Investigating the Roles of Mast Cells and Innate Activators in Oral Tolerance

by

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Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
June 2012

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Dated: June 26, 2012

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DALHOUSIE UNIVERSITY

DATE: June 26, 2012

AUTHOR: Matthew C. Tunis

TITLE: Investigating the Roles of Mast Cells and Innate Activators in Oral Tolerance

DEPARTMENT OR SCHOOL: Department of Microbiology & Immunology

DEGREE: Ph.D. CONVOCATION: October YEAR: 2012

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ABSTRACT

Oral tolerance is the state of immunologic non-responsiveness that is established following oral antigen consumption. Failures of oral tolerance can result in food allergy. The mechanisms regulating oral tolerance are not well understood, but similar mechanisms may control tolerance to foods and commensal microbes in the intestine. The specific roles of many pattern recognition receptors (PRRs) and innate cells have not been examined in the context of oral tolerance. Mast cells are innate sentinel cells positioned at mucosal surfaces, and have been identified as key regulators of peripheral tolerance to allografts. Toll-like receptor 2 (TLR2) is a PRR involved in bacterial responses and the regulation of intestinal inflammation. We evaluated the impact of mast cells, TLR2, immunoglobulin E (IgE)-mediated mast cell activation, TLR2 activation, and histamine receptor blockade in the development of oral tolerance in mice. Models of tolerance to ovalbumin, peanut butter, and cow’s milk were established. Oral tolerance was assessed in wild type, TLR2-deficient, or mast cell-deficient mice and was measured primarily by analysis of antigen-specific antibody levels after a systemic antigen challenge. The development of antigen-specific Tregs was also assessed. We observed that neither mast cells nor TLR2 were necessary for oral tolerance induction. Moreover, IgE-mediated mast cell activation and antihistamine treatment did not significantly alter oral tolerance induction. TLR2 activators, notably Pam3CSK4, were administered orally concurrent with food antigen and were found to impair oral tolerance to a later systemic antigen challenge. When Pam3CSK4 was administered as an oral adjuvant with ovalbumin, a profound selective enhancement of the IgA response to oral challenge was observed. These results highlight an important differential regulation of oral tolerance by TLR2. Oral TLR2 activation selectively promotes IgA responses to antigen upon repeated oral challenge but prevents the maintenance of oral tolerance upon a systemic challenge. Taken together these results suggest that mast cells are not essential regulators of oral tolerance, but TLR2 is involved in regulating IgA and IgE responses during oral and systemic challenges. These findings inform mechanisms of commensal tolerance and have implications for the potential therapeutic manipulation of oral tolerance to foods.
# LIST OF ABBREVIATIONS AND SYMBOLS USED

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C) ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>Cont</td>
<td>Sensitized control</td>
</tr>
<tr>
<td>CPE</td>
<td>Crude peanut extract</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>CTMC</td>
<td>Connective tissue type rodent mast cell</td>
</tr>
<tr>
<td>CSR</td>
<td>Class switch recombination</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment, crystallizable region</td>
</tr>
<tr>
<td>FcεRI</td>
<td>Fragment, crystallizable epsilon receptor I</td>
</tr>
<tr>
<td>FcγRIII</td>
<td>Fragment, crystallizable gamma receptor III</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead-box protein 3</td>
</tr>
<tr>
<td>FSL-1</td>
<td>Pam2CGDPKHPKSF</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>γδT</td>
<td>Gamma-delta T cell</td>
</tr>
<tr>
<td>GF</td>
<td>Germ free</td>
</tr>
<tr>
<td>HRH1</td>
<td>Histamine receptor 1</td>
</tr>
<tr>
<td>HRH2</td>
<td>Histamine receptor 2</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipopolysaccharide binding protein</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic-activated cell sorting</td>
</tr>
<tr>
<td>MadCAM-1</td>
<td>Mucosal addressin cell-adhesion molecule-1</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosal-associated lymphoid tissue</td>
</tr>
<tr>
<td>MCc</td>
<td>Chymase-producing human mast cell</td>
</tr>
<tr>
<td>MCCT</td>
<td>Chymase/tryptase-producing human mast cell</td>
</tr>
<tr>
<td>MCt</td>
<td>Tryptase-producing human mast cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>MMC</td>
<td>Mucosal type rodent mast cell</td>
</tr>
<tr>
<td>MMCP</td>
<td>Murine mast cell protease</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-B</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OIT</td>
<td>Oral immunotherapy</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalubumin from chicken egg</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>Pam3CSK4</td>
<td>Pam3CysSerLys4</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PB</td>
<td>peanut butter</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PPs</td>
<td>Peyer’s patches</td>
</tr>
<tr>
<td>pIgR</td>
<td>Poly-immunoglobulin receptor</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RALDH2</td>
<td>Retinaldehyde dehydrogenase 2</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-like receptor</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SLIT</td>
<td>Sublingual immunotherapy</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Tol</td>
<td>Tolerized</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNP</td>
<td>Trinitrophenyl</td>
</tr>
<tr>
<td>Tr1</td>
<td>T regulatory type 1 cell</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank Dr. Wade Watson and Dr. Ian Haidl for helpful discussions related to the manuscript presented in Chapter 3, Yisong Wei and Nong Xu for technical assistance, and Dr. Manel Jordana for his assistance with anaphylaxis assessment methodology.

I would like also to thank Jean Marshall for her help and support over the years. Jean, you have been the best supervisor I could ask for and I am certain that you will continue to be an inspiration to me long after I have left the lab. Your insight and analysis have been critical to all of this work, and I always walk out of our meetings feeling optimistic about the next experiment or the next stage of my life. I am so grateful that you were willing to invest in me despite the fact that I had never worked in a lab before.

I would also like to acknowledge my committee members; Wade Watson, Jun Wang, and Dave Hoskin. Your focus in helping me through this project has been much appreciated. Thanks also to Dave for bringing me into the world of teaching. Special thanks is due to all of the staff at the CACF. Without their energy and dedicated work none of these studies would have been possible. Also, thanks to Eva for taking the time to help me set up the OT-IIxFoxp3-GFP breeding colony and keeping a watchful eye on them when breeding was suboptimal. Thank you to John Rohde and Julie for helping us culture bacteria, we could not have done it without your help. All of the Marshall lab members have been exceptionally supportive of this work over the years. Thanks to Yisong and Nong for help learning new techniques. Thanks to Eric and Sarah for the good times, Ayhem and Ava for telling enough jokes that we had to start a quote wall. I would like to especially thank Ian for helping me keep the big picture in mind, in both my writing and analysis.

Kaitlyn Carson has been an integral part of this work, and without her much of this thesis would not have been possible. Specifically, Kaitlyn carried out the studies in Figure 4.2 and Table 4.1 under my supervision. Kaitlyn, I wish you all the best in this next stage of your life, and I am certain that you will be very successful.
Words can’t express my gratitude to Wojciech Dawicki. I would not have made it this far were it not for your examples of passion for life and discovery, both inside and outside of the lab. Working with you made me understand that research is as much an art as a science. Thank you for sitting with me through my first tail-vein injections and discussing the “finer” points of immunology, music, and bikes with me over a good porter. Sharon, ever since that first departmental function we knew that we would be buds for life. Thank you for always being there for me (and I don’t just mean feeding me). You have always been more than willing to give me your time and attention, whether I am trying to figure out a new assay or trying to figure out my life.

I would also like to thank my family, who have supported me with love and dedication through every stage of my life and at every juncture. Without their support and encouragement I would not be where I am today. Finally, to Erin: Thank you for supporting me through the final stages of this thesis by giving me the time and space I needed to make it happen. I owe you.
CHAPTER 1 INTRODUCTION
The human immune system has evolved over thousands of years to efficiently identify and eliminate pathogens. An equally important role of this complex system is to tolerate self antigens, harmless environmental antigens, and commensal microbes. Tolerance must be comprehensive and therefore involves epithelial cells, antigen presenting cells, regulatory T cells and B cells. Failures in tolerance can precipitate adverse health outcomes such as autoimmunity, inflammatory disease, and allergy. By characterizing cell types that regulate tolerance and identifying the specific receptor networks involved, we can better understand how these factors interact to influence immunologic tolerance. The analysis of these interactions will hopefully inform treatment strategies for autoimmunity and allergic disease.

1.1 Allergic Disease
Hypersensitivities are immune-mediated responses to innocuous environmental antigens. There are 5 classes of hypersensitivities, but Type I (immediate type) is typically referred to as classical “allergy”. Allergic disease is a spectrum disorder with a myriad of possible manifestations and severities within and between individuals. Atopic individuals are characterized by a Th2-polarized immune profile and many patients experience sequential development of several allergic disorders from atopic dermatitis to allergic rhinitis and asthma (1). This progression has been coined the “allergic march” and may stem from a failure in epithelial barriers, thus compromising tolerance to environmental antigens (1). The relationship between atopic disease and food allergy is not clear, and these may represent failures of tolerance in two distinct but related systems (2, 3). Food allergy is the most dangerous and enigmatic presentation of allergic disease.

1.1.1 Food Allergy and Oral Tolerance
Food allergy has been defined by the National Institute of Allergy and Infectious Diseases (NIAID) as an “adverse health effect arising from a specific immune response
that occurs reproducibly on exposure to a given food” (4). Despite the issues surrounding accurate reporting and measurement of allergic disease, estimates suggest that food allergy rates have risen in recent decades to reach the current prevalence of 3-6% (5). Not all food proteins are represented in the allergic population, with the seven most common categories of food allergy being egg, cow’s milk, peanut, tree nuts, wheat, crustacean shellfish, and soy (4). The characteristics distinguishing these food groups from others as allergenic have not yet been identified, but there is evidence that some allergens have inherent immunogenic properties. The major house dust-mite allergen DerP1 is known to have enzymatic activity that can act as an adjuvant to promote the Th2 response (6). Similarly, the major peanut allergen Arah1 has been shown to produce a Th2-polarized response (7) and peanut extract also activates complement via C3a, which may contribute to the severity of allergic responses (8). In one approach to reduce food allergies some groups have begun engineering “hypoallergenic” versions of some foods (9). An alternative strategy moving forward might be to better understand the immunological failure leading to food allergy, thereby allowing us to develop strategies for preventing food allergy without eliminating important foods from our diets and without relying on engineered foods.

Interestingly, some food allergies, such as cow’s milk, have an early onset in children whereas others such as shellfish develop in adult life (4). This points to an immunologic plasticity surrounding food allergy and reveals ongoing changes in the balance between tolerance and sensitivity. Indeed, it is hypothesized that most cases of food allergy result from a failure or breach in oral tolerance. Oral tolerance is typically defined as “specific suppression of cellular and/or humoral immune responses” to an oral antigen (10, 11), and tolerance is readily induced in mice and humans in response to oral treatment with most food antigens. When oral tolerance is incomplete, allergic patients can experience a wide range of symptoms from mild itching to life-threatening anaphylaxis.
1.1.2 Anaphylaxis

Anaphylaxis is the most severe and potentially fatal response to foods, and has been studied experimentally and clinically for over a century (12). When antigen is introduced systemically by injection or rapid intestinal absorption, the mast cells and basophils associated with mucosal tissues or heart tissue and blood vessels degranulate in response to immunoglobulin E (IgE) crosslinking to release high levels of histamine and platelet activating factor (PAF) (13, 14). Histamine and PAF, among others, then act on macrophages, causing further PAF release leading to changes in the vasculature and airways. Anaphylaxis is characterized by the involvement of multiple organ systems, and the hallmarks of anaphylaxis include urticaria, vomiting, abdominal cramping, diarrhea, vasodilation, hypothermia, and bronchoconstriction; although seizure, headache, chest pain, and arrhythmia may also occur (15). Currently, the standard of treatment for anaphylaxis includes the immediate administration of epinephrine, followed by a combination of HRH1 antihistamines, bronchodilators, supplemental oxygen, and i.v. fluids to control bronchoconstriction and hypotension (4). Despite these rescue measures and new immunotherapy treatment strategies, food allergy is reported to be responsible for over 30% of anaphylaxis in the United States. It has been estimated that there are approximately 29,000 cases of food-induced anaphylaxis annually in the United States causing 150 deaths (16).

