb; mean±SEM (n=3 and 5 mice per group, respectively; *P<0.05)], indicating that ICAM-1-binding integrins (β2-integrins) are involved in the constitutive homing of eosinophils into the colon.

The effect of blockade of the ICAM-1 pathway on eosinophil recruitment into the colon during colonic injury

To examine the role of the β2-integrin/ICAM-1 pathway in the migration of eosinophils into the colon during colonic injury, we employed WT and ICAM-1−/− mice. Administration of DSS to WT mice induced a colonic eosinophilic infiltrate (Fig. 5, a and b). In contrast, hapten-induced eosinophilic inflammation in ICAM-1−/− DSS-treated mice was significantly attenuated as compared with WT DSS-treated mice (Fig. 5b). Consistent with the reduction in eosinophil numbers in the absence of ICAM-1, luminal EPO activity was also reduced significantly in DSS-treated ICAM-1−/− mice as compared with WT mice (Fig. 5c). To demonstrate that this was mediated by an ICAM-1-dependent process, we challenged L-selectin−/− mice with DSS (Fig. 5c). DSS-induced eosinophil recruitment and EPO activity in L-selectin−/− mice were comparable with that observed in strain-matched WT mice (Fig. 5, b and c), suggesting that the observed attenuation of eosinophilic inflammation was

**Fig. 1.** β2-integrin+ CCR3+ cells in the colon following DSS administration. (a) Percentage of β2-integrin-positive cells in colon of control- and DSS-treated WT mice. (b) Representative histograms of isotype-matched control Ig (FITC and Alexa Fluor 647) on β2-integrin+–gated lamina propria cells from DSS-treated WT mice. Representative histogram of (c) αL-integrin- and (d) αM-integrin–gated colonic cells from DSS-treated WT mice. (a) Data represents the mean ± SEM of (n = 4–5 mice per group from duplicate experiments. Statistical significance of differences was determined using Student’s unpaired t-test. Significant differences (*P<0.05) between groups, *P<0.05, as compared with control.

Forbes et al. ICAM-1-mediated colonic eosinophilia 335
The lumen of the colon of these mice were elevated significantly. Eosinophil levels in the colon and the level of KI'O in the mice induced a colonic eosinophilic inflammation (Fig. 5d).

To demonstrate that the observed ablation of eosinophil recruitment into the colon by ICAM-1 blockade is directly a result of inhibition of eosinophil transmigration and not a result of the suppression of the recruitment of other inflammatory cells, we examined neutrophil levels in hapten-treated ICAM-1-/- mice. Neutrophils and not B and T cells have been shown to be important in the inflammatory response observed in hapten-induced colonic injury [49–51]. To quantitate neutrophil levels, we examined MPO activity in colon luminal washes from DSS-treated mice. To block residual EPO activity, we performed the MPO assay in the presence of an EPO-specific inhibitor resorcinol (60 μM) [34, 37]. DSS administration to WT mice promoted an increase in luminal MPO activity as compared with control-treated WT mice (Fig. 6). Similarly, administration of DSS to ICAM-1-/- mice induced a significant and comparable increase in luminal MPO activity, demonstrating no reduction in neutrophil recruitment into the colon in the absence of ICAM-1 (Fig. 6). The level of MPO activity is DSS-treated WT, and ICAM-1-/- mice were not reduced significantly in the presence of the EPO inhibitor resorcinol (0, 1, 10 (results not shown) and 60 μM; Fig. 6). These studies suggest that the β3-integrin/ICAM-1 pathway is involved directly in eosinophil but not neutrophil recruitment into the colon during colonic injury.

The effect of blockade of ICAM-1 in colonic injury

Recently, there has been increasing interest in eosinophils in the pathogenesis of colonic inflammatory disorders, particularly UC [52]. Elevated levels of eosinophils have been observed in colonic biopsy samples from UC patients, and increased numbers of this cell and eosinophil-derived granular proteins have been shown to correlate with morphological changes to the GI tract, disease severity, and GI dysfunction associated specifically with the ICAM-1 pathway. To exclude the possibility that inherit compensatory mechanisms associated with ICAM-1 gene deficiency can account for the ablation of eosinophil recruitment into the colon in ICAM-1-/- mice, we administered DSS-treated WT mice with Ig control or anti-ICAM-1 neutralizing mAb and examined colonic eosinophil levels. Administration of DSS to control Ig-treated WT mice induced a colonic eosinophilic inflammation (Fig. 5d). Eosinophil levels in the colon and the level of EPO in the lumen of the colon of these mice were elevated significantly compared with control-challenged anti-ICAM-1-treated mice (Fig. 5, d–e). In contrast, eosinophil and EPO levels in the colon of DSS-administered anti-ICAM-1-treated mice were reduced significantly as compared in DSS-challenged, control Ig-treated WT mice (Fig. 5, d–e). These studies confirm that the β3-integrin/ICAM-1 pathway is critical in the transmigration of eosinophils in the colon during colonic injury.

![Fig. 5. Blockade of ICAM-1 by gene deletion or neutralizing mAb inhibits eosinophil recruitment into the colon.](http://www.jleukbio.org)

![Fig. 6. MPO activity in the colon of DSS-treated WT and ICAM-1-/- mice.](http://www.jleukbio.org)
The observed reduction in eosinophil levels in the colon of hapten-treated ICAM-1<sup>−/−</sup> mice, as compared with WT mice, prompted us to examine the contribution of eosinophils to histopathological features of DSS-induced colonic injury. DSS treatment of strain-matched WT mice induced hapten-induced colonic injury characterized by increased DAI, diarrhea, rectal bleeding, and colon shortening (Fig. 7, and results not shown). Histological examination of the colonic tissue revealed extensive ulceration of the epithelial layer, submucosa edema, crypt damage, fibrotic thickening of the muscularis mucosa, and dense infiltration of the superficial layers of the mucosa with granulocytes and some mononuclear cells (Fig. 5, a–d). However, in ICAM-1<sup>−/−</sup> mice, DSS-induced colonic injury was significantly attenuated. Moreover, physical symptoms (DAI, diarrhea, and rectal bleeding) and histopathological features (ulceration of the epithelial layer, submucosa edema, and crypt damage) in DSS-treated ICAM-1<sup>−/−</sup> mice were reduced significantly as compared with either strain-matched WT mice (Fig. 7). This attenuation was associated with a reduction in eosinophil accumulation of the colon and the loss of EPO activity in DSS-treated ICAM-1<sup>−/−</sup> mice (Fig. 5). Thus, these studies demonstrate that ICAM-1 plays a central role in eosinophil recruitment into the colon and in the immunopathogenesis of colonic injury.

**DISCUSSION**

We have previously demonstrated that eosinophils express α<sub>4</sub>β<sub>7</sub>-integrin and that the β<sub>7</sub>-pathway is critical for eotaxin-mediated eosinophil recruitment into the small intestine [10]. In this study, we have examined the adhesion systems that regulate eosinophil recruitment into the large intestine and have demonstrated that eosinophil recruitment into the colon during colonic injury occurs via an α<sub>4</sub>- and β<sub>7</sub>-integrin-independent pathway; that murine splenic eosinophils express the ligands for ICAM-1 (β<sub>2</sub>, α<sub>4</sub>, and α<sub>m</sub>); eosinophils (CCR3<sup>+</sup>) isolated from colon of hapten-treated mice express the ICAM-1 counter-receptor integrins (β<sub>2</sub>, α<sub>4</sub>, and α<sub>m</sub>); and blockade of ICAM-1 activity by gene knockout or by neutralizing mAb attenuated eosinophil recruitment into the colon. Collectively, these studies suggest that the ICAM-1 adhesion pathway and not VCAM-1 and α<sub>4</sub>β<sub>7</sub>/MAdCAM-1 pathways is important for the development of colonic eosinophilic inflammation.

Leukocyte recruitment into the GI tract and gut-associated lymphoid tissue are thought to be critically regulated by β<sub>7</sub>-integrin [31, 32, 59, 60]. Indeed, the expression of MAdCAM-1 and α<sub>4</sub>β<sub>7</sub> expression on leukocytes in the intestinal mucosa is up-regulated significantly in GI diseases, including cows milk allergy and food allergy [61, 62]. Recently, experimental stud-
ies have demonstrated that leukocyte recruitment into the large intestine can occur in a β2-integrin-independent manner [32, 33, 60]. Moreover, no impairment in CD4+ T cell and mast cell infiltration into the colon of β2-integrin−/− mice was observed following helminth infection [32]. Furthermore, the concentration of mast cell progenitors in the large intestine of β2-integrin−/− mice was not reduced as compared with WT mice [60]. We demonstrate that eosinophil recruitment into colon during inflammatory conditions occurs predominantly via a β2-integrin-independent mechanism, through a β2-integrin/ICAM-1 pathway. The contribution of the β2-integrin pathway in leukocyte and mast cell recruitment into the colon are not yet fully elucidated.

In our previous investigations, we have demonstrated that eosinophil recruitment into the colon during colonic injury is critically regulated by the CCR3 ligand, eotaxin-1 [34]. It is notable that eotaxin-1 up-regulates the expression of ICAM-1-binding integrins αL and αm expression on lymphocytes [63]. Furthermore, we have recently demonstrated that in vitro stimulation of splenic eosinophils with eotaxin-1 in the presence of IL-5 up-regulates the surface expression of αL and αm-integrins on eosinophils (results not shown). In light of these findings and our demonstration of a role for the β2-integrin/ICAM-1 pathway in eosinophil recruitment, it is tempting to speculate that eotaxin-1 preferentially promotes β2-integrin/ICAM-1 interactions and eosinophil recruitment into the colon.

In different disease states, elevated eosinophil numbers are often restricted to specific GI compartments. For example, EE is characterized by elevated level of eosinophils restricted to the esophagus, whereas, eosinophilic colitis and UC are characterized by elevated levels of eosinophils in the colon without increased numbers in other GI compartments. Paradoxically, the recruitment of eosinophils into these various GI compartments (small and large intestine) is thought to be primarily regulated by eotaxin-1 [17]. How eotaxin-1 selectively orchestrates the trafficking of eosinophils into specific GI compartments remains unclear. It is possible that eotaxin-1 preferentially up-regulates αL, αm-integrins on eosinophils and the β2-integrin counter-receptor, ICAM-1, on colonic microvascular endothelial cells to promote eosinophil infiltration into the colon. Indeed, eotaxin-1 has been shown to stimulate expression of ICAM-1 on microvascular endothelial cells [64]. However, as we demonstrate that αL, αm, β2-integrins are expressed on eosinophils under noninflammatory conditions, the differential expression of integrins cannot fully account for the eotaxin-1-mediated selectivity for the ICAM-1 pathway. An alternate explanation is that chemokines such as eotaxin-1 differentially regulate integrin/adhesion molecule avidity, promoting β2/ICAM-1-dependent adhesion pathway interaction. Weber and colleagues [65] have demonstrated that RANTES and MCP-3, eotaxin-1 receptor (CCR3) agonists, can selectively down-regulate eosinophil VLA-4/VCAM-1 adhesion and promote β2/ICAM-1 adhesion. Furthermore, they showed that the differential regulation of adhesion occurred independently of integrin surface expression and directly involved modulation of integrin avidity [65]. We postulate that eotaxin-1-mediated recruitment involves a complex step of events involving integrin expression on leukocytes (increased αL, αm, β2), adhesion molecule expression on microvascular endothelium (ICAM-1), and increased integrin and integrin receptor avidity (LFA-1/ICAM-1, MAC-1/ICAM-1).