In addition to IgE-mediated anaphylaxis, complement activation by antigen complexes via IgG or IgM antibodies can activate the anaphylatoxins C3a and C5a which promote degranulation of mast cells via G protein-coupled receptor signaling (17). It has even been demonstrated that peanut protein alone can directly cause spontaneous production of C3a that may contribute to anaphylaxis responses (8). Mast cell granule-derived tryptase can be detected in high levels during anaphylaxis (18), although the functional contribution of these mast cell proteases to anaphylaxis are not clear. In mice, anaphylaxis is readily induced by injection of allergens but oral anaphylaxis can only be induced in some strains depending on intestinal mast cell levels (19).
1.1.3 Hygiene Hypothesis
The human immune system has coevolved for millennia with pathogens and commensal microbes alike. It is therefore likely that the human immune system has been calibrated through evolution to function optimally in the presence of a variety commensal bacteria and helminths. The “hygiene hypothesis” is a theoretical framework proposing that through the increased sterilization of human environments to protect us from pathogens, we have inadvertently eliminated important microbial signals that keep the immune system in a balance between immunity, excessive inflammation, and regulation. The validity of the hygiene hypothesis in allergic disease, first proposed 2 decades ago (20), has been thoroughly reviewed by Bloomfield et al. (21). There is more recent evidence supporting a relationship between bacteria (22, 23) or helminths (24-26) and low rates of allergy and asthma; however, the link between microbes and food allergy are less apparent. The hygiene hypothesis has now moved out of the realm of correlational epidemiology into experimental science, but the relationship between innate intestinal cell types, innate signaling pathways, and oral tolerance have not yet been well described.

1.2 Mechanisms of Oral Tolerance
Oral tolerance is the desired outcome of food exposure in healthy humans. Although the intestinal environment is a dynamic balance between immunity and tolerance (27), tolerance must be maintained in order for individuals to survive and continue to supply the body with nourishment in the absence of profound inflammatory responses.

1.2.1 Physiology of Oral Tolerance
Although oral tolerance has been studied for over a century, the physiological and immunological sites essential to the induction and maintenance of tolerance remain an active area of investigation. Ingested food antigens travel through the varied elements of the digestive tract where a dynamic network of regulatory cells are induced and traffic between many of these sites.
Ingested antigen is first exposed to the tongue and oral cavity. Although this represents the true first site of exposure, foods have not yet been digested and therefore the access of cells to protein antigens is minimal compared to sites following the stomach. The tongue contains DCs and CD4+ T cells (28). Beyond this, little is known about the relative importance of this site in oral tolerance induction and it is unclear how much antigen is absorbed or presented in the lingual environment as compared to later in the digestive process. However, sublingual immunotherapy trials have shown promising results in recent years (29). Of interest, antigen-specific Tregs have recently been identified in the human tonsils, and this may represent another early location for tolerance induction (30). The esophagus is also populated with lymphocytes, but little is known about their local contribution to oral tolerance. Although they may be involved, the tongue, tonsils, and esophagus are not essential sites for oral tolerance induction, as tolerance can be achieved in mice by gavage antigen delivery directly into the stomach.

1.2.1.1 Peyer’s Patches

The Peyer’s patches (PPs) are located along the small intestine and represent the first point of food antigen contact for many intestinal DCs and T cells. Given this position at the interface with the luminal environment, the PPs are an important site for initiating and dictating the response profile to a new oral antigen.

A diminished capacity for oral tolerance induction has been observed in aged animals that correlates with reduced levels of DCs in the PPs and impaired T cell tolerance upon antigen challenge (31). Despite this, oral tolerance can be induced in the absence of PPs, provided that the mesenteric lymph node (MLN) is intact (32, 33). While the PPs may not be necessary for the induction of oral tolerance, it is clear that DCs in the tolerized PP induce antigen-specific Tregs (34, 35) and many of these express the intestinal homing marker CCR9 (35). Active deletion of antigen-specific CD4+ T cells also occurs in the PPs of tolerized mice (36), which positions the PPs as important initial sites of antigen exposure where the course of oral tolerance could be altered if the regulatory balance is disrupted. The PPs are also an active source of IgA-producing B
cells which are implicated in tolerance to foods (37). Upon leaving the PPs, antigen specific Tregs induced by oral antigen move downstream to the MLN.

1.2.1.2 Mesenteric Lymph Node
The MLN is an important gatekeeper site where the balance of oral tolerance and immunity is critical. The MLN acts as a site of Treg expansion and regulation of responses to foods, and transplant of the MLN Tregs from ovalbumin (OVA)-tolerized mice can confer tolerance to naïve recipient mice, making them resistant to sensitization (38). The MLN is a highly tolerogenic environment that is host to a higher proportion of Tregs than other lymphoid organs. Oral tolerance cannot be induced in mice lacking both the PPs and MLN but can be induced in the absence of PPs (32, 39). The MLN is therefore both a necessary and sufficient mucosal lymphoid tissue for successful oral tolerance induction.

The MLN is a unique node, and structural elements of the MLN are likely responsible for the tolerogenic environment created therein. In node transplant studies, it was observed that the MLN stromal cells dictate the extent of Treg development in response to oral tolerance (40). While transplant of a peripheral lymph node into the MLN site does not preclude tolerance induction, the balance of Tregs is substantially dependent on the actual structure of the MLN. Tregs induced in the MLN disseminate throughout the body to maintain systemic tolerance to foods.

1.2.1.3 Liver
The function of the liver in oral tolerance is unclear, but all food is filtered and processed through this complex organ making it a likely participant in the induction and maintenance of tolerance. Injection of antigen directly into the liver of mice via the portal vein can result in systemic cellular non-responsiveness to the antigen upon later challenge (41, 42). Accordingly, active deletion of antigen-specific T cells occurs in the liver of tolerized mice (36). Interestingly, transplant of liver tissue from a food allergic patient can transfer allergy to the recipient (43, 44). Conversely, oral tolerance measured
by delayed-type hypersensitivity (DTH) response can be transferred to naïve mice by liver transplantation from tolerized mice, but removal of the liver did not break tolerance in the donor mice (45). These studies show that the liver is sufficient but not necessary for the maintenance of oral tolerance and may be more highly involved in situations of high antigen dose reactive T cell deletion than low dose Treg-driven scenarios.

1.2.1.4 Lamina Propria
The intestinal lamina propria (LP) is a lymphocyte-rich tissue compartment situated between the intestinal epithelium and the submucosa. As food is absorbed through the small intestine, antigen is sampled by DCs in the LP with a tolerogenic phenotype expressing CD103 and producing retinoic acid (RA) and indoleamine 2,3-dioxygenase (IDO) (46). These DCs migrate to the MLN where they present food antigen in the context of regulatory cytokines to T cells, creating a robust population of antigen-specific Tregs (47, 48). Recent evidence suggests that food-specific Tregs established in the MLN subsequently return to the LP to establish and maintain intestinal tolerance to foods (49, 50). Accordingly, oral tolerance is deficient in mice with T cells lacking the intestinal homing integrin α4β7 and the intestinal chemokine receptor CCR9 (50). Further to this, IgA-producing B cells derived in the MLN or PPs migrate into the LP via CCR9 in response to CCL25 (51) and interactions between the mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) with α4β7 on B cells (52). The LP is clearly an important site of initial antigen sampling and later maintenance of tolerance to foods via Tregs and IgA-producing B cells.

1.2.1.5 Spleen
The spleen is an important immunological organ involved in surveillance of the blood and systemic trafficking of lymphocytes. Removal of the spleen impedes the suppression of lymphocyte proliferation to oral antigen in rats (53). Furthermore, oral tolerance can be transferred to naïve mice by transplant of splenic cells, but not when CD11c+ cells were depleted from the transfer in an asthma challenge mouse model (54). This points to
splenic DCs as important intermediaries in the maintenance of systemic oral tolerance. The spleen is also host to a significant population of B cells that can be activated to produce antibody and undergo IgE class switch recombination (CSR) upon a systemic antigen challenge.

Oral tolerance induction is a complex immunologic process that implicates multiple secondary lymphoid organs at a number of physiological sites. Because of this, antigen-specific Tregs can be assessed in many of these organs in order to gain a comprehensive interpretation of oral tolerance progression. The PPs, MLN, and spleen are most commonly assessed in mice, and are the most widely accepted as key sites of oral tolerance induction and maintenance. The intestinal LP is also an emerging site for investigations of tolerance.

1.2.2 Tolerogenic Dendritic Cells

Dendritic cells (DCs) are potent antigen presenting cells identified 4 decades ago (55) that are critical in directing the final T cell responses to oral antigen. In the intestinal environment we now understand that DCs can take on a tolerogenic phenotype and produce regulatory cytokines.

The mucosal epithelial $\alpha_E$ integrin (CD103) is a component of the $\alpha_E\beta_7$ integrin heterodimer that binds to E-cadherin on the basolateral surface of epithelial cells. This integrin has been recognized as an important identifying marker for tolerogenic CD11c$^+$ DCs in the intestine and gut-associated lymphoid tissues (GALT). Many of these CD103$^+$ DCs migrate from the LP into the MLN carrying intestinal food antigens (47, 48), and CD103$^+$ DCs have been shown to potently drive T cell differentiation into suppressive Treg cells (47). CD103$^+$ DCs are predisposed to the production of IL-10 and TFG-\(\beta\), both of which can promote Treg differentiation. Other important regulatory cytokines directing Treg differentiation that are actively produced by CD103$^+$ DCs include IDO (56) and RA (47). In addition to their tolerogenic functions, CD103$^+$ DCs isolated from the MLN of mice and humans promote upregulation of the intestinal homing chemokine receptor CCR9 on T cells (48).
Not surprisingly, there is a close relationship between the functionalities of intestinal tolerogenic DCs and the metabolism of available food sources. IDO is an enzyme involved in the metabolism of dietary tryptophan (57), and production of IDO by DCs in the PPs drives Treg induction and tolerance to oral antigen (56). These IDO-producing DCs in tolerized nodes also have a less mature phenotype in terms of MHCII and CD80/86 expression, which may further facilitate Treg differentiation (56). Notably, when IDO is inhibited oral tolerance is deficient (58).

CD103+ DCs in the MLN also express high levels of retinaldehyde dehydrogenase 2 (RALDH2), which converts dietary vitamin A to RA. When RALDH2 activity drops during vitamin A deficiency (59), defects in oral tolerance are observed (50). RALDH enzymes are also expressed by intestinal epithelial cells (59), and the resulting RA likely promotes tolerogenic DC functions. The retinoic acid receptors (RAR) are transcription factors that are believed to control the function of TGF-β by directly regulating the relative expression of Th17 versus Treg genes (60). Vitamin D3 is also known to promote DCs with tolerogenic functions that in turn regulate T cell responses (61, 62). These tolerogenic DCs still retain high levels of TLR2 and TLR4 expression (62). IDO is also produced by plasmacytoid DCs (pDCs) in tumor draining lymph nodes (63), and liver-derived pDCs are known to contribute to oral tolerance (64). These pDCs have also been shown to have an important role deleting food-reactive CD8+ T cells (65).

Tolerogenic DCs are clearly a heterogeneous population with several described phenotypes and cytokine profiles that are dictated by both the inflammatory and dietary environment. The extent to which these critical regulators of mucosal tolerance are controlled and modulated by surrounding cells, such as mast cells, in the intestinal environment and innate signaling via PRRs has not been elucidated.

1.2.3 Regulatory T cells
Regulatory T cells are now well accepted as being important regulators of immune tolerance to self and foreign antigens. Although Treg immunology and differentiation
continues to be a rapidly expanding field, Tregs can be broadly classified into two groups: “natural Tregs” and “inducible Tregs”. In the thymus, “natural Tregs” (nTregs) are established that are specific to self antigen. These nTregs act primarily as a safeguard against autoimmunity, circulating throughout the body to suppress auto-reactive T cells that escaped deletion in the thymus. Thymus derived nTregs express the MHCII co-receptor CD4 and the IL-2 receptor α-chain CD25 on their surface, while inside the cells they express transcription factors Foxp3 and Helios (66). It is not clear what role, if any, nTregs play in tolerance to foreign antigens (67). In the periphery, CD4⁺ T cells with specificity for non-self antigens can be driven towards a suppressive phenotype by a variety of cytokine signals, resulting in “inducible” or “adaptive” Tregs (iTregs). This results in the expression of Foxp3 by most iTregs, while they are primarily negative for Helios (68). In vivo, oral tolerance induction results in antigen-specific CD4⁺, Helios⁻, Foxp3⁺ iTregs in mice (68).

While the precise function of Foxp3 and associated factors are not fully understood in gene regulation, Foxp3 has been shown to suppress genes coding lineage differentiation into Th1, Th2, and Th17 cells as well as the affiliated cytokines (69, 70). It was established early on that antigen dosage will influence the extent to which oral tolerance is mediated through active suppression of T cells versus anergy of reactive T cells (71), and low-dose antigen treatment is understood to be most effective at generating iTregs (72). A low strength of TCR activation by the MHC-antigen complex is believed to be a major determining factor for induction of Tregs (73, 74), and expression of the inhibitory molecule cytotoxic T-lymphocyte antigen 4 (CTLA-4) is another important factor in regulating Treg differentiation (75).