The histological presence of eosinophils in the GI mucosa of patients with IBD has long been recognized; however, the contribution eosinophils make to disease pathogenesis is still not well-understood. Recent clinical investigations of bowel biopsy specimens from UC patients have shown a correlation among the eosinophil numbers in the mucosa, the levels of eosinophil-derived granule proteins (MBP, EPO, eosinophil cationic protein, and eosinophil-derived neutrotoxin) in perfusion fluid samples, and disease severity [14, 56, 57, 66]. Previously, we have demonstrated that eosinophil mediated disease pathogenesis in experimental colitis through EPO-dependent pathways [34]. EPO catalyzes the oxidation of halides and pseudoaldehydes (Cl-, Br-, and SCN-) with the products of respiratory burst (O2 and H2O2) to generate cytotoxic oxidants. Elevated levels of reactive oxygen species (H2O2) have been reported in mucosal tissue samples from patients with UC [67, 68]. Furthermore, H2O2 has been shown to promote an up-regulation of β2-integrin expression on eosinophils [69].

Using L-selectin−/−deficient mice, we demonstrate that DSS-induced eosinophil recruitment and EPO activity could occur independently of L-selectin. It is notable that eosinophil levels in DSS-treated L-selectin−/−deficient mice were comparable with WT. However, although EPO activity levels were elevated significantly in DSS-treated L-selectin−/−/EPO−/− mice, the EPO activity levels were reduced by ~35%, as compared with DSS-treated WT mice. These studies suggest that eosinophil release of EPO is at least in part dependent on L-selectin, which binds cell-surface carbohydrates including sialylated, fucosylated, and sulfated lactosaminoglycans such as the sialyl LewisX tetrasaccharides. It is notable that liposaccharides of enteric pathogens have been shown to express LewisX antigens. It is possible that during DSS-induced colonic injury, activation of L-selectin via cell-surface carbohydrates on enteric bacteria promotes eosinophil degranulation and release of EPO. Consistent with this notion, eosinophil granule proteins have been shown to possess bactericidal activity [70, 71].

In the present study, we also examined the role of ICAM-1 in the recruitment of neutrophils into the colon during hapten-induced colonic injury. To study neutrophil levels, we examined MPO activity, a marker of polymorphonuclear neutrophil granules in the colon of DSS-treated WT and ICAM-1−/−/EPO−/− mice. Consistent with previous investigations, we show that hapten treatment enhances colonic neutrophil levels. Furthermore, we show no difference in MPO activity between DSS-treated WT and ICAM-1−/−/EPO−/− mice. To confirm that contaminating EPO activity was not contributing to the peroxidase activity, we performed the assay in the presence of an EPO inhibitor, resorcinol (60 μM), which has been shown to selectively inhibit EPO activity without neutralizing MPO [37, 72]. These studies suggest that neutrophil recruitment during hapten-induced colonic injury is mediated via ICAM-1-independent mechanisms. Consistent with this observation, Krieglstein and colleagues [49], using an identical model to what we have described in this investigation, have demonstrated that monocyte and neutrophil (measured by MPO activity) accumulation
into the colon is regulated by an αβ1-integrin-dependent pathway [49].

It is notable that although we observed a significant attenuation in physical symptoms and histopathological features of disease in DSS-treated ICAM-1−/− mice as compared with matched WT mice, we still observed a significant weight-loss over the course of the experimental regime. We cannot fully account for the observed weight-loss in DSS-treated ICAM-1−/− mice. However, although eosinophils and EPO levels are significantly attenuated, the level of eosinophils and EPO activity is two- to threefold higher than that observed at baseline in ICAM-1−/− control mice. It is possible that this residual level of eosinophils and EPO activity may contribute to the weight-loss, or alternatively, weight-loss may be driven by concurrent pathways, independent of ICAM-1, eosinophils, and EPO.

Clinical and experimental studies have provided evidence for a role of ICAM-1 in UC [73–76]. ICAM-1 levels are elevated in sonicated colonic tissue samples from UC patients, as compared with control patients, and in the colon of mice experimental colitis [73]. Furthermore, blockade of ICAM-1 function by neutralizing mAb or by antisense oligonucleotide against ICAM-1 ameliorates experimental colitis [74–76]. To our knowledge, this is the first demonstration of a critical role for ICAM-1 adhesion pathway in eosinophil accumulation in the colon during colonic injury. These findings are particularly important, given 1) that activated eosinophils and eosinophil-derivated granular proteins have been linked to the pathogenesis of diseases characterized by colonic injury, such as UC in humans and that therapeutic approaches targeting ICAM-1 including as antisense ICAM-1 oligonucleotide (ISIS-2302) therapy, are being examined for the treatment of IBD [77–79]; and 2) that recent clinical trials examining the use of a humanized antibody to α4β1 as a therapeutic treatment for IBD, particularly UC [80], suggest the involvement of adhesion pathways, independent of α4β1, in the regulation of cellular recruitment in UC.

In conclusion, we have shown that eosinophils express ICAM-1-binding integrins (β2, α4, and α5) and that recruitment into the colon occurs via an ICAM-1-dependent and not β1-integrin-dependent pathway. Furthermore, we demonstrate a pathogenic role for eosinophils and ICAM-1-mediated adhesion pathways in the development of experimental colitis. These studies highlight the importance of eosinophils in GI diseases and suggest that antagonism of ICAM-1/eosinophil pathways may be a significant, therapeutic approach for the treatment of UC and allergic colitis.

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Forbes et al. ICAM-1-mediated colonic eosinophilia 339


Forbes et al. ICAM-1-mediated colonic eosinophilia 341
IL-9/mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity

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Abstract

Previous mouse and clinical studies demonstrate a link between Th2-intestinal inflammation and induction of the effector phase of food allergy. However, the mechanism by which sensitization and mast cell responses occurs is largely unknown. We demonstrate that IL-9 has an important role in this process: IL-9-deficient mice fail to develop experimental oral antigen-induced intestinal anaphylaxis and intestinal IL-9 overexpression induces an intestinal anaphylaxis phenotype (intestinal mastocytosis, intestinal permeability and intravascular leakage). In addition, intestinal IL-9 overexpression predisposes to oral antigen sensitization, which requires mast cells and increased intestinal permeability. These observations demonstrate a central role for IL-9/mast cell-mediated experimental intestinal permeability in oral antigen sensitization and suggest that IL-9-mediated mast cell responses have an important role in food allergy.
Introduction

Allergic responses in the gastrointestinal (GI) tract were relatively uncommon several decades ago, however recent studies have demonstrated that food allergies now affect 2-6% of the population (1, 2). Clinical and experimental analyses suggest that initiation of food-induced intestinal anaphylactic responses is regulated by numerous inflammatory mediators, including Th2-cytokines. Indeed, peripheral blood and intestinal tissue from patients with food allergy contain elevated numbers of activated T-cells, which correlate with elevated levels of Th2-cytokines and the degree of GI inflammation and dysfunction (3, 4). Furthermore, in vitro allergen-stimulated T-cells and T-cell clones generated from food allergic patients produce Th2-cytokines (IL-4, IL-5 and IL-13) (5). These cytokines activate immunological pathways associated with the onset of allergic reactions including, Th2-cell differentiation, IgE synthesis, mast cell and eosinophil recruitment and activation.

IL-9 is a pleiotropic cytokine involved in Th2-inflammatory reactions (6). Transgenic expression of IL-9 in the lung promotes Th2-mediated allergic pulmonary disease characterized by elevated Th2-cytokines and immune pathology [mucus hypersecretion] and bronchial hyperresponsiveness (7, 8). In vitro studies demonstrate that IL-9 enhances IL-4-induced IgE production (9-11), and airway epithelial cell derived CC-chemokine expression [CCL11/eotaxin-1, CCL3/MIP-1α, CCL2/MCP1, CCL7/MCP-3, and CCL12/MCP-5]. IL-9 has also been implicated in the regulation of mast cell recruitment and effector function (6). Transgenic expression of IL-9 in the lung promotes a pulmonary mastocytosis (7, 8) and IL-9 stimulation of mast cells induces histamine release and promotes mast cell protease, IL-6 and FceRIα expression (12, 13). While IL-9 has been implicated in the regulation of a number of Th2-processes, the contribution of this cytokine to oral antigen-induced intestinal allergic responses has not been explored.
The molecular basis underlying the causality of food antigen sensitization in susceptible individuals is not currently understood. One predisposing factor that has long been suspected for GI diseases is impaired barrier function, termed "leaky gut" (14, 15). First degree relatives of inflammatory bowel disease (IBD) patients have increased intestinal permeability in the absence of clinical symptoms (16-19). Food allergy patients also have increased intestinal permeability, which correlates with the severity of their clinical symptoms (14). While constitutive abnormalities in intestinal permeability have not been consistently observed in food allergic individuals, it is postulated that environmental events including infection and stress may alter intestinal permeability and promote food antigen sensitization (15).

In this study, we evaluate the roles of IL-9 and mast cells in the oral antigen-sensitization and effector phases of experimental intestinal anaphylaxis. We demonstrate an IL-9-stimulated, mast cell-mediated increase in intestinal permeability is central to the induction of oral antigen sensitization.
Results

Experimental intestinal anaphylaxis is IL-9 dependent

The temporal expression of IL-9 mRNA in the jejunum following oral ovalbumin (OVA) challenge was evaluated by quantitative PCR analysis in OVA-sensitized mice that had received 1 and 3 oral OVA- or saline (SAL)-intragastric (i.g.) challenges. Jejunal IL-9 mRNA expression was upregulated in WT mice following one (~200-fold) and three (~150-fold) oral OVA challenges as compared to SAL-challenged mice (1 i.g. challenge: 200.8 ± 115.1 and 3 i.g. challenges 142.5 ± 29.3 fold change in IL-9/GADPH ratio; p<0.05 and p<0.01 respectively). Thus, oral antigen-induced intestinal anaphylaxis is associated with increased intestinal IL-9 mRNA expression.

To begin to elucidate the contribution of IL-9 to oral antigen-induced intestinal anaphylaxis, we employed IL-9 deficient mice (IL-9−/−) and WT BALB/c mice. i.g. challenges of OVA to OVA-sensitized WT mice induced the intestinal anaphylaxis phenotype [intestinal mastocytosis, mast cell activation and diarrhea] (Fig. 1.). WT mice started developing diarrhea acutely after the third i.g. challenge with greater than 75% of the mice suffering from diarrhea following the 7th challenge (Fig. 1 a). Diarrhea in the OVA-challenged and sensitized WT mice was also noted by direct observation of the colon and cecum following the 9th i.g. challenge; the liquid stool observed following OVA-challenge of WT mice contrasts with the solid pellets seen in the distal colon of SAL-challenged WT and OVA-challenged IL-9−/− mice (results not shown). Notably, intestinal mast cell and serum mMCP-1 levels were also significantly elevated compared to SAL-challenged OVA-sensitized WT mice (Fig. 1. b - e). In contrast, oral antigen-induced intestinal anaphylaxis was attenuated in IL-9−/− mice. Typically in any one experiment, 1/6 OVA-challenged OVA-sensitized IL-9−/− mice would have evidence of diarrhea 45-60 minutes following i.g. OVA-
challenge. No more than 20% of IL-9−/− mice developed diarrhea following i.g. OVA-challenges (Fig. 1 a). Consistent with the reduction in intestinal anaphylaxis, intestinal mast cell and serum mMCP-1 levels were significantly lower in oral antigen-challenged OVA-sensitized IL-9−/− mice than in WT mice (Figure 1 b, c and e). To determine whether the ablation of diarrhea in IL-9−/− mice was due to an attenuated OVA-CD4+ Th2 response, we examined splenic cytokine production and levels of OVA-specific IgE in OVA-challenged OVA-sensitized WT and IL-9−/− mice. These levels were equivalent in WT and IL-9−/− mice (Table S1 and Fig. 1. f).

The inhibition of intestinal mastocytosis in oral antigen-challenged IL-9−/− mice led us to examine basal intestinal mast cell levels in WT and IL-9−/− mice. These were decreased 2-fold in IL-9−/− mice compared to WT mice (Fig. 1 g). In contrast, the basal level of mast cell progenitors was similar in the intestines of IL-9−/− mice and WT mice (Figure 1. h).