Tregs can suppress effector T cells and APCs through a variety of mechanisms including cytokine actions and direct contact (76). The peripheral iTregs can be further classified based on cytokine profiles: Tr1 cells are potent sources of IL-10, whereas Th3 cells produce primarily TGF-β (77). TGF-β is a complex potent cytokine that presents in 3 different isoforms, β1 being the most common, and complexes with peptides that must be cleaved to convert TGF-β from the latent to the active form before it can signal through the multimeric TGF-β receptor (78). The suppression of antigen-specific T cells
in the PPs can be blunted by administration of anti-TGF-β antibodies (34, 79), and mice with CD4 T cells lacking the TGF-β type II receptor are refractory to oral tolerance (80). TGF-β is in fact so potent in establishing tolerance that the administration of just 3 oral doses of 5μg of TGF-β1 in mice was sufficient to improve oral tolerance induction to OVA (81). TGF-β can also be found bound to the surface of Tregs and thereby mediate suppression via direct cell contact (82). Tregs can also act to suppress responses in the absence of TGF-β (83), and are understood to be capable of suppression via other mechanisms. Human Tregs, for example, can kill surrounding cells by the release of granzyme A and perforin (84). Additional suppressive functions of Tregs, reviewed in (85), include sequestration of IL-2 from effector cells, regulation of adenosine, and targeting DCs to develop a tolerogenic phenotype via CTLA-4, allowing DCs to then act on other antigen-specific T cells. The expression of CTLA-4 on OVA-specific CD4+ T cells is indeed dramatically enhanced following oral tolerance induction to OVA in mice (86).

These multiple mechanisms of Treg immune suppression can all contribute to the induction and maintenance of oral tolerance to foods. Indeed, the transfer of Tregs from the MLNs of OVA-tolerized mice was sufficient to transfer tolerance to recipient mice, protecting them from anaphylaxis upon OVA sensitization (38). Importantly, Treg differentiation is not a terminal event and Tregs in the PPs can differentiate into T follicular helper cells to promote IgA antibody production (87). The suppressive functions of Tregs are diverse, making them resilient regulators of tolerance to microbes and food in the intestinal environment. However, their plasticity also makes them responsive and vulnerable to changing cellular environments.

### 1.2.4 Other Cell Types in Oral Tolerance

Oral tolerance is a complex and essential process that is certainly not restricted to the involvement of DCs and Tregs, although these are the most active participants that have been characterized to date. γδT cells are reduced in the PPs of allergic sensitized mice suggesting that they may contribute to allergen regulation (88), and a failure of oral
tolerance was reported in γδ-deficient mice (89). Similarly, invariant NKT cells have been implicated in allowing oral tolerance to progress in mice (90, 91), and NKT cells may be required for the initial induction of antigen-specific Tregs in oral tolerance (92).

TH17 cells are present in relatively high numbers within the small intestine LP (93) and their differentiation in the LP is dependent on adequate commensal colonization (94). Furthermore, TH17 cells have been shown to promote poly-Immunoglobulin receptor (pIgR) expression in the lung, thereby facilitating the transcytosis of IgA into the lumen (95). The intestinal presence of TH17 cells may therefore have an important undescribed role in the induction and maintenance of oral tolerance to commensals and to foods.

The role of CD8+ T cells in oral tolerance has not been fully elucidated, but transfer of CD8+ T cells from spleens of OVA-primed mice into OVA-sensitized mice prevents allergic diarrhea (96). Indeed, regulatory CD8+ T cells have been identified that can produce TGF-β (97). Although systemic humoral oral tolerance can be successfully induced in CD8−/− mice, the local cellular suppression is deficient (98). CD8+ T cells may therefore represent a meaningful cellular contribution to the suppression of responses to oral antigen, but they are not required for successful oral tolerance induction.

B cells are important cells in maintaining oral tolerance. The extent of B cell activation will determine the levels of food-specific antibodies produced, and the cytokine milieu can initiate class switch recombination (CSR) towards allergy-promoting antibodies (IgE) or towards classes associated with functional tolerance (IgA and IgG4 in humans). Oral tolerance can also be transferred by the transfer of unconventional B-1 cells from tolerized mice into B cell-deficient mice (99). Furthermore, regulatory IL-10 producing “B10” cells have been described in the GALT in response to intestinal inflammation (100), but these cells have not been investigated in the context of oral tolerance to foods.

Since multiple cells contribute to the intestinal responses to pathogens, it is not surprising that the tolerance response to food is equally complex. Only by directly investigating and understanding the roles and relative contributions of different intestinal
cells can we hope to understand the complete picture of oral tolerance and successfully modify tolerance with immunotherapy approaches.

1.2.5 Humoral Tolerance: Antibody Production and Class Switch

The profile of antibody responses to food antigen can regulate the balance between functional tolerance and allergy. IgE is the antibody class most commonly associated with food allergy. Upon allergen challenge, IgE antibodies bound to the FcεRI will crosslink the receptors and initiate degranulation of basophils and mature mast cells, resulting in allergic symptoms in sensitized patients. The primary class-switch recombination (CSR) factor for IgE has been confirmed as IL-4 (101), but IL-13 can also initiate the CSR event (102). This cytokine profile suggests that IgE levels are correlated with a dysregulated Th2 IL-4 type immune response to food antigen. CSR to IgE is also highly T cell dependent, relying on the CD40/CD40L interaction between B cells and T cells to provide a second activation signal (103, 104). Food-specific serum IgE is tested clinically, but levels vary significantly between individuals and no standard threshold IgE level can be established as a reliable predictor of anaphylaxis (4), as the balance between free and FcεRI-bound IgE will dictate the extent of allergic activation. Despite this, IgE is responsible for initiating anaphylaxis to foods and is often monitored and evaluated in patients as an indicator of the potential for allergic disease and the extent of oral tolerance in oral immunotherapy treatment (11, 105).

In the context of food oral tolerance IgA is the most important mucosal antibody. IgA and IgM are the only two antibody classes to be actively exported to the mucosal lumen environment via the pIgR, but IgA is present in higher levels and the average human excretes up to 5g of IgA in feces daily (106). IgA is found in serum in a monomeric form or as a dimer associated by a “J chain”. The pIgR interacts with the J chain in dimeric complexes to move them through epithelial cells and out into the mucosal lumen where these antibody complexes can efficiently bind to target antigens in the intestine, lungs, and other mucosal surfaces. Surprisingly, despite its important role in blocking attachment of pathogens to the intestinal epithelium, IgA deficiency is relatively common (107, 108).
The regulatory cytokine TGF-β is a CSR factor for IgA production in activated B cells (109). Interestingly, activated B cells may default to IgA production via autocrine TGF-β in the absence of external cytokine signals (110). The PPs are the primary location where IgA CSR occurs, and T cells transferred from the PPs of tolerized mice expand the number of antigen-specific IgA-producing cells in the PPs of naïve recipient mice (111). IgA CSR in conventional B cells occurs primarily in response to TGF-β, although RA and nitric oxide can also contribute (37, 112). Interestingly, in the PPs IL-21 suppresses the alternative CSR to IgG2b in response to TGF-β signaling, thereby ensuring IgA production in the mucosal environment (113). T-independent initiation of IgA CSR can also occur in the absence of CD40L stimulation in the presence of the cytokines APRIL, BAFF, and TSLP which can be released by intestinal epithelial cells in response to TLR activation (37). This may represent a significant pathway by which IgA is generated in response to commensal bacteria. Additionally, intestinal DCs carry live commensal bacteria to the MLN, where commensal-specific IgA-producing B cells are expanded and migrate to the intestine to manifest protective commensal tolerance by IgA secretion (114).

Elevated secreted IgA has also been correlated with improved tolerance to peanut challenge in allergic patients (115). A recent animal study by Strait et al. also showed that serum IgA can protect against oral anaphylaxis in sensitized mice (116). Similarly, elevated secreted IgA has been documented in mice treated with oral food antigen compared to naïve animals (111). Elevated antigen-specific IgA is also detected in the serum of mice upon OIT (117). However, in most animal models of tolerance and allergy the levels of serum and secreted food-specific IgA are typically higher in sensitized animals compared to tolerized mice; this is an inevitable response to the potent adjuvants, such as alum or cholera toxin, used in sensitization protocols. Relative levels of IgA between tolerized and sensitized animals reflect food-specific Treg suppression in the face of immunization, but do not necessarily reflect the state of typical mucosal antibody responses to food in a tolerant human. The role of IgA in oral tolerance to commensals and foods is an active area of research and discussion, and evidence currently supports a protective role for IgA in human tolerance.
It has been suggested that immunotherapy works to prevent allergic reactions by skewing the antibody response to food antigen from IgE towards IgG (118-120). CSR to IgG\textsubscript{1} is initiated by IL-4, whereas CSR to IgG\textsubscript{2a} is initiated specifically by IFN-\gamma. Both can be supported by IL-13 (121). Regardless of whether food-specific IgG\textsubscript{1} and IgG\textsubscript{2a} are protective against allergic disease, together these two antibodies can help describe the cytokine milieu and the T\textsubscript{H1} or T\textsubscript{H2} polarized response. Human IgG\textsubscript{4} is emerging as an important antibody in the interpretation of tolerance and sensitization. The cytokine IL-10 can potentiate IL-4-driven CSR to IgG\textsubscript{4}, which suggests that tolerogenic cells producing IL-10 may shift the IL-4 mediated IgE response in allergic individuals towards an IgG\textsubscript{4} antibody profile (122). IgG\textsubscript{4} shows promise as a potential correlate of successful oral immunotherapy treatment, and food-specific IgG\textsubscript{4} levels increase as IgE levels decrease upon oral immunotherapy in humans (105). Unfortunately there is no murine correlate to IgG\textsubscript{4}, meaning that many questions regarding the function and protective capacity of this interesting antibody cannot be easily addressed experimentally \textit{in vivo}.

1.3 Therapeutic Manipulation of Oral Tolerance

1.3.1 Immunotherapy

Allergen-specific immunotherapy is the controlled administration of low doses of antigen in allergic patients. Over the years, several different antigen delivery routes have been employed to this end and animal studies continue to inform our understanding of the basic mechanisms underlying oral tolerance induction and maintenance. These observations can be paired with data from clinical trials to help guide and optimize immunotherapy protocols to better help clinicians minimize disease in allergic patients.

1.3.1.1 Oral Immunotherapy

Oral immunotherapy has proven effective in the clinic to establish tolerance to several foods in allergic patients, most notably cow’s milk (123) and more recently peanut (105). The mechanisms are still being characterized, but oral immunotherapy to peanut can result in elevated peanut-specific Tregs, elevated protective IgG\textsubscript{4} antibodies, and an
elevated oral threshold dose of peanut antigen (approximately 5g) (105). Interestingly, peanut-specific IgE levels were not reduced following one year of OIT, although patients are significantly more resistant to allergic reactions to peanut. This finding suggests that the promotion of allergen-specific Tregs and IgG4 may be relatively more important in the progression of OIT than direct modulation of the IgE response. The permanence of OIT is not currently known, but in the coming decades we can expect to learn more about the long-term immunological changes and optimal clinical maintenance protocols for OIT.

1.3.1.2 Sublingual Immunotherapy
Sublingual immunotherapy (SLIT) has shown promising results in recent years (29, 124, 125). SLIT differs from OIT in the delivery site of antigen; in SLIT a drop of antigen is placed under the tongue for several minutes instead of immediately ingested. The immunologic outcomes of peanut SLIT resemble OIT in terms of enhanced antigen-specific Tregs and IgG4 antibodies in allergic patients and increased threshold doses of antigen that can be safely ingested (105, 124). However, in trials of SLIT and OIT for cow’s milk allergy, OIT proved a more effective therapy in terms of elevating the threshold oral challenge dose of milk (126).

1.3.1.3 Cutaneous immunotherapy
The skin is generally thought to be a site of sensitization to antigens (127), but cutaneous delivery of peanut and egg has recently been shown to prevent oral sensitization to these antigens (128). These divergent results may be explained by the state of disruption to the skin. In models involving the placement and removal of patches, skin can be disrupted or inflamed providing a cue to sensitization mechanisms, whereas painted antigen leaves the skin intact. Subcutaneous allergen immunotherapy by injection is used to treat asthma and hymenoptera venom allergy (129, 130), but it is not commonly employed in the treatment of food allergies.
While the many forms of allergen immunotherapy are promising and have the potential to significantly reduce morbidity and mortality of food sensitivities such as peanut allergy, it remains to be seen if allergen immunotherapy can induce lasting immunologic tolerance to allergens. It is possible that once oral tolerance has been compromised, a continuous regime of immunotherapy must be observed in order to maintain a state of functional tolerance.

1.3.2 Treatment of Autoimmune Disorders
For several decades Dr. Howard Weiner and colleagues have been pioneering the use of oral tolerance as immunotherapy for multiple sclerosis. Extensive work has also been done on other autoimmune disorders in several centers including rheumatoid arthritis, diabetes, myasthenia gravis, and uveitis. Much of the recent progress has been thoroughly reviewed by Faria & Weiner (10, 131). Clinical attempts at oral tolerance induction to collagen in rheumatoid arthritis patients have been most promising (10), exemplified by a recent phase III clinical trial in China which found significant improvements to joint function following oral administration of chicken type II collagen (132).

Early studies in a therapeutic mouse model of oral tolerance to myelin basic protein demonstrated TGF-β production and a suppressive phenotype in the PP of tolerized mice (133). Despite promising outcomes in animal models, clinical trails using bovine myelin basic protein have not been encouraging (131). The apparent failure of oral immunotherapy in some autoimmune contexts may relate to limitations in the analysis of patient subpopulations. In a clinical trial of oral immunotherapy to insulin in diabetic patients, it was found that patients with confirmed insulin autoantibodies were more responsive to oral immunotherapy (134). In future, careful analysis of the patient disease immune profile may prove important in assessing the true efficacy of these trials.

Optimizing the dosage, timing, and antigen source will likely be the big challenges facing therapeutic clinical oral tolerance for autoimmune disorders in the coming years. This underscores the urgent need for basic animal studies investigating
how these factors interact; and investigations into the functions of mucosal cell types and their activation by PRRs could point the way to important immunotherapy adjuvants.

1.3.3 Oral Adjuvants
Oral tolerance is the ideal outcome for responses to foods, but the induction of tolerance to oral antigens may present a significant barrier to oral vaccine development. Oral vaccines are desirable because they can establish specific mucosal immunity to oral pathogens and they require minimal training or equipment to deliver (135). Oral vaccines can include live attenuated microbes, inactivated microbes, or microbial antigen components, but in order for these vaccines to be effective the appropriate adjuvants must be employed to optimize the local secreted IgA response. Unfortunately, because of the barriers posed by vaccine tolerance and digestion of antigenic components we have only a small number of functional oral vaccines (135, 136). Mucosal vaccines can also be applied in the nasal cavity (137-139) and vaginal mucosa (140, 141); results from animal studies in these areas may translate to the development of better oral adjuvants. The relationship between tolerance induction and IgA production is not fully understood, but this axis is paramount to the development of effective mucosal vaccines. Investigations into the regulation of oral tolerance by PRRs and innate intestinal cells will be critical in the search for better mucosal adjuvants.

1.4 Murine Models of Oral Tolerance
1.4.1 Oral Tolerance Induction Models
Oral tolerance must be induced by oral exposure to an antigen. The two primary models used in mice are *ad libitum* oral antigen treatment and treatment by oral gavage. Oral treatment with antigen in a bolus dose by a gavage needle will bypass the upper digestive tract and deliver antigen directly to the stomach. This system is preferable when the exact dose of antigen must be controlled, when dose timing is critical, or when antigen dose must be concurrent with a costly second oral treatment. In systems of oral antigen provision and *ad libitum* treatment the daily dose is less controlled, but antigen exposure
includes all elements of the digestive tract and more closely reflects human exposure to foods.

1.4.2 Assessment Tools
Oral tolerance can be evasive to measure and quantify. Since tolerance is characterized by immunologic non-responsiveness, most facets of oral tolerance can only be observed upon a challenge to the immune system with the antigen in question. Measuring the induction of antigen-specific Tregs is currently the only metric of oral tolerance that can be reliably assessed in the absence of an antigen challenge system in mice. Antigen-specific Tregs and a variety of the challenge-induced readouts can be measured to determine the state of tolerance and help translate findings to the complex process of human allergic disease.

1.4.2.1 Antigen-specific Antibodies
Antigen-specific antibodies are present at low levels in tolerized animals, but upon immunization they can become an informative tool by which to assess tolerance. IgE is measured clinically as an important indicator of the potential for allergic responses (4). Food-specific IgE levels are suppressed in mice that were treated orally with antigen prior to sensitization and measuring the level of food-specific IgE in mice is a reliable tool to predict anaphylaxis. IgG\textsubscript{1} and IgG\textsubscript{2a} CSR are initiated by IL-4 and IFN-\gamma respectively, and therefore analysis of these antibodies inform the T helper cell polarization of a food response. Finally, IgA can be measured in the serum or secreted form. IgA is a natural response to oral antigen, but levels are typically suppressed in tolerized mice upon immunization compared to sensitized animals. Changes in the IgA compartment inform studies of mucosal tolerance.
1.4.2.2 Asthma and Immediate-type Skin Response

Oral tolerance is a systemic event and therefore can prevent airway hyperresponsiveness in mouse models of asthma (54). This readout can be useful to characterize systemic responses to oral antigen and to examine possible therapies for asthma, but it does not directly inform the progression of local intestinal tolerance and may implicate different mechanisms and cell subsets. Immediate-type skin responses can also be assessed as systemic measurements of oral tolerance (81). Allergen-specific IgE binds to mast cells in the skin and upon local challenge causes local inflammation and vascular changes. Allergen skin tests are a useful tool in the clinic, but a positive skin test can often lead to false positive results in the context of food allergy (4). Dermal manifestations of IgE-mediated mast cell activation therefore do not necessarily translate to intestinal allergy or local intestinal tolerance.

1.4.2.3 Oral Challenge

In human patients, an oral allergen challenge is the definitive measure of sensitization and anaphylaxis risk to a known allergen (4). In contrast to humans, anaphylaxis is not readily induced in sensitized mice from all strains upon oral allergen challenge, and therefore oral challenge alone is not a sufficient assessment tool to reflect the equivalent of human disease. Most oral challenge protocols involve initial sensitization of mice by antigen-alum immunization or oral challenge with antigen plus cholera toxin or staphylococcus enterotoxin B, followed by frequent high-dose oral antigen challenges over several weeks (19, 116, 142-144). Upon a final oral challenge, diarrhea can be observed in sensitized animals. Anaphylaxis and hypothermia can also be induced by such protocols in some strains of mice (19, 116). Oral anaphylaxis has not been published in C57BL/6 mice, and recent evidence suggests that oral anaphylaxis occurrence and severity is controlled by intestinal mast cell levels (19). Therefore, mouse strains rich in intestinal mast cells are more likely to respond to an oral challenge. Historically, models of oral sensitization have relied on the C3H/HeJ mouse strain (145), although many examples of BALB/c mice are currently in use (19, 116, 143). These strain variations make the oral allergen challenge readout problematic when attempting to
compare tolerance and anaphylaxis across strains of mice. Furthermore, the reliance on frequent high-dose oral antigen treatments to adequately sensitize mice implicates variables such as altered intestinal permeability and changes in antigen access between strains or treatment regimes. Despite these shortcomings, oral challenge models in mice are valuable new tools to model human allergic disease – particularly in terms of intestinal tolerance and diarrhea.

1.4.2.4 Peritoneal Challenge and Anaphylaxis
Peritoneal or intravenous allergen injection is preferred as a method to induce reactions in mouse strains that are not amenable to anaphylaxis from an oral challenge. Sensitized mice undergo classic IgE-mediated mast cell degranulation upon allergen challenge resulting in histamine release and production of PAF (146). The ensuing drop in blood pressure and core temperature can be measured as indicators of anaphylaxis, and mast cell protease can be measured as a specific indicator of mast cell degranulation. Diarrhea is not a common outcome of injection-induced anaphylaxis in mice.

It has been well documented that basophils and/or macrophages can bind IgG antibodies and cause alternative IgG-mediated anaphylaxis in mice (146-148). This is not known to be a significant mechanism of anaphylaxis in humans and complicates comparisons of animal models to human allergy and anaphylaxis. Mice deficient in the FcγRIII receptor are protected from IgG-mediated anaphylaxis and present a powerful tool for making isolated physiological assessments of IgE levels and anaphylaxis.

1.4.2.5 Antigen-specific Tregs
Increased levels of antigen-specific Tregs can be detected in patients following oral immunotherapy and these Tregs correlate with improved tolerance upon challenge (105). In mice, antigen-specific Tregs can also be observed upon oral antigen treatment; the OT-II and DO11.10 transgenic mouse strains are powerful tools for investigations of Tregs and oral tolerance as their TCRs respond only to the OVA\textsubscript{323-339} peptide fragment. Oral treatment with OVA expands Tregs in both the MLNs and spleens of OVA transgenic
mice (86), and T cells from these transgenic mice have been used in adoptive transfer systems to study oral tolerance in the GALT since the 1990’s (149). Several groups have now crossed Foxp3-GFP mice to the transgenic strains, which allows transferred OVA-specific T cells to be used in tracking the development of the Treg component of oral tolerance (49).

1.5 Mast Cells
Mast cells are highly granulated cells positioned at interfaces with the external environment (skin, lungs, intestine) and also associated with blood vessels and nerve endings. Mast cells were first described by Paul Ehrlich in his doctoral thesis in 1878, and much progress has since been made in characterizing and understanding the functionality of these important cells (150, 151).

Mast cells are now understood to have a variety of sentinel, regulatory, and protective roles. Mast cells are implicated in bolstering immunity to bacteria (152), recruitment of effector cells during viral infections (153, 154), and mediating the elimination of some parasites (155). In the last decade mast cells have also been studied almost as thoroughly in their newly described roles as immune regulators (156-159). Mast cells are a heterogeneous population of cells that, like many innate cells, are specialized according to their tissue distribution. In rodents, mast cells present as mucosal type mast cells (MMCs) or connective tissue type mast cells (CTMC). Human mast cells can be classified into three functional categories based on their capacity to produce high levels of tryptase (designated MC$_T$), high levels of chymase (designated MC$_C$), or the active production of both tryptase and chymase (designated MC$_{TC}$) (160, 161). As with many immune cells the translation between species is not perfect, but human MC$_T$ correlate functionally to rodent MMCs while human MC$_{TC}$ approximate rodent CTMCs (161). Accordingly, in mice MMCs and CTMCs release distinct profiles of murine mast cell proteases (MMCP). All mast cells express the stem cell factor (SCF) receptor c-kit (CD117) as SCF is essential for mast cell differentiation and development in combination with IL-3 and its continued presence is required for long term mast cell survival (161). Another important phenotypic feature of mast cells is the surface
expression of the high affinity IgE receptor FcεRI. This allows mast cells to respond to antigens via specific antibody responses, thus bridging innate and adaptive immunity. Mast cells are also equipped with a host of TLRs, thereby allowing them to serve as potent innate sentinel cells in the early stages of infection.

1.5.1 Allergic Activation of Mast Cells

When antigen crosslinks IgE bound to the FcεRI on mast cells, 3 discreet responses are observed: 1) release of preformed granule contents (e.g. histamine and proteases); 2) de novo synthesis of lipid mediators (e.g. prostaglandins and leukotrienes); 3) gene activation and de novo synthesis of cytokines (e.g. IL-6 and TNF) (13, 151). These 3 outcomes may be adaptive responses to pathogenic infection but they are also the source of tissue damage and adverse health outcomes when initiated by harmless environmental antigens in allergic patients. The relative levels of mast cells in the intestine have been correlated with susceptibility to oral anaphylaxis in mice (19) and it is understood that histamine release from activated mast cells is directly involved in initiating the symptoms of allergy and anaphylaxis (15, 148, 162).

Histamine is an amine that acts on a group of 7-transmembrane G-protein coupled histamine receptors (163). Histamine signaling has a wide range of outcomes depending on the tissue site and cellular target; notable effects in allergic disease include vasodilation, vascular permeability, and itching. Histamine receptor 1 (HRH1) is most commonly associated with these allergic symptoms, but antihistamines have been developed for all 4 histamine receptors. The “first generation” HRH1 antihistamines, such as pyrilamine, were developed before rigorous clinical testing standards were established, but “second generation” HRH1 antihistamines are now used more commonly because they do not readily cross the blood brain barrier and therefore have fewer off-target effects such as drowsiness and weight gain (164). HRH2 antihistamines, such as ranitidine, can be used in allergic disease, but are more commonly applied in gastroesophageal reflux disease. Clinical trials continue to investigate the potential of HRH3 and HRH4 antihistamines as allergy therapeutics (164).
1.5.2 Mast Cells in Innate Immunity

Despite their unfortunate involvement in allergic disease, mast cells are versatile innate sentinel and effector cells (155, 165). Mast cells express a range of toll-like receptors and respond to both bacterial and viral components (152). Mast cells can recruit effector cells to sites of viral infection via chemokine production (153, 154), and also carry out antimicrobial functions (166) and neutrophil recruitment in bacterial infection (167).