**Intestinal overexpression of IL-9 promotes intestinal mastocytosis**

In an attempt to delineate the IL-9-regulated inflammatory pathways associated with oral antigen-induced intestinal anaphylaxis, we took a transgenic approach utilizing the intestine-specific promoter of the rat fatty acid-binding protein (iFABPp) gene. This promoter has been extensively used to direct the expression of genes specifically in enterocytes of the small intestine (20, 21). Lightcycler PCR analysis employing murine IL-9 specific primers revealed an increase in the intestinal mIL-9 mRNA expression in the iFABPp-IL-9Tg mice compared to WT mice (Fig. 2 a). Furthermore, IL-9 protein in serum and intestinal tissue was significantly elevated in iFABP-IL-9Tg mice compared to WT mice (Fig. 2 b and c). To assess the consequence of IL-9 expression in the small intestine, we performed histological analysis on the small intestine from WT and iFABPp-IL-9Tg mice. The gross morphology and architecture of the small intestine of iFABPp-IL-9Tg mice
were similar in appearance to those of WT mice (Fig. 3 c and d). Furthermore, epithelial cell subpopulation numbers (goblet cell, enteroendocrine, Paneth and enterocytes) and intestinal epithelial cell proliferation in the small intestine were equivalent between WT and iFABPp-IL-9Tg mice (results not shown). Mast cell levels, however, were significantly elevated in iFABPp-IL-9Tg mice compared to WT mice (Fig. 3 a). Mast cells were predominantly localized to intraepithelial, intercryptic and lamina propria regions of the small intestine of IL-9 intestinal transgenic mice (Fig. 3 a and d). The level of mMCP-1 in the serum of iFABPp-IL-9Tg mice was ~6-fold greater than that of WT mice (Fig. 3 b). In contrast, levels of mast cell progenitors in the small intestine and other tissues including lung, spleen and bone marrow of iFABPp-IL-9Tg and WT mice were similar (Fig 3 e). To determine the effect of intestinal expression of IL-9 on other intestinal immune parameters, we examined CD4⁺, CD8⁺ T-cell, B (B220) cell, regulatory T-cell (CD4⁺, CD25⁺, CD45RBlow and FoxP3⁺) and dendritic cell (DC) levels in the mesenteric lymph node (MLN) of iFABPp-IL-9Tg and WT mice (Table S2). We observed no difference in the number or percentages of these cells in the draining mesenteric lymph nodes of iFABPp-IL-9Tg and WT mice and no difference in T-cell activation status (CD44, CD62L, and CD69) or DC subpopulations between groups. Finally, we examined serum IL-4 and IFNγ and total Ig levels in WT and iFABPp-IL-9Tg mice and observed no significant differences between groups [IL-4; 103.9 ± 28.8 vs 134.2 ± 47.4 ng/ml; IFNγ; 717.4 ± 134.3 vs 736.4 ± 133.1 ng/ml; mean ± SD; n=4-5 mice per group, WT and iFABPp-IL-9Tg, respectively and results not shown]. Collectively, these studies reveal that overexpression of IL-9 in the small intestine selectively promotes intestinal mastocytosis and mast cell activation.
Intestinal overexpression of IL-9 induces an experimental intestinal anaphylaxis transcriptome and phenotype

To gain a further understanding of the consequence of elevated intestinal IL-9 and mast cells on the intestine, we performed a genome-wide expression profile analysis using Affymetrix oligonucleotide chips on small intestinal RNA from WT and iFABPp-IL-9Tg mice. Using a criterion of 2.0-fold change, we identified 176 genes altered in the iFABPp-IL-9Tg mice (Table S3). Out of these transcripts, 126 were up regulated and 52 were down regulated. Functional classification of the altered transcripts revealed a significant predominance of mast cell-associated genes including phospholipase A2, group IVC (26.9-fold), carbonic anhydrase 3 (12.7-fold), mMCP-2 (7.4-fold), mMCP-1 (6.8-fold) and Fc receptor IgE, high affinity alpha polypeptide (Fc^RI) (Fig. 4 b). To validate the whole genome-wide findings, we performed real-time PCR analysis on select genes. Levels of mMCP-1 (~50-fold), mMCP-2 (~100-fold), mMCP-4 (~10-fold), and Fc^RI (~100-fold) in the small intestine of iFABPp-IL-9Tg mice were significantly elevated compared with WT mice (Fig. 4 a). Notably, we observed no increase in the levels of mMCP-5 mRNA expression (Fig. 4 a). These studies demonstrate that IL-9 overexpression upregulates mast cell gene expression in the intestine. Previous whole genome-wide analysis of oral antigen-induced intestinal anaphylaxis (22) demonstrated that the most upregulated genes associated with oral antigen-induced intestinal anaphylaxis were the mast cell associated genes mMCP-2, mMCP-1, and Fc receptor, IgE, high affinity, alpha polypeptide, and Th2-immunity genes, including RELMβ and small proline-rich protein 2A (22) (Fig. 4 b). Remarkably, comparison of the upregulated genes in these studies and the upregulated genes in the intestine of syngeneic iFABPp-IL-9Tg mice revealed a similar profile (Fig. 4 b). Thus, intestinal expression of IL-9 is sufficient to induce a genetic profile that overlaps with that observed in oral antigen-induced intestinal anaphylaxis.
Oral antigen-induced intestinal anaphylaxis in WT mice is associated with increased intestinal permeability (22). The similarities in the intestinal phenotype and gene profile between iFABPp-IL-9Tg mice and WT mice led us to examine this parameter in WT and iFABPp-IL-9Tg mice (Fig. 5). Resistance, a measure of tissue permeability, was significantly decreased in iFABPp-IL-9Tg mice compared to WT mice, indicating increased intestinal permeability (Fig. 5 a). To confirm altered epithelial cell barrier function in iFABPp-IL-9Tg mice, we examined intestinal permeability by analyzing FITC-dextran and HRP transport in jejunum segments ex vivo. Compared with control mice, iFABP-IL-9Tg jejunum had increased intestinal permeability to FITC-dextran and HRP (Fig. 5 b and c).

Because previous studies employing models of parasitic infestations demonstrated a role for mast cells in intestinal permeability (23, 24), the observed intestinal mastocytosis in iFABPp-IL-9Tg mice led us to assess the role of mast cells in intestinal permeability in these mice. Initially, we treated iFABPp-IL-9Tg and WT mice with the anti-c-kit (ACK2) (25) neutralizing antibody and demonstrated that mast depletion abrogated intestinal permeability in iFABPp-IL-9Tg mice (results not shown). c-kit, however, is also expressed on interstitial cells of Cajal (ICC), which play a central role in the regulation of intestinal epithelial cell barrier function (26, 27). To confirm that the increased intestinal permeability in iFABPp-IL-9Tg mice is mast cell mediated, we treated mice with the mast cell stabilizer cromolyn. Intraperitoneal (i.p.) administration of cromolyn reduced serum mMCP-1 levels, confirming mast cell stabilization (Fig. 5 d). Notably, reduction in mast cell activity correlated with a reduction in HRP and dextran-FITC intestinal permeability in iFABPp-IL-
9Tg mice (Fig 5, e and f). Collectively, these studies indicate that intestinal permeability in iFABPp-IL-9Tg mice is mast cell-mediated.

A major manifestation of food allergy, cardiovascular dysfunction, is primarily due to increased vascular leakage (VL) (28-30). Initially, we assessed whether multiple oral OVA challenges induced VL in our experimental model of intestinal anaphylaxis. As VL causes hemoconcentration, we measured venous hematocrit in mice with oral antigen-induced intestinal anaphylaxis. Previous studies have demonstrated an increase in hematocrit during systemic anaphylaxis in humans, rats, dogs and mice (31-34). We show that oral sensitization and oral antigen challenge induces a significant increase in hematocrit following 4 and 6 challenges (Fig. 5, g). We next examined naïve iFABPp-IL-9Tg and WT mice and found that hematocrit levels in iFABPp-IL-9Tg mice were significantly greater than those observed in WT mice, suggesting increased VL (Fig. 5 h). To confirm VL, we measured Evan's blue extravasation in the tissue of naïve iFABPp-IL-9Tg and WT mice. Three hours following i.v. injection, Evans blue concentration in the small intestine of iFABPp-IL-9Tg mice was significantly greater than in WT mice. Notably, increased VL directly correlated with intestinal mastocytosis and IL-9 transgene expression in the jejunum (Fig. 5 i). These studies demonstrate that intestinal expression of IL-9 was sufficient to promote an intestinal anaphylaxis-like phenotype.

**Intestinal expression of IL-9 is sufficient to predispose to intestinal anaphylaxis**

The intestinal anaphylaxis phenotype in iFABPp-IL-9Tg mice prompted us to determine whether overexpression of IL-9 in the intestine increases susceptibility to oral antigen-induced intestinal anaphylaxis. We performed i.g. OVA-challenge in OVA-sensitized WT and iFABPp-IL-9Tg mice.
Susceptibility to intestinal anaphylaxis in iFABPp-IL-9Tg mice was significantly increased as compared to WT mice (Fig. 6 a). Greater than 80% of OVA-sensitized iFABPp-IL-9Tg mice developed diarrhea by the third OVA challenge compared to <25% of WT mice (Fig. 6 a). The increased acute diarrhea in iFABPp-IL9-Tg mice correlated with increased intestinal mast cell numbers and serum mMCP-1 levels compared to WT mice (Fig. 6. b and c). Notably, we observed no significant difference in OVA-specific IgE (Fig. 6 d) suggesting that the increased susceptibility was not due to enhanced antigen-specific Th2 immunity. Remarkably, OVA-challenge of non-sensitized iFABPp-IL-9Tg mice induced diarrhea in 25% of iFABPp-IL-9Tg mice by challenge 6, and greater than 80% by challenge 9 (Fig. 6 a). In contrast, i.g. OVA-challenges of non-sensitized WT mice did not induce diarrhea. We confirmed that the persistence of diarrhea in unsensitized iFABPpIL-9Tg mice was antigen-specific by administering consecutive i.g. OVA challenges to iFABPp-IL-9Tg mice and WT mice until they developed diarrhea and then administering i.g. bovine serum albumin (BSA) (Fig 6. e). BSA failed to induce acute diarrhea in iFABPp-IL-9Tg mice. Notably, subsequent i.g. OVA challenge re-induced diarrhea (Fig. 6 e). These results and the observation that unsensitized WT mice did not develop diarrhea—demonstrate that diarrhea in unsensitized iFABPp-IL-9Tg mice was not solely the result of an osmotic load in the gut, but rather an antigen-specific acute immunological response.

Intestinal anaphylaxis in non-sensitized iFABPp-IL-9Tg mice is dependent on STAT6 and IL-4Rα-pathways

IgE-mediated anaphylaxis is IL-4Rα-dependent (35). IL-4 and IL-13 signal through IL-4R α-chain via STAT6 to promote CD4+ Th2-differentiation and IgE antibody production and exacerbate the effector phase of anaphylaxis by increasing target cell responsiveness to vasoactive mediators.
To confirm that oral antigen induced intestinal anaphylaxis is mediated by STAT6 and IL-4Rα-dependent pathways, IL-4Rα and STAT6⁻/⁻ mice were i.p. sensitized to OVA and subsequently received repeated i.g. OVA challenges. We demonstrate that oral antigen-induced intestinal anaphylaxis was dependent on STAT6 and IL-4Rα expression (Table S4). Notably, the absence of intestinal anaphylaxis in these mice was linked to diminished intestinal mast cell and serum mMCP-1 levels (Table S4) and OVA-specific IgE (Fig. S1). To determine if OVA-induced intestinal anaphylaxis in non-sensitized iFABPp-IL-9Tg mice was dependent on an IL-4Rα/STAT6/IgE-mediated pathway we crossed the iFABPp-IL-9Tg mice onto IL-4Rα- and STAT6-deficient (BALB/c) backgrounds. OVA-induced intestinal anaphylaxis (diarrhea) in naïve iFABPp-IL-9Tg mice was ablated in the absence of IL-4Rα and STAT6 (Table 1). Thus, intestinal anaphylaxis in iFABPpIL-9Tg mice is largely dependent on IL-4Rα-, STAT6-, and mast cell/IgE-pathways.