Physiologically, mast cells in the intestine are important in maintaining appropriate peristalsis and barrier function (168, 169), and acute or chronic stress can compromise intestinal barrier integrity in a mast cell-dependent manner (170, 171). Recent work shows mast cells can degranulate in response to several fungi independent of specific IgE (172-174), but the protective function of this response has not yet been characterized. IgE-mediated mast cell activation may also be an important component of immunity to some intestinal parasites. Mast cells are dramatically increased in the intestine of mice infected with nematodes (175), and elimination of *Trichinella spiralis* is delayed in mice lacking mast cell protease-1 (176, 177). Mast cells are now also being investigated for roles promoting and directing helper T cell responses.

1.5.3 Mast Cell Modulation of T Cell Responses

Recent years have seen a flurry of research exploring the interaction between mast cells and T cells. As mast cells have potent inflammatory roles when activated as innate sentinels and allergic effector cells, it is useful to consider separately their resting and activated states in relation to T cells.

In the resting state, mast cell-derived TGF-β1 can promote Treg differentiation *in vitro* (178) and in May of this year Nakano *et al.* identified TGF-β-producing mast cells colocalized with Tregs and associated with liver allograft survival in rats (179). This adds to a growing list of mast cell involvement in peripheral tolerance and Treg function in the context of solid organ transplants (180-182).
When activated, mast cells can produce histamine and IL-6 which both have potent immunomodulatory roles on DC and T cell function (183, 184). Tregs have been shown to suppress mast cell degranulation via OX40/OX40L interactions (185), yet new evidence paradoxically suggests that surface-bound TGF-β on Tregs promotes IL-6 production in mast cells (186). Mast cell-derived IL-6 can suppress Treg activity and ultimately drive Th17 differentiation following the OX40/OX40L interaction (187). Therefore, it would appear that mast cells and Tregs can engage in a complex balance of mutual suppression and inactivation via receptor interactions and cytokine production. Not surprisingly, mast cells can drive Th1 and Th17 differentiation in vivo via IL-6, IFNγ, and TGF-β production by mast cell-primed DCs (183).

While there is a general bias towards mast cell suppression of Tregs in vitro, it is clear that the tissue site, cellular microenvironment, and type of mast cell activation are all factors that can dictate the outcome of mast cell activation on T cell responses. Care must be exercised when predicting the outcome of mast cell activation, as different contexts can yield divergent outcomes for responding T cells. For this reason, it is critically important that investigations of mast cell presence and activation continue in disease models like food allergy and oral tolerance in vivo.

1.5.4 Murine Mast Cell Models
Several strains of mast cell-deficient mice have been developed in order to examine the role of mast cells in immunity and disease. The two most common and best-characterized of these models are the KitW-sh/W-sh and KitW/W-v mice, both of which were established by defects in the c-kit receptor for stem cell factor (SCF). Since SCF is essential for mast cell development and survival, mice failing to express c-kit are deficient in mast cells.

Perhaps the most controversial application of mast cell-deficient mice has been to investigate the role of mast cells in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Divergent reports on the susceptibility of KitW-sh/W-sh and KitW/W-v mice have raised concerns about the reliability and relevance of these models
(188-191), but new models of conditional mast cell deficiency have been developed that have already shed light on some of these issues (192). Specifically, one conditional knock-out model has shown that mast cells are not involved in preventing or exacerbating EAE in mice (193). In these early days, conditional mast cell deficiency models do not appear to present as many phenotypic abnormalities as mice reliant on c-kit mutations (193, 194). As new mouse models of conditional mast cell deficiency are developed, we may find that our current understanding of mast cell roles in mouse models of disease are incomplete and warrant revision. However, despite other defects, both the KitW-sh/W-sh and KitW/W-v mice are confirmed to be severely deficient in mast cells (169). As such, any immunologic process that successfully occurs in these mice does not require mast cells. Further confirmation as to the role of mast cells in such models can be obtained by selective reconstitution of relevant tissue sites with cultured mast cells.

1.6 Pattern Recognition Receptors

Every immune system is presented with the critical task of identifying and eliminating foreign pathogens. The idea that the immune system has evolved to recognize pathogens and provide “danger” signals was proposed in 1989 by Dr. Charles Janeway (195), and in the short time that has passed since then microbiologists and immunologists have characterized a list of pathogen-associated molecular patterns (PAMPs) and corresponding pattern recognition receptors (PRRs). Notable PAMPs include bacterial cell wall components, flagellin, viral and bacterial nucleic acids, lipids, and lipopeptides. These PAMPs are molecular signatures identifying bacteria, viruses, and fungus, and can be readily detected by PRRs including Toll-like receptors (TLRs), Nod-like receptors (NLRs), and RIG-1-like receptors (RLRs) and Lectin-like receptors (196, 197).

It should be noted that “danger” signals can also arise in response to tissue damage, causing some to believe the paradigm of PAMPs and PRRs is inadequate to describe the immunologic process of discriminating self from non-self (198). As our awareness and characterization of the human microbiome accelerates, this relationship and the functional consequences of discriminating self from non-self appear increasingly complex and nuanced. Indeed, it has been proposed that PAMPs be renamed as
microorganism-associated molecular patterns (MAMPs) in order to better capture the spectrum of organisms, pathogenic and otherwise, that initiate PRR signaling (199). In a healthy person food antigens are treated not unlike commensal organisms, and it is likely that by looking to the commensal relationship and the regulation of PRRs we will find clues as to the mechanisms of successful food tolerance and uncover new therapeutic targets for food allergy.

PRRs allow for a targeted initial response and customized direction of the ensuing adaptive responses to foreign antigen. TLRs set the stage for response profiles through modification of innate cell responses and APC functions, in addition to their direct actions on T and B cells.

### 1.6.1 Toll-like Receptors (TLR)

TLRs comprise a large and thoroughly studied family of PRRs. Originally described with roles in *Drosophila* fungal immunity (200), the first human TLR was identified (TLR4) by Mehdzitov *et al.* in 1997 (201). Twelve unique TLRs have now been identified, with TLRs 1-9 being shared by both mice and humans (197).

TLRs are expressed widely in the body and can be broadly classified by cellular location: surface or intracellular expression. TLRs 1, 2, 4, 5, 6, and 11 are found on the surface of cells, while TLRs 3, 7, 8, and 9 are found inside cells in vesicle compartments (197). The intracellular TLRs are primarily responsible for recognition of foreign nucleic acids, while the extracellular TLRs are tasked to identify extracellular elements of pathogens such as bacteria and fungus (Figure 1.1 adapted from (202)). The desired outcomes of TLR signaling are the production of inflammatory cytokines, chemokines, and interferons, and these responses can be accomplished via the MyD88 intracellular adaptor molecule or TRIF alternative signaling depending on the TLR in question (203). In the context of controlling oral tolerance and responses to foods, surface-expressed TLR2 and TLR4 are the most studied and are likely the most influential. Furthermore, neither TLR2 or TLR4 respond exclusively to foreign stimuli, and both are known to
respond to self-derived heat shock proteins and other factors produced by tissue damage (204).

1.6.2 TLR2 and Oral Tolerance

TLR2 is important in identifying bacterial (205) and fungal wall components (206), but it must first combine as a heterodimer with TLR1 or TLR6. The TLR1/2 heterodimer responds to diacyl lipopeptides while the TLR2/6 heterodimer responds to triacyl lipopeptides and peptidoglycan (207), and both heterodimers of TLR2 signal through the MyD88-dependent pathway leading to transcriptional activation of NF-κB (Figure 1.1 adapted from (202)) (197). Both TLR2 and TLR4 require the additional adaptor molecule TIRAP in order to signal via MyD88 and activate NF-κB (208). NF-κB gene expression initiates production of a number of inflammatory cytokines, notably IL-6, IL-12p40, and TNFα (197). TLR2 is expressed by a wide range of cells relevant to mucosal immunity and tolerance, including intestinal epithelial cells, mast cells, macrophages, DCs, T cells, and B cells. While the activation of these TLR2-mediated inflammatory responses are adaptive in the context of pathogenic infection, it is not clear how this axis impacts oral tolerance to food and commensal bacteria.

Intestinal epithelial cells are bathed in an environment replete with TLR2 agonists such that these cells are probably calibrated to function amid a constitutive level of activation. Accordingly, in TLR2−/− animals the intestinal epithelial cell tight junctions are compromised (209, 210). Furthermore, TLR2 stimulation promotes tight junctions (209), which could have implications for food processing and antigen dosing presented to T cells. Defects in TLR2 are associated with inflammatory bowel disease (210, 211) and TLR2 polymorphisms have been associated with some forms of allergic disease (212-214).

TLR2 activation can result in IL-10 production (215), which could influence local DC, T cell, or B cell responses in the LP. Recently, Wang et al. demonstrated that TLR2 and MyD88 are necessary for DCs to imprint T cells with intestinal homing markers α4β7 and CCR9 (216). Similarly, TLR2 activation of B cells also activates CCR9 in addition
to promoting IgA production (217). Direct TLR2 activation of T cells and B cells can directly modify their functions, but the functional role of TLR2 in oral tolerance to foods has not yet been explored.

1.6.3 TLR4 and Oral Tolerance

Like TLR2, TLR4 is also important in the recognition of bacteria in the intestine and other environments. TLR4 uniquely recognizes lipopolysaccharide (LPS) which is found abundantly in the cell membranes of gram-negative bacteria (218). TLR4 complexes with MD-2 to form an LPS-binding complex that binds LPS with assistance from the cofactors CD14 and LPS-binding protein (LBP) (207). TLR4 signaling can then progress through both the MyD88-dependent and the MyD88-independent TRIF pathway (Figure 1.1 adapted from (202)), which represents a major difference between the known signaling of TLR4 and TLR2 (219). The MyD88-dependent pathway also requires the TIRAP adaptor and results in NF-κB and inflammatory cytokine expression, while the TRIF pathway leads to expression of IFN-β (219).

While TLR4 activation promotes immunity to bacteria, the function of TLR4 in regulating intestinal tolerance has not been well-described. DCs in oral mucosal sites express TLR2 and TLR4 receptors (220), and LPS activation of human Langerhans cells from oral mucosal sites can promote a tolerogenic environment resulting in Treg induction in vitro (221). Similarly, allergic sensitization is more profound in TLR4−/− mice (222) and LPS stimulation of DCs leads to a Th2-polarized response in the absence of MyD88 (223).

However, intestinal mucosal macrophages do not express the TLR4 signaling component CD14 and are therefore less responsive to LPS (224). Furthermore, oral food sensitization to peanut or β-lactoglobulin was not altered in TLR4−/− mice, suggesting that TLR4 may have limited importance in regulating the balance between oral tolerance and sensitization (222). Current evidence is not conclusive regarding a role for TLR4 in regulating intestinal immunity and tolerance, whereas several reports point to TLR2 as an important regulating PRR in this microbe-rich environment.
1.7 Microbiology of Oral Tolerance

The human intestine is a dynamic environment host to a myriad of bacteria. It is unclear how these and parasites such as helminths regulate immunologic responses to food antigens, but there is mounting evidence that the microbiological environment of the intestine has a profound influence on oral tolerance. In addition to the commensal bacteria residing in the intestine, food products are often contaminated by a wide array of bacteria and fungi. It is likely that contaminating cultures can shape oral tolerance to foods.

The microbial composition of the human intestinal microflora is exceptionally diverse. It has been estimated that this microflora is composed of close to 400 different phylotypes of bacteria, primarily clustered in the Bacteroidetes and Firmicutes phyla (225). While resident bacterial populations can vary greatly between individuals, common groupings are observed and these are approximately conserved between mice and humans (226). Furthermore, it has long been known that various populations of resident bacteria colonize differentially based on intestinal region (227, 228), thereby reducing the diversity at key immunologic sites in the small intestine. For instance, Lactobacilli and Streptococci (both of the phylum Firmicutes) are preferentially associated with the small intestine in mice (228, 229).

We now understand that commensal bacteria in the gut are important inducers of peripheral Treg differentiation (230). This may represent a significant mechanism by which colonic commensals survive the intestinal environment without activating inflammation. Many studies also support a role for preferential induction of IgA production from intestinal LP plasma cells in response to commensal bacteria, as the secreted IgA response can present a neutralizing barrier sequestering the commensals to the lumen and preventing epithelial barrier breach (114). This barrier system protects the host from breach and infection, which in return protects the commensals from inflammation and robust commensal-specific responses needed to clear infections.
Commensals and pathogenic bacteria appear to have divergent effects on mucosal DCs, T cells, and the resulting immune responses. Stimulation of human MLN DCs by commensal *Bifidobacterium* and *Lactobacillus* bacteria promotes the production of the regulatory cytokines TGF-β and IL-10, whereas MLN DCs treated with pathogenic *Salmonella* bacteria results in an inflammatory IL-12 and TNF cytokine response (231). In recent years, there has been a flourish of activity seeking to identify the influence of different human commensals on intestinal Treg populations.

To date, species of commensal human *Bacteroides* (232, 233), *Clostridium* (234), and the multi-microbe benign Altered Shaedler Flora (235) have all been shown to induce Treg responses and reduce inflammation in the mouse colon. Elegant studies with *Bacteroides fragilis* in mice have shown that the Tregs induced by TLR2 activation with the unique Polysaccharide A are necessary for successful colonization (232, 233). Similarly, the probiotic *Bifidobacterium infantis* promotes Tregs and regulatory cytokine production in humans and functions through TLR2 (236). Commensal regulatory responses are particularly remarkable by contrast to the inflammatory responses observed upon colonization of the small intestine by segmented filamentous bacteria that embed in the intestinal epithelium and promote T<sub>H17</sub> differentiation in the LP and PPs (237, 238). Understanding how microbiological factors and the corresponding PRRs dictate the balance between Tregs or inflammatory T<sub>H17</sub> responses may provide clues to the etiologies of inflammatory intestinal diseases and possible probiotic treatment modalities.

While recent studies show a clear relationship between some commensals and immunologic tolerance, the specificity of these Treg responses have not been adequately characterized. Studies exploring commensal Treg induction and the resulting suppression of inflammation primarily examine colonic responses and little attention has been paid to the relationship between commensals and Tregs in the small intestine. The small intestine is an important site in food tolerance induction, and few studies have addressed the role of commensal colonization on tolerance to foods. Commensal bacteria are required for appropriate levels of Tregs to be established in the MLN, and without them oral tolerance is inadequate in germ-free (GF) mice (239). Furthermore, it has been proposed that the inability of GF mice to establish oral tolerance may be directly related
to the failure of these mice to establish adequate T cell populations in the PPs (240). Several studies have also shown that GF mice display a more Th2-polarized response to oral antigens, which results in IgE and IgG1 antibody production to oral antigen and a failure to tolerize (241, 242). The changes in Treg and humoral responses to food antigen in the absence of commensals are likely to implicate PRRs, but more directed investigations must be carried out to fully understand the precise role of TLR signaling in oral tolerance.

Recent evidence shows that a probiotic strain of *Lactobacillus* can prevent the effector stage of allergy by interacting with mast cells (243). Similarly, treatment with *Bifidobacterium* components or TLR2 activation of mast cells by the synthetic triacyl lipopeptide Pam3CSK4 suppress IgE-mediated mast cell degranulation *in vitro* and *in vivo* (244). This points to probiotics and TLR activation as having important regulatory roles in the effector phase of tolerance and allergy, in addition to potential roles in oral tolerance induction.

The roles of viruses in oral tolerance are largely undescribed, but a recent study examining oral tolerance induction to OVA in mice during an intestinal norovirus infection found that OVA-specific IgE was modestly enhanced (245). Oral stimulation of endosomal TLR9 can also promote food-specific secreted IgA (246). Similar to some commensal bacteria, several recent studies document that helminths can also promote intestinal Treg differentiation, likely for purposes of immune evasion, that consequently contributes to a regulatory intestinal environment (247-251). Interestingly, TLR2 was identified as necessary for Treg enhancement during infection with *Schistosoma mansoni* (250).

The known Treg-inducing properties of commensals and the deficits in food tolerance induction in the absence of commensal colonization underscores the immediate need for targeted studies exploring the relationship between innate pattern recognition receptors, bacteria, and food tolerance induction.
1.8  Rationale and Hypotheses

1.8.1  Mast Cell Modulation of Oral Tolerance Induction

Mast cells are positioned to interface with environmental microbes or antigens and have recently been identified as a critical regulators of peripheral tolerance to solid allografts (180-182). This evidence and the physical location of mucosal mast cells positions them as likely candidate cells for regulation of oral tolerance induction. Further to this, the progressive development of food allergies has been observed clinically (252), suggesting that IgE-mediated mast cell activation in allergic patients may alter their immunological responses to bystander foods. We set out to comprehensively assess the role of mast cells, resting and activated, in oral tolerance induction. We hypothesized that mast cells would not be essential for the induction of oral tolerance, but their activation by IgE and antigen would interfere with oral tolerance.

1.8.2  TLR2 Modulation of Oral Tolerance Induction

In addition to the unknown roles of innate immune cells, the relative importance of PRRs has not been well-characterized in the context of oral tolerance. TLR2 is an important component of intestinal immunity and defects in TLR2 can lead to dysregulated local inflammatory responses such as inflammatory bowel disease (209, 211). We sought to understand how the elimination or activation of TLR2 might manipulate oral tolerance induction and oral immunization. Our hypothesis was that oral TLR2 activation would prevent tolerance to bystander food antigens and act as an effective oral adjuvant in the context of oral immunization.
Figure 1. Schematic representation of TLR ligands and key signaling adaptor molecules.

This figure details interactions between different TLRs and their respective ligands. The subsequent signaling through key adapter molecules and cellular responses are also depicted. Pam$_3$CSK$_4$ and FSL-1 are synthetic lipopeptides commonly used in examinations of TLR2 signaling. This Figure is adapted from (202).
CHAPTER 2 MATERIALS AND METHODS

2.1 Mice
Only male mice were used in experiments, and mice were normally used between 6-8 weeks of age. All animal studies were performed in a minimum of 2 independent experiments, except where indicated. C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbour, ME) or Charles River Laboratories (Québec, Canada), or bred on site at the Carleton Animal Care Facility (CACF) from C57BL/6J mice. BALB/c mice were purchased from Jackson Laboratories or Charles River Laboratories. WBB6F1/J-KitW/KitW-v (KitW/W-v) mice were purchased from Jackson Laboratories. KitW-sh/HNxhrJaeBsmJ (KitW-sh/W-sh), B6.129P2-Fcgr3tm1Sjv/SjvJ (FcγRIII-/-), B6.129-Tlr2tm1Kir/J (TLR2-/-), and C57BL/6J mice were supplied by Jackson Laboratories or bred from stock obtained from Jackson Laboratories. B6.SJL-Ptprc Pepcb/BoyJ (CD45.1+) mice and B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II+) mice were purchased from Jackson Laboratories. These mice were then crossed and bred on site at the CACF. Mice were genotyped by flow cytometry of peripheral blood T cells for CD45.1, CD45.2, Vα2, and Vβ5. Homozygous males were then crossed with homozygous Foxp3-GFP females (originally acquired from Dr. Mohamed Oukka, Harvard Medical School, Cambridge, MA), genotyped by PCR, and bred on site. The Foxp3-GFP locus in these mice is X-linked, so all male offspring from the cross with OT-II/CD45.1+ mice possessed one copy of Foxp3-GFP. Approval for all animal studies was granted by the Dalhousie University Committee on Laboratory Animals.

2.2 Antibodies and Reagents
Anti-mouse IgG1 (clone: RMG1-1), IgG2a (clone: RMG2a-62), CD4-PerCP (clone: RM4-5), and CD138-APC (clone: 281-2) antibodies were purchased from BioLegend (San Diego, CA). Anti-mouse Vα2-PE (clone: B20.1) was purchased from BD Biosciences (Franklin Lakes, NJ). Anti-mouse IgE was purchased from eBiosciences (San Diego, CA) and Southern Biotech (Birmingham, AL). All other antibodies were
obtained from eBiosciences. Mouse mast cell protease-1 (mMCP-1) ELISA kit was purchased from eBiosciences. Magnetic Activated Cell Sorting (MACS) was performed with “CD4” T cell isolation kit II” and “CD62L (L-selectin) microbeads” from Miltenyi Biotec Inc. (Auburn, CA). 4S FITC-dextran, ranitidine, pyrilamine, LPS derived from E.coli, 45% fish gelatin, β-lactoglobulin, and ovalbumin from chicken egg white (OVA) were purchased from Sigma (Oakville, Canada). Trinitrophenylated bovine serum albumin (TNP-BSA) was purchased from Biosearch Technologies, Inc. (Novato, CA). Anti-TNP IgE was enriched from TIB141 (ATCC, Manassas, VA) supernatant by ammonium sulphate precipitation followed by high performance liquid chromatography. Endotoxin free RPMI-1640 medium and Nunc 96-well plates were purchased from Thermo Fisher Scientific (Nepean, Canada). Bovine serum albumin (BSA) Fraction V was purchased from Roche Diagnostics (Laval, Canada). Crude peanut extract (CPE) was purchased from Greer Technologies (Lenoir, NC). OVA323-339 peptide was purchased from C S Bio (Menlo Park, CA). Pam3CSK4 (Pam3CysSerLys4) and FSL-1 (Pam2CGDPKHPKSF) were obtained from EMC Microcollections (Tübingen, Germany). Collagenase A and DNAse I were purchased from Roche (Laval, Canada). NaHCO₃ was purchased from BHD Inc. (Toronto, Canada). MALP-2 was generously provided by Professor Guenther Jung from the University of Tübingen, Germany.

2.3 **Flow Cytometry**

FACS buffer was used in all assays and contained PBS, 1%BSA, and 0.01% sodium azide. Fc interactions and other non-specific interactions were blocked by treating samples with 2% v/v rat and/or mouse serum as appropriate for 15-20 min prior to staining. Staining was performed in 1% v/v rat and/or mouse serum, as appropriate, with a 1/200 dilution of primary anti-mouse antibodies: CD45.2-FITC (clone: 104), CD69-FITC (clone: H1.2F3), CD138-APC (clone: 281-2), CD117-PE (clone: 2B8) (1/1200 dilution), CD19-PerCP (clone: eBio1D3), CD62L-PE, Vα2-PE (clone: B20.1), CD4-biotin (clone: L3T4), CD4-APC (clone: GK1.5), CD4-PerCP (clone: RM4-5), CD45.1-PE (clone: A20), CD45.1-APC (clone: A20), mIgA-PE (clone: 11-44-2), CD62L-PE, or appropriate isotype controls. Secondary staining was performed for 15-20 min with
streptavidin-PerCP (BioLegend) was performed in conditions with biotinylated primary antibodies. Cells were washed 2 times after staining. In some studies, cells were treated with Cell Proliferation Dye eFluor® 670 as per the manufacturer’s protocol (eBiosciences) prior to adoptive transfer. Samples were fixed in 1% paraformaldehyde in PBS prior to data collection, and samples were read using the FACSCalibur from BD Biosciences (San Jose, CA). Analysis of flow cytometry was performed with FCS Express 3 software (De Novo Software, Los Angeles, CA). For analysis of adoptively transferred populations, cells were analyzed first through a gate of live lymphocytes. These cells were then gated on CD45.1 expression and CD4 expression. Cells were then gated on Foxp3 expression and/or proliferation dye. For analysis of in vivo cell proliferation, “undivided cells” were gated based on levels of Cell Proliferation Dye eFluor® 670 in CD4+/CD45.1+ cells recovered from control mice that had not been treated with OVA. For analysis of B cells and plasma cells, cells were first gated on FSC$^{hi}$/SSC$^{hi}$ or FSC$^{lo}$/SSC$^{lo}$ populations. These two discreet populations were then gated on CD19, CD138, CD69, IgM, or IgA expression.

2.4 Aluminum Hydroxide (Alum) Precipitation

Food proteins, OVA (Sigma), crude peanut extract (Greer Technologies), or β-lactoglobulin (Sigma) were precipitated to alum for use in immunizations. Protein was prepared at 1mg/mL in PBS and mixed 2.22:1 with 84mg/mL NaHCO$_3$ (BHD Inc.) in H$_2$O. The resulting solution was then mixed 1.45:1 with 94mg/mL aluminum potassium sulphate (Sigma) in H$_2$O by stir bar. The resulting precipitate was left to stand for 1h at room temperature, then spun for 15min at 350g. The supernatant was removed and measured, then the precipitate was washed 3 times and resuspended in PBS. The % of precipitated protein was calculated by analysis of the starting and final supernatants at 280nm on a spectrophotometer (GENESYS 10uv, Thermo Scientific; Fischer Scientific Limited, Nepean, Canada).
2.5 Oral Tolerance Induction

2.5.1 Induction and Assessment of Tolerance to OVA

2.5.1.1 Ad Libitum Tolerance to OVA

In order to induce oral tolerance to chicken egg ovalbumin (OVA), mice were provided with 4mg/mL OVA in drinking water *ad libitum* for 7 days while a control group was provided normal drinking water (Figure 2.1,A). Water was changed every 2 days. On day -2, all mice were returned to normal water. All groups were immunized by i.p. injection of 100μl on day 0 with 50μg or 10μg of OVA precipitated to alum in PBS, as indicated. All groups were boosted by i.p. injection of 10 or 1 μg as indicated of soluble OVA (in 100μl of PBS) on day 14 and blood was harvested by cardiac puncture in heparin-coated syringes on day 21. Following centrifugation for 15 min at 1,900g, plasma was removed and stored at -20°C. In some groups, fecal samples were taken on day 21 and homogenized in PBS with 0.01% sodium azide (100mg feces/mL) for later IgA analysis. These samples were centrifuged for 15 min at 10,000g and supernatants stored at -20°C.