Intestinal expression of IL-9 predisposes to oral antigen sensitization

The demonstration that oral antigen challenge induced intestinal anaphylaxis in unsensitized iFABPp-IL-9Tg mice and that this response depends on Th2-signalling led us to hypothesize that oral antigen challenge of iFABPp-IL-9Tg mice promotes sensitization rather than oral tolerance. To test this hypothesis, we orally challenged WT and iFABPp-IL-9Tg mice with OVA and examined intestinal IL-4 and CD4⁺ IL-4⁺ T-cell and OVA-specific IgG₁ levels (Fig 7 a-c). Oral antigen challenge of WT mice induced no significant change in intestinal IL-4 mRNA or protein levels compared to naïve WT mice (Fig 7 a and results not shown). Furthermore, we observed no significant difference in the level of jejunal OVA-specific IgG₁ (Figure 7 c). In contrast, repeated oral antigen challenge of iFABPp-IL-9Tg mice increased the level of intestinal IL-4 mRNA and
protein (Fig. 7a and results not shown). This was associated with increased levels of intestinal OVA-specific IgG1 and total IgE (Fig. 7c and d). To examine whether the IL-4 producing cells included CD4+ T-cells, we used flow cytometry to evaluate anti-CD3/CD28-stimulated lamina propria (LP) cells from the small intestine of OVA-challenged WT and iFABPp-IL-9Tg mice. The percentage of CD4+ IL-4+ T-cells in the LP of iFABPp-IL-9Tg mice was approximately 2-3 fold greater than in WT mice (Fig 7b). Thus, intestinal expression of IL-9 appears to predispose to oral antigen-induced CD4+ Th2-type sensitization.

**Intestinal IL-9-mast cell mediated intestinal permeability (“leaky gut”) predisposes to oral antigen sensitization**

We next evaluated the involvement of mast-cell-mediated intestinal permeability in oral antigen sensitization and predisposition to intestinal anaphylaxis in iFABPp-IL-9Tg mice. To distinguish the involvement of mast cells in the sensitization and effector phases of intestinal anaphylaxis, we blocked mast cell activity during the initial i.g. sensitization stage, and reconstituted mast cell activity to evaluate effector function. To accomplish this, we used the mast cell stabilizer cromolyn that we had previously have shown to block mast cell activity and abrogate intestinal permeability in iFABPp-IL-9Tg mice (Fig. 5d-f). Mice were treated with vehicle or cromolyn for 3 days to block intestinal permeability and were subsequently challenged i.g. with OVA while continuing cromolyn treatment for another 6 days. The control and cromolyn-treated iFABPp-IL-9Tg mice received 6 additional i.g. OVA challenges without cromolyn over the following two weeks (Fig. 8a). Consistent with our previous observations, i.g. OVA-challenged of vehicle treated iFABPp-IL-9Tg mice induced intestinal anaphylaxis (Fig. 8b). However, OVA-challenge of cromolyn treated iFABPp-IL-9Tg mice did not induce intestinal anaphylaxis (Fig. 8b). Studies performed to
determine whether the attenuation of intestinal anaphylaxis was due to inhibition of oral sensitization demonstrated elevated levels of IgE in OVA-challenged, control-treated, iFABPp-IL-9Tg mice compared to OVA-challenged, cromolyn treated, iFABPp-IL-9Tg mice (Fig. 8 c). To confirm that the lack of diarrhea occurrence in these mice was not due to cromolyn-suppression of mast cell activity during the effector phase, we performed passive sensitization in control- and cromolyn-treated iFABPp-IL-9Tg mice. Following the 9th i.g. OVA challenge, mice were intravenously (i.v.) administered anti-2,4,6-trinitrophenyl (TNP)-IgE and 24 hours later subsequently challenged i.v. with BSA-TNP and body temperature, an indicator of systemic anaphylaxis, was measured over 60 minutes (Fig. 8 d at 20 minutes). i.v. BSA-TNP administration to naïve iFABPp-IL-9Tg mice did not affect body temperature (Fig. 8 d). In contrast, i.v. administration of BSA-TNP to naïve iFABPp-IL-9Tg mice that had previously been injected i.v. with IgE anti-TNP induced a significant decrease in body temperature. Importantly, BSA-TNP administration to vehicle- or cromolyn-treated, OVA-challenged, iFABPp-IL-9 mice that received IgE anti-TNP also induced a rapid body temperature decrease demonstrating functional mast cell effector activity (Fig. 8 d). Thus, lack of diarrhea occurrence in cromolyn-treated iFABPp-IL-9Tg mice was not due to cromolyn-suppression of mast cell activity during the effector phase. Furthermore, these studies indicate that mast cell mediated intestinal permeability promotes oral antigen sensitization and subsequent predisposition to intestinal anaphylaxis.
Our studies demonstrate a non-redundant role for IL-9 in the induction of the effector phase of intestinal anaphylaxis. The observed comparable levels of oral antigen-specific IgE and IL-4, IL-5 and IL-13 in IL-9−/− and WT mice suggest that the reduction in disease is not due to an attenuated Th2 response. Consistent with this observation, previous studies have demonstrated no role for IL-9 in the development and differentiation of CD4+ Th2-T-cells or antigen-driven antibody responses (36). Attenuation of oral antigen-induced intestinal anaphylaxis in IL-9−/− mice was linked to a reduction in intestinal mast cell number and degranulation, which is required for development of the effector phase of intestinal anaphylaxis (22). Our studies in IL-9 deficient and IL-9 transgenic mice demonstrate that IL-9 is critical for the induction of intestinal mastocytosis.

The molecular basis of IL-9-mediated intestinal mastocytosis is not fully elucidated. Previous studies show that IL-9 is insufficient to induce mast cell growth and differentiation of mast cell progenitors, but can enhance stem cell factor (SCF)-dependent mast cell growth (37). Consistent with this, we observed no difference in intestinal mast cell progenitor levels among IL-9 deficient, iFABPp-IL-9Tg and WT mice, suggesting that IL-9 promotes recruitment of mature mast cells from other tissues or enhances mature mast cell survival. However, we did not observe an alteration in mature mast cell levels in tissues other than the intestine (results not shown) and IL-9 has previously been shown to be insufficient to support survival of mature mast cells (37). An alternate explanation is that IL-9 may promote intestinal mastocytosis via enhancing intestinal mast cell maturation and that immune mechanisms independent of IL-9 critically maintain intestinal mast cell progenitor number.
We also demonstrate that IL-9 transgene-induced intestinal mastocytosis is reduced but can occur in the absence of IL-4Rα and STAT6 (Table S4). Moreover, intestinal mast cell levels in iFABPp-IL-9Tg mice backcrossed onto the IL-4Rα or STAT6 backgrounds were significantly higher than those observed in IL-4Rα or STAT6 deficient mice. Consistent with this observation, previous studies have demonstrated intestinal mastocytosis in the combined absence of IL-4- and IL-13, and that this was dependent on IL-9 (38). Importantly, while mast cell levels in iFABPp-IL-9Tg/IL-4Rα-deficient or iFABPp-IL-9Tg/STAT6-deficient mice were elevated as compared to the IL-4Rα or STAT6 deficient mice, the levels were reduced by ~50% as compared to iFABPp-IL-9Tg mice that express IL-4Rα and STAT6 normally. These studies suggest that optimal intestinal mastocytosis requires factors dependent on IL-4Rα and STAT6 signaling in our model. Antibody neutralization of IL-3 and IL-4 has been shown to block Nippostrongylus brasiliensis-induced intestinal mastocytosis by 85-90% (39). We speculate that IL-9 is a potent inducer of intestinal mastocytosis, however IL-9 may act additively or synergistically with other factors, including IL-3 and IL-4 to induce an optimal intestinal mast cell response. Furthermore, the importance of a particular mast cell stimulatory factor or signaling pathway may vary in different models. Consistent with this possibility, STAT6 signaling has been shown to enhance mastocytosis in some systems but suppress it in others (40, 41).

Our demonstration that IL-9-induced mastocytosis is associated with elevated mMCP-1 and mMCP-2 (enzymes associated with mucosal mast cells) suggests that IL-9 selectively stimulates generation of intestinal mucosal mast cells. Consistent with this, we observed a significant increase in inter-epithelial and inter-cryptic mast cells in the iFABP-IL-9Tg mice. Furthermore, in vitro studies demonstrate that IL-9 stimulates mouse bone marrow derived mast cells (BMMC) to
express high steady-state levels of mMCP-1 and mMCP-2 transcripts (42, 43). Observations that mast cell lines, human CD34+ cord blood cells and 12-week-cultured mast cells all express IL-9Rα mRNA suggest this may be direct effect of IL-9 on mast cells (37, 44).

Recent studies that employed IL-9 transgenic mice in which IL-9 is constitutively expressed in all tissues (cIL-9Tg mice) (45), IL-9R deficient mice and a passive model of systemic anaphylaxis have demonstrated that IL-9/IL-9R signaling can potentiate, but is not essential for systemic anaphylaxis (46). In contrast, we demonstrate that oral antigen-induced intestinal anaphylaxis is critically dependent on IL-9. A possible explanation for these contrasting findings is the distinct molecular pathways central to the induction of systemic and intestinal anaphylaxis. In the present study, we show that the effector phase of intestinal anaphylaxis in iFABPp-IL-9Tg mice is dependent on IL-4Rα and STAT-6-signaling and mast cells. Consistent with this observation, we have previously demonstrated that the effector phase of intestinal anaphylaxis is mediated via mast cell-, FcεRI- and IgE-dependent pathways (22). In contrast, murine systemic anaphylaxis can occur via a mast cell, FcεRI, and IgE-independent pathway, via IgG antibody, macrophages, FcγRIII and PAF (47-49). Therefore, signaling pathways independent of IL-9 can induce systemic anaphylaxis whereas IL-9 dependent signaling mediates intestinal anaphylaxis.

Induction of intestinal allergic responses, including OVA-induced intestinal anaphylaxis in WT mice, is dependent on antigen sensitization with adjuvant and subsequent multiple antigen challenges (22, 50, 51). Remarkably, intestinal overexpression of IL-9 was sufficient to predispose mice to intestinal anaphylaxis in the absence of systemic sensitization. Notably, we show that the development of intestinal anaphylaxis in the absence of systemic sensitization was associated
with development of an antigen-specific CD4\(^+\) Th\(_2\)-cell response and dependent on CD4\(^+\) Th\(_2\)-signalling pathways (IL-4R\(\alpha\)- and STAT6). These studies suggest that intestinal overexpression of IL-9 promotes the generation of antigen-specific CD4\(^+\) Th\(_2\)-cell responses. Consistent with this observation, recent investigations employing a model of *Leishmania major* infection demonstrated a role for IL-9 in the promotion of a detrimental Th\(_2\) type intestinal response (52). While in this study the mode of IL-9 action was not defined, we demonstrate that IL-9 promotion of an oral antigen-specific CD4\(^+\) Th\(_2\)-cell response is primarily via induction of mast cell-mediated intestinal permeability. Notably, mast cell-mediated intestinal permeability has been shown to be primarily regulated via IL-4 and IL-13 (24). The increased intestinal permeability in iFABPp-IL-9Tg mice occurred in the absence of elevated IL-4 and IL-13. Defining the molecular mechanisms involved in IL-9/mast cell-mediated increase in intestinal permeability will be important for understanding of oral sensitization and subsequent development of intestinal anaphylaxis.

The demonstration that increased intestinal permeability in iFABPp-IL-9Tg mice predisposes to oral antigen sensitization suggests that a constitutive defect in barrier function could predispose to oral sensitization and subsequent development of food allergy. Impairment of intestinal barrier function has been implicated as a critical determinant in the predisposition to a number of GI diseases including IBD and food allergy (53, 54). Indeed, patients with atopic diseases, including food allergy have increased intestinal permeability (15, 55, 56). Furthermore, increased intestinal permeability in IBD is predictive of clinical relapse and 10-25\% of first-degree relatives of patients with IBD have increased intestinal permeability (54). Recently, a mutation in the caspase recruitment domain family member 15/nucleotide binding oligomerisation domain 2 (CARD15/NOD2) 3020insC has been linked to increased intestinal permeability in quiescent
Crohn’s disease (CD) patients and their first degree relatives (53). Genetic mapping studies in both humans and mice demonstrated a linkage between the atopic phenotype and the IL-4 and IL-9 gene (57, 58). Furthermore, gene-gene interactions between IL-4Rα and IL-9 SNPs, in particular IL4RA Q576R and IL-9R rs731476 have been observed in asthma-related diseases (59).

An alternate explanation to a congenital abnormality in intestinal permeability predisposing to oral sensitization and food allergy is that perturbations of environmental factors, such as stress, may induce intestinal barrier dysfunction and leaky gut. Notably, recent clinical studies have demonstrated an increase in food protein sensitivity in pediatric liver transplant patients treated with the immunosuppressive tacrolimus (60, 61). Tacrolimus, a calcineurin inhibitor, increases intestinal permeability by uncoupling mitochondrial oxidative phosphorylation, leading to a disruption in intercellular junctional integrins (62, 63). Two recent studies examining heart and liver transplant patients taking Tacrolimus revealed they have an increased risk of developing food allergy (64, 65). Experimental investigations have also provided corroborative evidence supporting a role for intestinal barrier dysfunction and leaky gut, predisposing to oral sensitization and subsequent development of food allergy. Malnutrition-increased β-lactoglobulin-specific IgE and intestinal anaphylactic responses in guinea pigs, demonstrating an effect of environmental factors in intestinal permeability and oral antigen sensitization (66, 67). Furthermore, intestinal infection that leads to increased intestinal barrier dysfunction may also predispose to oral antigen sensitization. Notably, *Helicobacter pylori* gastric infection has been shown to positively correlate with food allergy (68) and mast cells and the mast cell-derived mediator, chymase, are elevated in human *H. pylori*-associated gastritis (69).
Clinical and experimental studies have previously demonstrated a role for mast cells in the end stage effector mechanisms of food allergy (22, 51). We provide corroborative data supporting a role for mast cells in exacerbation of the intestinal anaphylactic phenotype. Importantly, we have identified a role for mast cells in oral antigen sensitization. Interestingly, patients with systemic mastocytosis often present with intestinal manifestations and some of these patients have impaired small intestinal absorption (70). Clinical studies have demonstrated that cromolyn is a successful treatment modality for intestinal symptoms of systemic mastocytosis (71). A major draw back of cromolyn is its poor absorption properties, thus it is not unreasonable to speculate that the ability of cromolyn to successfully treat the intestinal symptoms in systemic mastocytosis may be at least in part to increased intestinal permeability (72).

In conclusion, we demonstrate a central role for IL-9 in the regulation of oral antigen-induced intestinal anaphylaxis and identify a previously unappreciated role for mast cell-induced intestinal permeability in oral antigen sensitization and predisposition to intestinal anaphylaxis. These studies demonstrate the importance of intestinal barrier function in oral antigen sensitization and identify a role for IL-9-driven mast cells in this process.
Materials and Methods

Mice—6-8 week old IL-9-deficient mice (N10 BALB/c) as previously described (36) were a gift from Andrew McKenzie (MRC, Laboratory of Molecular Biology, Cambridge, UK). BALB/c mice were obtained from the National Cancer Institute (Bethesda, MD, USA). All mice were maintained in a barrier facility, and animals were handled under IACUC-approved protocols.

Generation of Transgenic Mice—cDNA was a gift from Andrew McKenzie (MRC, Laboratory of Molecular Biology, Cambridge, UK). The IL-9 cDNA was amplified by PCR employing oligonucleotides containing BamHI sites (5’-ggatccatgttggtgacatacatccttg and 3’-ggatcctcatggtcggcttttctgcc) and the 446-bp fragment containing the entire coding region of the murine IL-9 cDNA was ligated into pCR2.1 TOPO TA cloning vector. The IL-9 cDNA was digested with BamHI, and the IL-9 DNA was ligated into the BamHI site of the PBSIF1178-hGHpgkNeo plasmid, which contained a 3.5-kb EcoRI fragment containing nucleotides −1178 to +28 of rat Fabpi promoter linked to nucleotides +3 to +2150 of human growth hormone (hGH) gene (except for its 5’ regulatory sequences). The transgene plasmid was propagated in Escherichia coli DH5α cells, and the transgene fragment was liberated from the vector sequences by EcoRI endonuclease digestion and gel electrophoresis, and then purified using the QIAEX DNA extraction kit (Qiagen Inc., Chatsworth, CA). After extensive dialysis, 5 μg of the linearized fragment was co-electroporated with 5 μg of circular neomycin resistance plasmid (pMC1Neo, Stratagene, La Jolla, CA) into BALB/c embryonic stem (ES) cells, a generous gift of Dr. Birgit Ledermann (University of Zurich). Positive selection was performed with G418 for 10 days and 7 surviving clones were screened for integration of the transgene by PCR. Of three Tg-positive ES colonies, one was injected into 3.5-day-old blastocysts from C57BL/6 mice and implanted into pseudopregnant females. Chimeric mice were bred with wild-type BALB/c females and germline
(white) mice genotyped to identify positive transgenic mice. Heterozygous positive Tg mice were crossed to wild-type BALB/c mice for 2 generations to remove any possible random modifications due to tissue culture. Transgenic mice were identified by Southern blot analyses after restriction fragment digestion with BamHI, using the hGH genomic fragment to ensure specificity for identification of the transgene. Mice transgenic for IL-9 were also identified by PCR using a forward primer (P1) (5'-ggatccatgttggtgacatacttgc-3') specific for the IL-9 cDNA in the transgenic construct and a reverse primer specific for the hGH (P2) (5'-gtgagctgtccacaggacc-3'); the transgenic band was ~484 bp. STAT-6 or IL-4Rα-deficient mice expressing the iFABPPp-IL-9 transgene were generated by mating STAT-6 or IL-4Rα-deficient mice of the BALB/c background (73-75) with iFABPPp-IL-9 mice and subsequently mating iFABPPp-IL-9F1 mice with STAT-6 or IL-4Rα-deficient mice. The resulting F2 mice were screened by PCR analysis for the presence of the IL-9 transgene and for the homozygous deficiency of the STAT-6 or IL-4Rα gene employing the primers previously described. Control mice were matched wild-type mice derived from both original backgrounds.

**Experimental intestinal anaphylaxis**- 6-8 week old mice were sensitized twice, 2 weeks apart, with 50 μg of OVA (grade V, A-5503; Sigma-Aldrich, St. Louis, MO) in the presence of 1 mg of aluminum potassium sulfate adjuvant [alum: AlK(SO₄)₂-12H₂O] (A-7210; Sigma-Aldrich) in sterile saline or sterile saline by i.p. injection. Two weeks later, mice were held in the supine position three times a week (every other day) and orally administered 250 μl of OVA (50mg) in saline. Before each intragastric (i.g.) challenge, mice were deprived of food for 3–4 hours with the aim of limiting antigen degradation in the stomach. Challenges were performed with i.g. feeding needles (01-290-2B; Fisher Scientific Co., Pittsburgh, Pennsylvania, USA). Diarrhea was assessed by visually monitoring mice for up to 1 hour following i.g. challenge. Mice demonstrating profuse liquid
stool were recorded as diarrhea-positive animals. Sometimes multiple observers blinded to the experimental protocol scored the occurrence of diarrhea. To stabilize mast cell activity mice were administered (i.p.) 100mg/kg (200μl) cromolyn sodium (Sigma, St. Louis, MO) every 12 hours (final volume 200μl) for 2.5 days. Mast cell stabilization was determined by serum mMCP-1 levels.

**Ussing chambers.** 1cm segments of mucosa were mounted in U2500 Dual Channel Ussing chambers that exposed 0.30 cm² of tissue to 10 ml of Krebs buffer. Agar-salt bridges and electrodes were used to measure the potential difference. Every 50 s the tissues were short-circuited at 1 V (EC 800 Epithelial Cell Voltage Clamp; Warner Instruments, Hamden CT), and the short-circuit current (Isc) was monitored continuously. In addition, every 50 s the clamp voltage was adjusted to 1 V for 10 s to allow calculation of tissue resistance using Ohm's law. After the preparation had stabilized for 10 minutes and baseline potential difference and resistance had been established, FITC-dextran (2.2 mg/ml, molecular mass 4.4 kDa; Sigma-Aldrich) were added to the mucosal reservoir. Medium (0.25 ml out of 10 ml) was removed from the serosal reservoir and replaced with fresh medium every 20 minutes over a period of 180 minutes for measurement of FITC-dextran. The concentration of HRP was measured by a kinetic enzymatic assay. Briefly, 120 μl of sample were added to 800 μl of phosphate buffer containing 0.003% H₂O₂ and 80 μg/ml o-dianisidine (Sigma Aldrich, St. louis, Mi), and the enzymatic activity was determined from the rate of increase in optical density at 460 nm during a 1.5-min period. The luminal-to-serosal flux was calculated using a standard formula and expressed as ng/ml. FITC-dextran concentration was determined from analysis of standard curve of dextran-FITC using a FLx800 96-well microplate fluorescence reader (excitation, 490 nm; emission, 530 nm).
**Solutions and drugs.** Krebs buffer contained 4.70 mM KCl, 2.52 mM CaCl$_2$, 118.5 mM NaCl, 1.18 mM NaH$_2$PO$_4$, 1.64 mM MgSO$_4$, and 24.88 mM NaHCO$_3$ on each side. The tissues were allowed to equilibrate for 15 min in Krebs buffer containing 5.5 mM glucose. All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

**Ribonuclease protection assay.** Jejunum RNA was obtained using Trizol reagent (Life Technologies Inc., Grand Island, NY) following the manufacturer’s protocol. The ribonuclease protection assay (RPA) was performed by making a radioactive probe from the mCk-1b multiprobe template (Riboquant multi-probe RPA System; BD Biosciences PharMingen, San Diego, CA). RNA from OVA- and saline-challenged Balb/c WT mice was then hybridized overnight with the radioactive probe, purified, and finally run on an urea-acrylamide gel at 75 W as described in the Riboquant protocol from BD Biosciences PharMingen.

**Northern blot analysis.** RNA was extracted from the lung tissue using Trizol reagent following the manufacturer’s protocol. 20$\mu$g of total RNA was used for Northern blot analysis, as previously described (76).