The 4mg/mL 1 week OVA oral treatment dose for tolerance induction was used based on a pilot experiment examining different doses of OVA (4mg/mL, 0.4mg/mL, and 0.04mg/mL) over a 4-week feeding period (Figure 2.2). It should be noted that different strains of mice consume different volumes of water on a daily basis, and therefore the relative ingested antigen dose varies between strains (Table 2.1). Water consumption was determined in mouse groups by measurement of the remaining water volume every 2 days.

2.5.1.2 Assessment of Anaphylaxis in Actively Sensitized or Tolerized Animals

In order to determine the physiological relevance of oral tolerance and antibody levels in mice, groups were challenged i.p. with 10 mg OVA or 1mg CPE in 100μl PBS, as indicated, on day 21 of treatment (Figure 3.1). Rectal temperature was measured before, 10, 30, and 50 min after challenge by insertion of a “Traceable® expanded-range
thermometer” (VWR; Texas) 1cm into the rectum while mice were held in the supine position. In mice challenged with CPE, blood was collected 60 min post-challenge and mast cell protease-1 (mMCP-1) levels were assessed in plasma by ELISA as per manufacturer’s protocol (eBioscience). Samples below the limit of detection for the assay were assigned a value of that limit, based on the standard curve (115.98 pg/mL), for the purposes of statistical analysis.

2.5.1.3 Gavage Tolerance to OVA

Two models of gavage tolerance were used to manipulate OVA tolerance induction. OVA containing water was provided ad libitum at 4mg/mL from day -9 to -2. In studies examining mast cell activation, mice were also treated with OVA by gavage 2 times during the week of ad libitum OVA containing drinking water provision (described in section 2.8). In studies examining the role of TLR activators on tolerance, mice were treated with OVA by gavage (1mg in 100μl PBS) 3 times during the week of ad libitum OVA containing water (days -9, -6, and -3) (Figure 2.3,A). In some groups, OVA gavage treatments were supplemented with one of the following: 10μg of the TLR2/1 heterodimer activator Pam3CSK4, 5μg of the TLR2/6 activator MALP-2, 5μg of the TLR2/6 activator FSL-1, or 10μg of the TLR4 activator LPS. These groups were compared to tolerized mice receiving 3 gavage treatments of OVA in PBS alone, and control mice receiving 3 gavage treatments of PBS. Oral treatment with FSL-1 was performed at a 5μg dose due to toxic effects observed in a pilot experiment using a 10μg dose (data not shown). Oral treatment with MALP-2 was performed at a 5μg dose due to commercial product availability.

On day -2 all mice were returned to normal water. All groups were immunized by i.p. injection of 100μl on day 0 with 50μg or 10μg of OVA precipitated to alum in PBS, as indicated. All groups were boosted i.p. with 100μl of soluble OVA (10 or 1 μg as indicated) in PBS on day 14 and blood was harvested by cardiac puncture in heparin-coated syringes on day 21. Following centrifugation for 15 minutes at 1,900g, plasma was removed and stored at -20°C. In some groups, fecal samples were taken on day 21
and homogenized in PBS with 0.01% sodium azide (100mg feces/mL). Samples were centrifuged for 15 min at 10,000g and supernatants stored at -20°C.

### 2.5.2 Induction and Assessment of Tolerance to Peanut

Mice were treated as detailed in Figure 3.1. One group of mice was fed peanut butter *ad libitum* (KRAFT® “All Natural Peanut Butter”; Don Mills, ON) for 7 consecutive days, followed by 2 days of regular chow. A control group was fed regular mouse chow devoid of peanut protein. C57BL/6 mice consumed an average of 1.9g ± 0.1 SEM of PB/mouse/day, which is equivalent to 501mg of peanut protein (n = 19 daily measurements pooled from 5 cages) (Table 2.1). Mice were immunized on day 0 with 10μg of crude peanut extract (CPE)-precipitated to alum. All groups were boosted with 1μg CPE in 100μl PBS on day 14. Blood was harvested by cardiac puncture with heparin-coated syringes following anaesthesia on day 21. Blood samples were centrifuged at 1900g for 15 min, then plasma was removed and stored at -20°C.

### 2.5.3 Induction and Assessment of Tolerance to Milk

#### 2.5.3.1 Bacterial Supplementation of Ad Libitum Tolerance to Milk

Skim milk was prepared from powder (no name® LOBLAWS INC., Toronto, Canada) at 27.8mg/mL (equivalent to 10mg/mL protein) in filtered water. β-lactoglobulin represents approximately 10% of cow’s milk protein (253), therefore mice consumed an average of approximately 5mg β-lactoglobulin/day based on 5mL/day water intake (Table 2.1). Optimal milk dosing for tolerance induction was established based on pilot studies examining different dosage and timing protocols (Figure 2.4).

We supplemented one milk stock with 2.5x10⁶ heat killed CFU/mg protein of the two most common yogurt bacterial cultures, *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. delbrueckii*) and *Streptococcus thermophilus* (*S. thermophilus*) to match reported bacterial levels in yogurt (254). Milk was provided to BALB/c mice at 10mg/mL protein *ad libitum* for one week (Figure 2.5). After 2 days rest from antigen treatment, mice
treated with supplemented milk, milk alone, and untreated control mice were immunized i.p. with 50µg β-lactoglobulin precipitated to alum. Two weeks later, all mice were boosted i.p. with 1µg β-lactoglobulin, and blood was harvested after 1 week. Oral tolerance to β-lactoglobulin was assessed by ELISA for β-lactoglobulin–specific IgE, IgA, IgG1, and IgG2a antibody levels (see below).

2.5.3.2 Bacterial Culture

*Lactobacillus delbrueckii* subsp. *bulgaricus* (ATCC 11842) was grown in deMan Rogosa Sharpe broth (Oxoid Ltd, Basingstoke, England) and incubated at 37°C in 5% CO₂ without shaking. *Streptococcus thermophilus* (ATCC 19258) was grown in brain heart infusion broth (Becton, Dickinson and Company, Sparks, MD) and incubated at 37°C with shaking. Cell growth was measured by optical density (O.D.) at 570 nm for *S. thermophilus* and 600 nm for *L. delbrueckii* subsp. *bulgaricus*. Bacterial numbers were calculated based on 2 x 10⁸ CFU/mL being equivalent to 1 OD₆₀₀ for *S. thermophilus* and 1 OD₅₇₀ for *L. delbrueckii* subsp. *bulgaricus* (255). Following incubation for 24 hrs, bacteria were harvested by centrifuging (3220g for 15 min) and washed twice with sterile PBS. Cells were resuspended in PBS, heat killed at 100°C for 20 min, aliquoted, and stored at -80°C. Broth cultures were inoculated with heat killed bacteria and incubated for 24 h to ensure bacteria were killed. No growth was observed.

2.6 Antigen-specific Antibody Enzyme-linked Immunosorbent Assay (ELISA)

2.6.1 OVA-specific and Peanut-specific Antibody ELISA

OVA-specific and peanut-specific antibodies were measured by an antigen binding ELISA assay. Plates were coated overnight with anti-mouse IgE, IgG₁, IgG₂a, or IgA in 0.2M borate buffered saline pH 8.3. After washing plates in phosphate buffered saline pH7.4 (PBS) with 0.01% Tween-20 wash buffer, samples were blocked for 1h at room temperature in PBS with 2% BSA. Plates were washed and plasma or fecal samples were serially diluted in PBS with 0.2% BSA and 0.005% Tween-20, then added to the wells of plates overnight. Sample dilutions of plasma, to detect IgE, were performed at 1/5 and
1/10, IgA at 1/5 and 1/15. 1/3 serial dilutions of plasma to detect IgG1 were performed from 1/200 to 1/4.37x10^5, while 1/3 dilutions of IgG2a were performed from 1/25 to 1/5.47x10^4. A standard positive control plasma sample was run on all plates. Plates were washed, and biotinylated OVA or crude peanut extract (CPE) was added to the wells for 1h. Plates were then washed again and 50μl of streptavidin conjugated to alkaline phosphatase was added for 30min (Invitrogen). Plates were washed and color developed with a commercial ELISA amplification system (Invitrogen). Plates were normalized between assays with positive control standard. OVA-specific IgG1 and IgG2a antibody levels were determined by titre threshold. To assess titres, the dilution curve of each sample was Log transformed and the point of intersection with a titre threshold was reported as the inverse Log value. Samples that failed to reach the titre threshold were designated as non-responders and assigned a -Log titre value of 0.01. In studies assessing the IgG1/IgG2a class ratio, ODs were used from dilutions in the dynamic range of IgG1 (1/16200) and IgG2a (1/675). OVA-specific and peanut-specific IgE and IgA levels were assessed by comparisons of final optical density (O.D.) at a 1/5 plasma dilution, relative to standard since the low levels of antibody observed were not appropriate for titre analysis in many experimental groups. Similarly, peanut-specific IgG1 and IgG2a levels were compared by O.D. due to low levels that were not appropriate for titre analysis. OVA-specific IgE O.D.s were standardized relative to a commercial OVA-specific IgE standard (Chondrex, Inc.; Redmond, WA) and reported as ng/mL.

2.6.2 β-lactoglobulin-specific Antibody ELISA

β-lactoglobulin-specific antibodies were measured by an antigen binding ELISA assay. Plates were coated overnight with anti-mouse IgE, IgG1, IgG2a, or IgA in 0.2M borate buffered saline pH 8.3. After washing plates in phosphate buffered saline pH7.4 (PBS) with 0.01% Tween-20 wash buffer, samples were blocked for 1h at room temperature in PBS with 2% fish gelatin. Fish gelatin was used in buffers to avoid interference with the assay through the use of BSA, which constitutes 1% of cow’s milk protein (253). Plates were washed and plasma or fecal samples were serial diluted in PBS with 0.2% fish gelatin and 0.005% Tween-20, then plated overnight. Plasma samples were diluted to
detect IgE at 1/5 and 1/10, IgA at 1/5 and 1/15. 1/4 serial dilutions of plasma to detect IgG_1 were performed from 1/1800 to 1/2.95x10^7, while 1/4 dilutions of IgG_2a were performed from 1/180 to 1/2.95x10^6. A standard immunized plasma sample was run on all plates. Plates were washed, and biotinylated β-lactoglobulin was added to the wells for 1h. Plates were then washed and streptavidin-alkaline phosphatase was added for 30min (Invitrogen). Plates were washed and color developed with a commercial ELISA amplification system (Invitrogen). Plates were normalized between assays to an immunized standard. β-lactoglobulin-specific IgG_1 and IgG_2a antibody levels were determined by titre threshold. To assess titres, the dilution curve of each sample was Log transformed and the point of intersection with a titre threshold was reported as the inverse Log value. Samples that failed to reach the titre threshold were designated as non-responders and assigned a -Log titre value of 0.01. β-lactoglobulin-specific IgE and IgA levels were compared by optical density (O.D.) at a 1/5 plasma dilution since the low levels of antibody observed were not appropriate for titre analysis in many experimental groups.