**Mononuclear cell preparation and MCp assessment**

Mice were killed by CO$_2$ asphyxiation and the small intestine, lungs, spleen, and BM were harvested. The entire organs were removed except for BM, where a single femur was taken from each mouse. Individual tissues from 2 mice were pooled, placed in 20 mL RPMI 1640 complete (RPMI 1640 containing 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, 10 $\mu$g/mL gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% heat-inactivated fetal calf serum), and processed essentially as previously described (77). Briefly, the intestines (flushed out and rinsed...
twice in HBSS) were finely chopped with a scalpel blade and transferred separately to 50 mL plastic tubes with 30 mL RPMI 1640 complete plus 1 mg/mL collagenase Type 4 (Worthington, Lakewood, NJ). There were 3 enzymatic digestions carried out for approximately 20 minutes each at 37°C. The undigested tissue clumps were collected after each digestion period and were subjected to another enzymatic digestion, while the liberated cells were pelleted, resuspended in 44% Percoll (Sigma-Aldrich, St Louis, MO), overlayed on a 67% Percoll layer, and spun at 400g for 20 minutes at 4°C. The cell collection procedure for BM and spleen omitted the digestion steps. BM was extruded from one femur of each animal using a 25-gauge syringe and 5 to 10 mL complete RPMI 1640. Spleen cells were obtained from crushed whole spleens suspended in complete RPMI 1640. The collected cells were pelleted and resuspended in 44% Percoll before centrifugation over 67% Percoll as described. The mononuclear cells (MNCs) were harvested from the interfaces of the 3 digestions of the lung and intestine, pooled by separate tissue source, and washed in complete RPMI 1640. The numbers of viable cells were determined by trypan blue dye exclusion with a hemocytometer. Cells were serially diluted in complete RPMI 1640, and 100 µL samples of the MNC dilutions were added to each well of standard 96-well flat-bottomed microtiter plates (Corning, Corning, NY). Typically, 24 wells were plated for each cell concentration. Intestinal or BM MNCs were plated starting at 5000 to 10 000 cells/well, and lung or spleen MNCs starting at 20,000 to 40,000 cells/well. Then, each well received 100 µL gamma-irradiated (30 Gy) splenic feeder cells plus cytokines (recombinant mouse interleukin-3 [IL-3] at 20 ng/mL and recombinant mouse stem cell factor [SCF] at 100 ng/mL). The cultures were incubated in humidified 37°C incubators with 5% CO₂ for 12 to 14 days, and positive wells containing mast cell colonies were identified and counted with an inverted microscope. The MC colonies were easily distinguished as large colonies of nonadherent small- to medium-sized cells. The MCp
concentration is expressed as the number of MCps per 10^6 MNCs isolated from the tissue. The number of MCps/tissue is derived by multiplying the concentration of MCps by the MNC yield/organ.

**ELISA measurements.** mMCP-1 serum levels were measured by ELISA according to the manufacturer's instructions (respectively, Moredun Scientific, Midlothian, United Kingdom, and BD Biosciences PharMingen, San Jose, CA). ELISA determined serum and jejunal OVA-specific IgE. Briefly, plates were coated for 2 hours with 100 µl of anti-IgE Ab (EM-95; 10 µg/ml, BD Pharmingen), blocked with 200 µl of 10% Fetal Bovine Serum (FBS diluted in sterile PBS) before adding serial dilutions of plasma samples (100 µl/well). After overnight incubation, plates were washed, and biotinylated OVA was added (2.5 µg/ml, 100µl/well). After one hour of incubation, streptavidin-HRP (1µg/ml, Biosource, Camarillo, CA, USA) was added. Prior to the initiation of each step, plates were washed with 0.05% Tween-20 in PBS. Finally, after one-hour incubation, 100 µl of substrate (TMB Substrate Reagent Set, BD OptEIA, San Diego, CA) was added. Colorimetric reaction was stopped with 1M H_2SO_4 and was quantified by measuring optical density with an ELISA plate reader at 450 nm. Jejunal OVA-specific IgG_1 levels were determined by ELIZA. In brief, wells were coated with OVA (100 µg/ml) and blocked with 10% FBS in PBS. The wells were washed with 0.05% Tween-20 in PBS and 100µl of plasma samples (diluted 1/100) were added and incubated for 2 hours at room temperature. Plates were washed and HRP-conjugated anti-mouse IgG_1 (X56; 0.5µg/ml, BD Biosciences-PharMingen) or biotin-conjugated rat anti-mouse IgG_1 (A85-1; BD Biosciences PharMingen) followed by streptavidin, HRP conjugate (1µg/ml, Biosource, Camarillo, CA). 100 µl of substrate (TMB Substrate Reagent Set; BD OptEIA, San Diego, CA) was added. Colourimetric reaction was stopped with 1M H_2SO_4 and was quantified by measuring optical density with an ELISA plate reader at 450 nm. IL-4, IL-5, IL-9, IL-
and IFN-γ in jejunum was measured by ELISA according to the manufacturer's instructions (BD Biosciences PharMingen, San Jose, CA; R&D systems, Minneapolis, MN). For jejunal lysate samples, whole jejenum was excised and snap frozen at -70°C. Frozen jejunum sections were mechanically disrupted and suspended in 1 mL of PBS containing protease inhibitors. The jejunal pellet was vigorously vortexed and the suspension was centrifuged at 12,000g for 10 minutes, supernatant removed, aliquoted and stored at -20°C until analysis. Tissue protein samples were quantitated using a BCA protein assay kit following manufacturer's instructions (Pierce Chemicals, Rockford, IL). In vivo IL-4 and IFNγ levels were determined by IVCCCA as previously described (78). In brief, iFABPp IL-9 Tg and BALB/c WT mice were i.v. injected with biotinylated rat IgG neutralizing monoclonal antibody anti-mouse IL-4 (BVD-1D11, (10 μg/mouse) and biotinylated rat IgG neutralizing monoclonal antibody anti-mouse IFNγ (R4-6A2, (10 μg/mouse) and bled 24 hours later. Serum levels of IL-4 and IFNγ were determined by ELISA as previously described (78).

Intestinal mast cell quantification. Jejunum tissue was collected 7-10 cm distal to the stomach, while ileum and colon samples were collected 1 cm proximal or distal of the cecum. All samples were fixed in 10% formalin and processed by standard histological techniques. The 5-μm tissue sections were also stained for mucosal mast cells with chloroacetate esterase (CAE) activity as described elsewhere (22) and lightly counterstained with hematoxylin. At least four random sections per mouse were analyzed. Quantification of stained cells was performed by counting the number of chloroacetate positive cells from 25-50 fields of view (magnification 40x).

Microarray hybridization. Following TRIzol purification, RNA was repurified with phenol-chloroform extraction and ethanol precipitation. Purified RNA from four WT and four iFABPp-IL-9Tg mice were then pooled together and processed at Cincinnati Children’s Hospital Medical
Center Affymetrix Gene Chip Core facility, using the murine MOE430_2, a whole genome expression chip encoding 45,101 genes as previously described by the manufacturer (Affymetrix, Santa Clara, CA). Differences between WT and iFABPpIL-9 mice were also determined using the GeneSpring software (Silicon Genetics, Redwood City, CA). Data were normalized to WT mice, and genes were screened for a greater than twofold change over SAL. A further description of the methodology, according to MIAME (minimum information about a microarray experiment) guidelines are available at (www.mged.org/Workgroups/MIAME/miame.html).

**Lightcycler PCR.** BALB/c wild-type and iFABPp-IL-9Tg mice were obtained and sacrificed. Intestinal samples were harvested. RNA was isolated from intestinal samples and cDNA was generated by standard procedures. The RNA samples (500 ng) were subjected to reverse transcription analysis using Iscript reverse transcriptase (Bio-Rad Laboratories) according to manufacturer's instructions. GAPDH, mMCP -1, -2, -4, -5, and FCεR1α were quantified by real-time PCR using the LightCycler instrument and LightCycler FastStart DNA Master SYBR Green I as a ready-to-use reaction mix (Roche Diagnostics Corp. Indianapolis, IN). Results were then normalized to GAPDH amplified from the same cDNA mix and expressed as fold induction compared with the controls. cDNAs were amplified using the following primers; GAPDH forward 5'-tggaaatcccatcaccatc-t-3', reverse 5'-gtcttctgggtgcgtgcatgat-3'; mIL-9 forward 5'-agctgtctgtgtctctccgtc-3', reverse 5'-tcacccgatggaacacgaa-3', mMCP-1, -2, -4 forward 5'-gctggagctgaggagattattg-3', mMCP-1 reverse 5'-gattaaaaacagcatactgg-3', mMCP-2 reverse 5'-cctcctcctcagcctcgtta-3', mMCP-4 reverse 5'-gaggcctgtttaaatctatggc-3', mMCP-5 reverse 5'-gaactacctgctgcgtcagc-3', mMCP-5 forward 5'-ttgcaactgctgaaggtg-3' and FcεR1α forward 5'-ttgcaactgctgaaggtg-3' and FcεR1α reverse 3'-acatgagttggctttgacagt-5'. mIL-4 primers were used as described by the manufacturer (PPM3013A; Superarray Frederick, MD).
Quantitative expression data from each gene of interest was normalized to GAPDH expression and then expression in IL-9 TG mice was compared to expression in WT mice.

**Fluorescent-activated cell sorter (FACS) analysis.** Single cell suspensions from indicated organs were washed with FACS buffer (PBS/1% FCS) and incubated with combinations of the following antibodies: All antibodies were from BD Biosciences PharMingen unless indicated: PerCP anti-mouse CD4 (I3T4) (RM4-5); PE anti-mouse CD8a (53-6.7); APC anti-mouse CD62L (MEL-14); FITC anti-mouse CD44 (IM7), APC anti-mouse CD25 (PC61), PE anti-mouse CD45RB (16A) and FITC anti-mouse FoxP3 (FJK-16S). PE anti-mouse B220 (RA3-6B2), FITC anti-mouse CD23 (B3B4) and PE-Cy7 anti-mouse IgM (R6-60.2). APC anti-mouse CD11c (HL3), APC-Cy7 anti-mouse Gr-1 (RB6-8C5) and PE-Cy7 anti-mouse CD11b (M1/70); PE anti-mouse CD4 (L3T4) and PE-Cy7 anti-mouse IL-4 (BVD6; ebiscience). The following antibodies were used as appropriate isotype controls: PerCP rat IgG2a (R35-95), PE rat IgG2a, (53-6.7), APC rat IgG2a (R35-95) and FITC rat IgG2a (R35-95). PerCP rat IgG2a, (R35-95), APC rat IgG1, (R3-34), PE rat IgM (R4-22), and FITC rat IgG2a, (R35-95), PE rat IgG2a (R35-95), FITC rat IgG2a (R35-95), PE-Cy7 rat IgG2a (R35-95) respectively. 7-AAD was used to identify non-viable cells (BD Biosciences PharMingen). Cells were analyzed on FACScalibur (BD Immunocytometry Systems, San Jose, CA) and analysis performed using FlowJo software.

**Vascular permeability.** Peripheral blood samples were collected in EDTA microtainer tubes (Becton Dickinson, Franklin Lakes, NJ) by retro-orbital bleeding. Automated total cell counts and differential counts were performed according to manufacturer's instructions (Fisher Diagnostics, Middletown, VA). Evan's blue tissue extravasation was performed as previously described (79).
Briefly, mice received i.v. Evan's blue dye in PBS; 20 mg/kg and 3.5 hours later, mice were anesthetised with pentobarbital (20 mg/kg, i.p.) and heart perfusion was performed (10 ml PBS arterial perfusion). Jejunum and colon were harvested and Evan's blue extravasation was measured in OD at 650 nm. Tissue protein levels were quantified using a BCA protein assay kit, following manufacturer's instructions (Pierce, Rockford, IL).

**Mononuclear cell isolation from jejunal tissue.** Approximately 5 cm of jejunum was excised and flushed with 1ml of calcium and magnesium free Hank's buffered salt solution (CMF-HBSS). The jejunum was dissected longitudinally and placed in 5 ml CMF-HBSS and shaken vigorously for 30 seconds at room temperature to remove luminal debris. Tissue was then incubated in CMF-HBSS containing 10% fetal calf serum (FBS), 25 mM HEPES and 5 mM EDTA for 10 minutes at 37°C and shaken in 5 minute intervals to remove epithelia and intraepithelial lymphocytes. The tissue was then washed and incubated in CMF-HBSS to block any remaining EDTA activity. The remaining tissue was cut into small pieces and incubated with incomplete RPMI-1640 supplemented with Collagenase A (2.4 mg/ml) for 30 minutes at 37°C. The cell suspension was filtered using sterile gauze, washed in incomplete RPMI-1640, centrifuged and the remaining pellet was resuspended in RPMI-1640 + 10% FCS. Mononuclear cell suspension was used in in vitro stimulation assays.

**In vitro stimulation of jejunum mononuclear cells.** Mononuclear cells were plated at $5 \times 10^5$ cells/ml for 6 hours in the presence of IL-2 (10 ng/ml, BD Pharmingen) and $\alpha$CD3/$\alpha$CD28 (5 $\mu$g/ml and 1$\mu$g/ml respectively, BD Pharmingen) and monensin (ebioscience, San Diego, CA) to block
Intracellular protein transport. Mononuclear cells were then examined for CD4 and IL-4 expression by flow cytometry as described.

**Passive anaphylaxis model.** Mice were primed i.v. with 10 μg of IgEαTNP then challenged i.v. 24 hours later with 100 ng TNP-BSA. The severity of the anaphylactic shock was assessed by change in rectal temperature employing rectal probe (Physitemp Model BAT-12) as previously described (32, 80).

**Statistical Analysis.** Data are expressed as mean ± Standard error (SEM), unless otherwise stated. Statistical significance comparing different sets of mice was determined by Student's t-test. In experiments comparing multiple experimental groups, statistical differences between groups were analyzed using the one-way ANOVA nonparametric and a Bonferroni post-test. P<0.05 was considered significant. All analyses were performed using Prism 4.0 software.
Online Supplemental Material

Supplementary Table S1. Splenocyte cytokine production from OVA-sensitized OVA-challenged IL9−/− and BALB/c WT mice. Anti-CD3/CD28 stimulated splenocyte IL-4, IL-5, IL-13 and IFNγ production from OVA-sensitized and challenged WT and IL-9−/− mice.

Supplementary Table S2. Characterization of mesenteric lymph node immune profile in WT and iFABPp-IL-9Tg mice. Characterization of T- (CD4, CD8, CD44, CD62, FoxP3, CD45RB), B- (B220), dendritic (CD11c, GR-1 and CD11b) cell levels in mesenteric lymph nodes of WT and iFABPp-IL-9Tg mice by flow cytometry analysis.

Supplementary Table S3. Gene Profile analysis of the small intestine of WT and iFABPp-IL-9Tg mice. Whole genome wide scan using the murine MOE430_2, a whole genome expression chip encoding 45,101 genes. Differences between WT and iFABPp-IL-9 mice were also determined using the GeneSpring software (Silicon Genetics, Redwood City, CA). Data were normalized to WT mice, and genes were screened for a greater than twofold change over WT.

Supplementary Table S4. Experimental intestinal anaphylaxis in OVA-sensitized and nonsensitized Saline- and OVA-challenged IL-4Ra and STAT6 factor deficient mice. Diarrhea, mean number of mast cells per high power field of view and serum mMCP-1 in IL-4Ra or STAT6 following intraperitoneal sensitization with OVA or saline and subsequently nine intragastric OVA or saline challenges.
Supplementary Figure S1. OVA-specific IgE in OVA-sensitized and non-sensitized Saline- and OVA-challenged IL-4Rα and STAT6 factor deficient and iFABP-IL-9Tg/IL-4Rα and STAT6 factor deficient mice. (a) Serum antigen-specific IgE in Sal- and OVA-sensitized and OVA-challenged BALB/c WT, STAT6−/− and IL-4Rα−/− mice. (b) Serum antigen-specific IgE in SAL- and OVA-sensitized and OVA-challenged iFABPp-IL-9Tg, iFABPp-IL-9Tg x STAT6−/− and iFABPp-IL-9Tg x IL-4Rα−/− mice. Data represented as mean ± SEM, 4-7 mice per group from at least n=2 experiments.
Acknowledgements

The authors would like to thank Anne Prins for her histopathology expertise, Dan Friend for guidance on mast cell histochemistry and Andrea Lippelman and Lisa Roberts for their editorial assistance. The authors would also like to thank Amal Assa'ad, Nives Zimmermann, Yoshi Yamada and Ariel Muntz for helpful discussions. This work was supported in part by Fulbright New Zealand MoRST award (E.F.), Australia NHMRC Program Grant (224207) (P.S.F., K.I.M and S.P.H), Cincinnati Children’s Hospital Medical Center Trustee Grant 2005 and the Academy of Allergy and Asthma and Immunology Interest Section Award 2007 (S.P.H).
References


37 Forbes et al.,


**Figure 1**

*Oral antigen-induced intestinal anaphylaxis is attenuated in IL-9 deficient mice.*

(a) Diarrhea occurrence and (b) mean number of mast cells per high power field (hpf) in the intestine of OVA-sensitized and subsequently i.g. SAL- or OVA-challenged BALB/c WT and IL-9\(^{-/-}\) mice. Photomicrograph of chloroacetate esterase-stained jejunal sections from OVA-sensitized and OVA-challenged BALB/c WT (c) and IL-9\(^{-/-}\) (d) mice. (e) serum mouse mast cell protease-1 and (f) serum OVA-specific IgE in OVA-sensitized and subsequently i.g. SAL- or OVA-challenged BALB/c WT and IL-9\(^{-/-}\) mice. (g) Mean number of mast cells per high power field (hpf) and (h) mast cell progenitor numbers in the intestine under basal conditions in BALB/c WT and IL-9\(^{-/-}\) mice. (a) Data represented as percentage of diarrhea occurrence over number of OVA challenges. (b, e and f) Data represented as mean ± SEM; 4-5 mice per group from n=3 experiments. (c and d) Photomicrograph 10x magnification; insert 40x magnification. SAL/OVA indicate saline sensitized i.g. OVA challenged mice and OVA/OVA indicate OVA sensitized i.g. OVA challenged mice. (g) Data represented as mean ± SEM, 4-5 mice per group from n=4 experiments. (h) Data represented as mean ± SEM, 4 mice per group.
Figure 2

*Increased systemic and intestinal IL-9 in iFABPp-IL-9Tg mice.* (a) Quantitative PCR analysis of IL-9 mRNA expression in the jejunum and IL-9 protein levels in the (b) sera and (c) jejunum of WT and iFABPp-IL-9Tg mice. Data represented as mean ± SEM; 4-5 mice per group from n=3 experiments. (a) open circle represents an individual mouse. AU: arbitrary units.
Forbes et al., Figure 2

(a) 

(b) 

(c) 

p<0.01

Average GADPH Quantity (AU 10^5): 3.29 ± 2.44 BALB/c WT 5.48 ± 4.96 iFABPp IL-9 Tg

IL-9 (pg/ml): ND BALB/c WT iFABPp IL-9 Tg

IL-9 (ng/ml/mg protein): BALB/c WT 2.1 ± 0.07 iFABPp IL-9 Tg 2.3 ± 0.07
Figure 3

Intestinal mastocytosis in iFABPp-IL-9Tg mice. (a) Localization of mean number of mast cells in the small intestine and (b) serum mMCP-1 in WT and iFABPp-IL-9Tg mice. (c and d) Photomicrograph of chloroacetate esterase (CAE)-stained jejunum sections of BALB/c (c) WT and (d) iFABPp IL-9 Tg mice. (e) mast cell progenitor levels in the intestine, lung, spleen and bone marrow of iFABPp IL-9 Tg and BALB/c WT mice. (a-e) Data represented as mean ± SEM; 4-5 mice per group from n=4 experiments. (a) Pictorial representation of localization determination. (c and d) Photomicrograph 10x magnification; insert 40x magnification. (e) Data represented as mean ± SEM; 4 mice per group.
Forbes et al., Figure 3
Figure 4

Overexpression of IL-9 in the intestine induces features of an intestinal anaphylaxis genotype.

(a) Quantitative PCR analysis of mast cell gene mRNA expression in the jejunum of iFABPp IL-9 Tg and BALB/c WT, (b) genome wide expression gene profile comparative analysis of iFABPp IL-9 Tg and BALB/c WT mice compared to OVA-sensitized, OVA-challenged BALB/c WT mice (6). (a) Results expressed as gene/GADPH ratio with respect to fold change over BALB/c WT. Gene expression was normalized to GADPH expression in each individual sample. (a) Circles represent individual mice and black line represents mean value in each group. (b). value represents fold increase over respective control.
Forbes et al., Figure 4
Figure 5

Overexpression of IL-9 in the intestine induces features of an intestinal anaphylaxis phenotype including mast cell-dependent increased intestinal permeability and intravascular leakage.

(a) Transepithelial resistance and intestinal permeability measured by FITC-dextran (b) and HRP (c) transport in jejunal segments ex vivo for iFABPp IL-9 Tg and BALB/c WT mice, (d) Serum mouse mast cell protease-1 and mean number of mast cells per high power field (hpf) and intestinal permeability measured by FITC-dextran (e) and HRP (f) transport in jejunal segments ex vivo for iFABPp IL-9 Tg and BALB/c WT mice treated with control or the mast cell stabilizing agent, Cromolyn Sodium. (g) % hematocrit before and after 4x or 6x i.g. SAL- or OVA-challenges of OVA-sensitized BALB/c WT mice. (h) % hematocrit in iFABPp IL-9 Tg mice compared to BALB/c WT and (i) Evan’s blue extravasation in the jejunum and colon of iFABPp IL-9 Tg and BALB/c WT mice.

(b) Data represents genes found to be upregulated from profile analysis. (c - g) Data represented as mean ± SEM, 4-5 mice per group. (h) Data represents Evan’s Blue concentration in jejunum and colon normalized per mg of tissue protein. Black line represents mean value in each group.
Forbes et al., Figure 5
Figure 6

Overexpression of IL-9 in the intestine increases susceptibility to oral antigen-induced intestinal anaphylaxis.

(a) Diarrhea occurrence, (b) mean number of mast cells per high power field (hpf), (c) serum mouse mast cell protease-1 and (d) serum antigen-specific IgE in OVA or saline (SAL) sensitized and subsequently OVA-challenged BALB/c WT and iFABPp IL-9 Tg mice. (e) Diarrhea occurrence in OVA-challenged BALB/c WT and iFABPp IL-9 Tg mice and subsequently challenged with BSA. (a) Data represented as percentage of diarrhea occurrence over number of OVA-challenges. (b - d) Data represented as mean ± SEM, 4-5 mice per group from n=3 experiments. (e) Data represented as percentage of diarrhea occurrence over number of OVA-challenges and then subsequent BSA challenge.
Forbes et al., Figure 6
Figure 7

Overexpression of IL-9 in the intestine increases local Th2 responses after OVA i.g. challenge.

(a) IL-4 protein levels in jejunal lysates and (b) percentage of CD4^+ IL-4^+ cells in the lamina propria of the jejunum of BALB/c WT and iFABPp IL-9 Tg mice after i.g. OVA-challenges. (c) antigen-specific IgG1 and (d) total IgE protein levels in jejunal lysates from BALB/c WT and iFABPp IL-9 Tg mice under basal conditions and after 5 i.g. OVA-challenges. (a) Data expressed as protein level in pg/ml per mg protein. Each circle represents an individual mouse and the black line represents the mean value in each group. (a - d) Data represented as mean ± SEM, 4-5 mice per group from at least n=2 experiments.
Forbes et al., Figure 7
Figure 8

Treatment with mast cell stabilizing agent Cromolyn Sodium blocks intestinal permeability and protects against antigen sensitization.