2.6.3 Total IgA ELISA

Plates were coated overnight with 1μg/mL anti-mouse IgA (Biolegend) in 0.2 M borate buffered saline pH 8.2, 50μl/well. Plates were washed in PBS + 0.01% Tween-20 and blocked for 1h at room temperature with 100μl/well of 2% fish gelatin or 2% BSA in PBS. Plates were washed, then fecal or plasma samples were diluted 1/500 and 1/2500 in ELISA dilution buffer (0.01% Tween-20 and 0.2% Fish gelatin or 0.2% BSA) and applied to wells overnight at 4°C. A standard curve of mouse IgA (Biolegend) was applied parallel to samples with 1/10 serial dilutions from 10μg/mL to 1x10^-6 μg/mL. Plates were washed, then anti-mouse IgA-biotin (Biolegend) was added to wells at 1μg/mL in 50μl dilution buffer for 1 hour at room temperature. Plates were washed, and plates were treated with 50μl/well streptavidin-alkaline phosphatase (Invitrogen) for 30min in TBS. Plates were washed and color developed with a commercial ELISA amplification system (Invitrogen). Total IgA from plasma samples was expressed as μg/mL, while total IgA from fecal samples was expressed as μg/100mg feces.
2.7 Antihistamine Treatment
Mice were fed either pyrilamine (0.04 mg/mL) or ranitidine (0.04 mg/mL) ad libitum in drinking water for one day prior to the initiation of tolerance induction. During the feeding stage of OVA tolerance induction, mice were provided with pyrilamine or ranitidine, at the doses above, mixed in OVA-water (4mg/mL) or in normal drinking water. After a 2 day period in which all mice receive normal drinking water, mice were immunized and boosted with OVA as described above. Mice were weighed daily. Based on water consumption of approximately 5mL/day for a 20g mouse, pyrilamine and ranitidine were provided at an approximate oral dosing of 10mg/kg/d.

2.8 Passive Mast Cell Sensitization and Activation
Experiments were performed in an OVA model of tolerance. The Fc epsilon bearing cells of three groups of mice were sensitized in vivo by i.p. injection on day -9 with 4.4µg of anti-trinitrophenylated (α-TNP) IgE in 100µL PBS. On day 0, one antigen-fed group was injected i.p. with 62.5µg TNP-BSA in PBS to induce sub-clinical mast cell activation, while the other two groups received 62.5µg BSA in PBS concurrent with antigen immunization. Mice were later boosted with the relevant antigen in PBS. In a second model designed to assess the effects of mast cell activation during oral tolerance induction, mice were sensitized with anti-TNP IgE on days -16 and -5. Seven days later TNP-BSA was delivered by i.p. injection concurrent with gavage feeding of 1mg OVA in 100µl PBS on days -9 and -3 of OVA treatment.

2.9 Assessment of Passive IgE Sensitization and Sub-anaphylactic Activation
Wild type C57BL/6 mice were sensitized i.p. with 4.4 µg of anti-TNP IgE. Seven days later, mice were challenged by i.p. injection of 62.5 µg TNP-BSA or BSA control. Blood was collected after 60 min for mMCP-1 analysis in plasma by ELISA. In other experiments, wild type C57BL/6 mice were sensitized i.p. with 4.4 µg of anti-TNP IgE. Control mice received PBS injections. Eight to ten minutes after TNP-BSA activation,
mice received 1 mg of 4S FITC-dextran i.v. Blood and peritoneal lavage were harvested 10 min later. FITC-dextran influx into the peritoneum was assessed by spectrofluorimetry (RF-1501 Spectrofluorimeter Shimadzu, Mandel Scientific Co. Ltd., Guelph, Canada). Rectal temperature was assessed before and 10, 30, and 50 min after TNP-BSA administration.

**2.10 Adoptive Transfer and Antigen-specific Treg Assessment**

Spleens and peripheral lymph nodes were removed from OT-II/CD45.1+/ Foxp3-GFP+ mice. Naïve CD4+/ CD62L+ T cells were isolated by MACS using the “CD4+ T cell isolation kit II” and “CD62L (L-selectin) microbeads” as per the manufacturer’s protocol. Cells were treated with Cell Proliferation Dye eFluor® 670 as per the manufacturer’s protocol (eBioscience). Purity was assessed by flow cytometry to be greater than 75% naïve CD4+/CD62L+ cells, and 1x10^6 of these cells were injected i.v. into recipient C57BL/6, KitW-sh/W-sh, or TLR2/-/- male mice. One day later, mice were provided with OVA at 4 mg/mL *ad libitum* in drinking water; controls received normal drinking water (Figure 2.1,B). In some studies, *ad libitum* OVA treatment was supplemented with 3 gavage treatments (Figure 2.3,B). After 7 days, the Peyer’s patches (PPs), mesenteric lymph nodes (MLN), and spleens were harvested. Nodes and spleens were homogenized with a scalpel or syringe plunger and passed through 100μm filters (Fischer Scientific). Spleens were treated for 3 min with 3mL red blood cell lysis buffer (0.14 M NH4Cl, 20 mM TrisCl, pH 7.2), then washed and counted along with MLNs. Whole PP homogenates were stained (approximately 2x10^6 live cells), while 5x10^6 live MLN and splenocytes were stained to assess CD4+/CD45.1+/Foxp3-GFP+ Tregs as a percentage of the total CD4+/CD45.1+ T cells by flow cytometry. For analysis of *in vivo* cell proliferation, “undivided cells” were gated based on levels of Cell Proliferation Dye eFluor® 670 in CD4+/CD45.1+ cells recovered from mice that had not been treated with OVA.
2.11 B Cell Assessment of GALT

To investigate the levels of B cells in tolerized GALT, C57BL/6 mice were treated orally *ad libitum* and by gavage with PBS or OVA ± Pam3CSK4 as described in section 2.5.1.3. (Figure 2.3,A). On day -2 of treatment the small intestine, PPs, MLN, and spleens were removed aseptically from all 3 groups of mice. PPs, MLNs, and spleens were homogenized with a scalpel or a 3mL syringe plunger and passed through 100μm filters. To isolate LP lymphocytes, the small intestine was opened longitudinally and washed in PBS then placed in 10% FBS RPMI-1640 media. Intestinal sections were then rinsed in HBSS then incubated for 3 x 15 minutes in 5 mL HBSS with 10% FBS and 2mM EDTA at 37°C to remove epithelial cells. After each 15 min incubation, tubes were shaken for 10 sec and supernatant with debris were discarded. The remaining tissues were incubated for 45 min at 37°C in 5 mL RPMI-1640 media with 10% FBS, 0.24mg/mL collagenase A (Roche), 40 U/mL DNAse I (Roche). Following incubation, tubes were shaken and supernatants were collected then diluted in 10%FBS RPMI-1640 media. Tissues were then incubated a second time, then supernatants were pooled. LP supernatants were filtered through a 100μm filter, then spun for 5 min at 500g and resuspended in 5mL of 40% Percoll (Sigma) PBS solution. Separation by density gradient was performed by spinning a 40%/70% gradient for 20 min at 1000g, 20°C without brakes. LP cells were removed from the gradient interface and washed in FACS buffer for staining. All samples were stained for CD19, CD138, CD69, IgM, and IgA. During analysis, LP plasma cells were gated using a FSC<sup>hi</sup>/SSC<sup>hi</sup> gate of total cells.

2.12 Reconstitution of Mice with Murine Bone Marrow-derived Mast Cells (BMMCs)

*Kit<sup>W-sh/W-sh</sup>* mast cell-deficient mice were reconstituted with bone marrow-derived cultured mast cells (BMMC) from C57BL/6 donor mice by i.p. and i.v. injection of 4x10<sup>6</sup> and 2x10<sup>6</sup> BMMC, respectively, and used in experiments 12 weeks later to allow mast cells to mature *in situ*. 
2.12.1 Bone Marrow-derived Mast Cell Culture

Bone marrow-derived mast cells (BMMCs) were generated from the bone marrow of male C57Bl/6 mice. Mice were euthanized by CO₂ asphyxiation and submerged in 70% ethanol. Tibias and femurs were removed aseptically, and marrow was harvested by flushing the bone shaft with sterile endotoxin-free RPMI-1640 medium from a 25G needle. The cell suspension was passed through sterile wire mesh to remove bone fragments. Cells were centrifuged at 4°C and 250g for 20 min, then resuspended at 0.5 x 10⁶ cells/mL in complete BMMC culture medium containing RPMI-1640 medium, 10% FBS, 10% WEHI 164 conditioned media (containing IL-3), 1% penicillin/streptomycin, 50 µM 2-mercaptoethanol and 200 µM PGE₂. All media was filtered at 0.22 µm prior to use. Cells were transferred to fresh medium every 3-4 days. Cells were used when they reached >95% purity as determined by low pH Alcian Blue/Safranin O dichromatic staining of cytospin preparations.

2.12.2 Evaluation of BMMC Reconstitution

The efficacy of peritoneal reconstitution was assessed by flow cytometry for CD117⁺/SSC<sup>high</sup> cells in a lavage (4mL cold PBS) of the peritoneal cavity. All relevant peritoneal cavities were successfully reconstituted, yielding an average 9.18 ± 1.54 % SEM CD117⁺ve/SSC<sup>high</sup> mast cells. Wild type C57BL/6 peritoneal cavities contained 2.62 ± 0.25 % mast cells, and Kit<sup>W-sh/W-sh</sup> mice contained 0.07 ± 0.02 % mast cells. Evaluation of reconstitution of jejunal tissues was performed by evaluation of mMCP-1 content, although previous studies have suggested very few mast cells at this site (169). Briefly, jejunal tissue was sonicated in 20mL PBS/g wet tissue. The mMCP-1 ELISA was performed on supernatants. Wild type C57BL/6 jejunum contained a mean 1215 ± 586.3 SEM ng mMCP-1/ g tissue. Kit<sup>W-sh/W-sh</sup> and reconstituted Kit<sup>W-sh/W-sh</sup> jejunum contained 86.36 ± 17.79 and 37.08 ± 5.72 ng mMCP-1/ g tissue, respectively.
2.13 Ex vivo MLN Co-culture With OT-II CD4+ T cells

Isolation of MLN cells and OVA pulse for ex vivo co-culture: To investigate the suppressive capacity of tolerized MLNs, C57BL/6 mice were treated orally ad libitum and by gavage with PBS or OVA ± Pam3CSK4 as described in section 2.5.1.3 (Figure 2.3,A). On day -2 of treatment, MLNs were harvested aseptically from all 3 groups of mice, homogenized with a scalpel, and passed through a 100μm filter. Then 1x10^6 live cells from each mouse were pulsed with 10μg/mL OVA_{323-339} peptide with or without 1μg/mL LPS for 4h at 37°C in complete medium, while 1x10^6 control cells from each mouse were treated with complete media alone or complete media with 1μg/mL LPS. After 3h, cells were washed thoroughly in sterile RPMI-1640 medium with 10%FBS and plated at 5x10^5 cells/mL in a 2mL coculture with OT-II CD4+ T cells. Complete RPMI-1640 medium contained 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, 1mM sodium pyruvate, 100 μM MEM non-essential amino acids and 50 μM 2-mercaptopethanol.

Isolation of OT-II CD4+ T cells for co-culture: Spleens were removed aseptically from OT-II+/CD45.1+/Foxp3-GFP+ mice and homogenized through a 100μm filter with a 3mL syringe plunger. Red blood cells were eliminated from spleens by treatment for 3 min with 3mL lysis buffer (0.14 M NH4Cl, 20 mM TrisCl, pH 7.2), then washed with RPMI-1640 medium with 10%FBS. Splenocytes were enriched for CD4+/CD25- cells and depleted of Tregs by MACS using the “CD4+CD25+ Regulatory T Cell Isolation Kit” according to the manufacturer’s protocol (Miltenyi). The remaining cellular fraction was assessed for purity by flow cytometry of CD4+ T cells and determined to have greater than 75% purity of CD4+/CD62L+/Foxp3-GFP+ T cells and less than 2% CD4+/Foxp3-GFP+ Tregs. Cells were treated with Cell Proliferation Dye eFluor® 670 as per the manufacturer’s protocol (eBioscience). Finally, 3x10^5 CD4+/CD62L+/OT-II+/CD45.1+/Foxp3-GFP+ T cells were plated in complete medium in a 1.3mL coculture with 1x10^6 OVA_{323-339}-pulsed or control ex vivo MLN cells at a final concentration of approximately 1x10^6 cells/mL. After 3.5 days, 20,000 polystyrene microspheres were added to wells to facilitate cell quantification, then cells and supernatants were harvested.
from wells. Flow cytometry was performed to determine the proportion of divided cells and absolute numbers of OT-II CD4\(^+\) T cells and Tregs.

2.14 Statistical Analysis

All data sets were tested for normality using the Kolmogorov-Smirnov test. OVA-specific or β-lactoglobulin-specific IgG\(_1\) and IgG\(_{2a}\) antibody titres were not normally distributed, therefore comparisons of antibody titres were made between two groups by Mann Whitney test or between multiple groups by Kruskal-Wallis non-parametric analysis followed by Dunn’s Multiple Comparison Test. IgG\(_1\) and IgG\(_{2a}\) titres were represented as median with interquartile range (IR). Where normally distributed, mean ng/mL or OD for IgE and IgA were compared between two groups