(a) experimental regime, (b) diarrhea occurrence, (c) total serum IgE for iFABPp IL-9 Tg and BALB/c WT mice treated with control or the mast cell stabilizing agent, Cromolyn Sodium and subsequently OVA-challenged, (e) Passive anaphylaxis (Maximum temperature decrease- 20 minutes) in iFABPp IL-9 Tg mice treated with control or the mast cell stabilizing agent, Cromolyn Sodium, subsequently OVA challenged or control and administered TNP-BSA and IgE-anti-TNP. (a – d) Data represented as mean ± SEM, 4-5 mice per group. (a) Data represented as percentage of diarrhea occurrence over number of OVA-challenges in iFABPp IL-9 Tg and BALB/c WT mice treated with control or the mast cell stabilizing agent, Cromolyn Sodium.
(a) i.g. OVA challenge (50mg/250μl)

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</table>

-3-2-1 0 1 2 3 4

i.p. cromolyn Na
300μg/200μl or
PBS control (200μl)
every 12 hours

IgE-α TNP
TNP-BSA

(b) Diarrhea Occurrence (%)

Number of OVA challenges

0 1 2 3 4 5 6 7 8 9

- BALB/c WT i.p. PBS i.g. OVA
- IFABPp IL-9 Tg i.p. PBS i.g. OVA
- IFABPp IL-9 Tg i.p. Cromolyn Na i.g. OVA

(c) BALB/c WT
IFABPp IL-9 Tg
p<0.05

(d) TNP-BSA
IgEαTNP
Ig. OVA challenged
cromolyn Na
p<0.05

Forbes et al., Figure 8
Forbes et al.,

Table 1. Experimental Intestinal anaphylaxis in iFABPp-IL-9Tg mice is IL-4Ra and STAT6 dependent.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Treatment</th>
<th>% Diarrhea Occurrence (on day 9)</th>
<th>Mast cells/HPF (mean + SEM)</th>
<th>Serum mMCP-1 (ng/ml) (mean + SEM)</th>
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<tbody>
<tr>
<td>BALB/c WT</td>
<td>Vehicle</td>
<td>0/12</td>
<td>0.68 ± 0.07</td>
<td>27.46 ± 3.90</td>
</tr>
<tr>
<td>BALB/c WT</td>
<td>OVA</td>
<td>14/14</td>
<td>31.29 ± 0.57</td>
<td>16260.57 ± 4817.16</td>
</tr>
<tr>
<td>IFABPp IL-9 Tg</td>
<td>Vehicle</td>
<td>15/15</td>
<td>26.68 ± 0.82</td>
<td>19319.34 ± 3961.13</td>
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<tr>
<td>IFABPp IL-9 Tg</td>
<td>OVA</td>
<td>12/16</td>
<td>97.52 ± 2.19</td>
<td>83399.48 ± 7880.93</td>
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<tr>
<td>IL-9Tg/STAT6−/−</td>
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<td>2.87 ± 0.31</td>
<td>37.90 ± 1.60</td>
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<tr>
<td>IL-9Tg/STAT6−/−</td>
<td>Vehicle</td>
<td>0/9</td>
<td>2.93 ± 0.21</td>
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<td>IL-9Tg/STAT6−/−</td>
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<td>1/8</td>
<td>11.91 ± 0.66</td>
<td>1168.17 ± 268.46</td>
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<td>IL-9Tg/IL-4Ra−/−</td>
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<td>2.75 ± 0.01</td>
<td>26.20 ± 0.90</td>
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<td>Vehicle</td>
<td>0/10</td>
<td>3.04 ± 0.14</td>
<td>100.26 ± 15.01</td>
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<tr>
<td>IL-9Tg/IL-4Ra−/−</td>
<td>OVA</td>
<td>0/9</td>
<td>8.05 ± 0.19</td>
<td>808.07 ± 154.09</td>
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Diarrhea, mean number of mast cells per high power field of view and serum mMCP-1 for iFABPp IL-9 Tg and BALB/c WT mice deficient in IL-4Ra or STAT-6 following intraperitoneal sensitization with OVA or saline and subsequently nine intragastric OVA or saline challenges.
Supplementary Table S1. Splenocyte cytokine production from OVA-sensitized OVA-challenged IL9^-/- and BALB/c WT mice.

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<th>IL-4 pg/ml</th>
<th>IL-5 pg/ml</th>
<th>IL-13 pg/ml</th>
<th>IFN-γ pg/ml</th>
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<tr>
<td>BALB/c WT</td>
<td>58.75 ± 7.896</td>
<td>94.24 ± 19.42</td>
<td>2193 ± 740.8</td>
<td>3736 ± 1082</td>
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<tr>
<td>IL-9^-/-</td>
<td>91.67 ± 10.01</td>
<td>89.31 ± 19.55</td>
<td>2340 ± 647.8</td>
<td>4205 ± 891.7</td>
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</table>

1.5x10^6 cells/ml were plated in 24 well tissue culture plates and incubated with either media alone, 100μg/ml OVA or αCD3 (5μg/ml)/αCD28 (1μg/ml) and cultured for 72 hours. Supernatant cytokine levels were measured by ELISA as described in material and methods. Cytokine levels were normalised by removing background cytokine levels from cell cultures with media alone.
Supplementary Table S2. Characterisation of mesenteric lymph node immune profile in WT and iFABPp-IL-9Tg mice.

<table>
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<tr>
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<th>BALB/c WT Mean % ± SEM</th>
<th>iFABPp IL-9 Tg Mean % ± SEM</th>
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<td><strong>CD4⁺ T-cells</strong></td>
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<tr>
<td>CD4⁺ CD8⁻</td>
<td>53.8±0.46</td>
<td>54.93±2.23</td>
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<tr>
<td><strong>Naive % CD4⁺</strong></td>
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<tr>
<td>CD44&lt;sub&gt;low&lt;/sub&gt; CD62&lt;sub&gt;high&lt;/sub&gt;</td>
<td>13.77±1.72</td>
<td>17.03±1.46</td>
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<td><strong>Memory:</strong></td>
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<tr>
<td>CD44&lt;sub&gt;high&lt;/sub&gt; CD62&lt;sub&gt;low&lt;/sub&gt;</td>
<td>12.13±2.41</td>
<td>13.8±0.36</td>
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<tr>
<td><strong>T&lt;sub&gt;reg&lt;/sub&gt; % CD4⁺ CD25⁺:</strong></td>
<td></td>
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<tr>
<td>CD45RB&lt;sub&gt;low&lt;/sub&gt; FoxP3⁺</td>
<td>14.95±2.33</td>
<td>15.33±0.80</td>
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<td><strong>CD8⁺ T-cells</strong></td>
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<tr>
<td>CD4⁺ CD8⁺</td>
<td>20.27±0.85</td>
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<td><strong>B220⁺ cells</strong></td>
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<tr>
<td>B220⁺</td>
<td>22.17±0.57</td>
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<tr>
<td>DC</td>
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<td><strong>Lymphoid % of total DC:</strong></td>
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<tr>
<td>7AAD⁻ CD11c⁺ Gr-1⁻ CD11b⁻</td>
<td>71.32±3.13</td>
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<td><strong>Myeloid % of total DC:</strong></td>
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<tr>
<td>7AAD⁻ CD11c⁺ Gr-1⁻ CD11b⁺</td>
<td>13.68±3.55</td>
<td>15.54±3.7</td>
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n = 4-6 mice per group.
## Supplementary Table S3. Intestinal IL-9 induced Gene Profile

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<td>toxicity accelerating factor 1</td>
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<td>actin, alpha, cardiac</td>
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<td>2.631</td>
<td>lacz1l4f1 Mus musculus kidney m1k Mus musculus cDNA clone WAG3:1971577 3 similar to gi:1063680 Mus musculus 3'-untranslated region 1 mRNA (partial cds (MUSCLE) mRNA sequence</td>
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<tr>
<td>1421685_a_at</td>
<td>2.619</td>
<td>kallikrein 3, apolipoprotein related cysteine protease</td>
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Transcribed sequences

aderK>nucleotide A2b receptor

Transcribed sequences

syntaxin binding protein 6 (amisyn)
Transcribed sequences with weak similarity to protein pir:T00380 (H.sapiens) T00380 KIAA0637 protein • human
ribosomal protein S6 kinase, polypeptide 5
Transcribed sequences
doosin, doosin-like protein 1
Transcribed sequences
16 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A3109702J19 product:unknown EST, full insert sequence
U2AF-1 m. region 2
Transcribed sequences
ribosomal protein L38
AV323579, RIKEN full-length enriched, adult male medulla oblongata Mus musculus cDNA clone: E3305351111 product:unknown EST, full insert sequence.
general transcription factor 1B
hypothetical protein LOC252747
RIKEN cDNA 2410166105 gene
Transcribed sequences
ribosomal protein L38
AV323579, RIKEN full-length enriched, adult male medulla oblongata Mus musculus cDNA clone: E3305351111 product:unknown EST, full insert sequence.
AV323579, RIKEN full-length enriched, adult male medulla oblongata Mus musculus cDNA clone: E3305351111 product:unknown EST, full insert sequence.
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AV323579, RIKEN full-length enriched, adult male medulla oblongata Mus musculus cDNA clone: E3305351111 product:unknown EST, full insert sequence.
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AV323579, RIKEN full-length enriched, adult male medulla oblongata Mus musculus cDNA clone: E3305351111 product:unknown EST, full insert sequence.
AV323579, RIKEN full-length enriched, adult male medulla oblongata Mus musculus cDNA clone: E3305351111 product:unknown EST, full insert sequence.
AV323579, RIKEN full-length enriched, adult male medulla oblongata Mus musculus cDNA clone: E3305351111 product:unknown EST, full insert sequence.
AV323579, RIKEN full-length enriched, adult male medulla oblongata Mus musculus cDNA clone: E3305351111 product:unknown EST, full insert sequence.
solute carrier family 34 (sodium phosphate), member 2
complement receptor 2
chemokine (C-X-C motif) ligand 13
membrane-spanning 4-domains, subfamily A, member 1
**Supplementary Table S4.** Experimental Intestinal anaphylaxis in iFABPp-IL-9Tg mice is IL-4Rα and STAT6 dependent.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Treatment</th>
<th>% Diarrhea Occurrence (on day 9)</th>
<th>Mast cells/HPF (mean + SEM)</th>
<th>Serum mMCP-1 (ng/ml) (mean + SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c WT</td>
<td>Vehicle</td>
<td>0/12</td>
<td>0.13 ± 0.01</td>
<td>23.91 ± 3.64</td>
</tr>
<tr>
<td>BALB/c WT</td>
<td>OVA</td>
<td>14/14</td>
<td>31.29 ± 0.57</td>
<td>16260.57 ± 4817.16</td>
</tr>
<tr>
<td>STAT6+/−</td>
<td>Vehicle</td>
<td>0/9</td>
<td>0.10 ± 0.03</td>
<td>10.70 ± 0.24</td>
</tr>
<tr>
<td>STAT6−/−</td>
<td>OVA</td>
<td>0/10</td>
<td>5.92 ± 0.15</td>
<td>88.44 ± 31.28</td>
</tr>
<tr>
<td>IL-4Rα+/−</td>
<td>Vehicle</td>
<td>0/8</td>
<td>0.11 ± 0.03</td>
<td>19.6 ± 2.37</td>
</tr>
<tr>
<td>IL-4Rα−/−</td>
<td>OVA</td>
<td>0/11</td>
<td>5.74 ± 0.24</td>
<td>302.18 ± 44.42</td>
</tr>
</tbody>
</table>

Diarrhea, mean number of mast cells per high power field of view and serum mMCP-1 for iFABPp IL-9 Tg and BALB/c WT mice deficient in IL-4Rα or STAT-6 following i.p. sensitization with OVA or saline and subsequently nine i.g. OVA or saline challenges.
Supplementary Figure S1. OVA-specific IgE in OVA-sensitized and non-sensitized Saline- and OVA-challenged IL-4Rα and STAT6 factor deficient and iFABP-IL-9Tg/IL-4Rα and STAT6 factor deficient mice.

(a) BALB/c WT - □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ ^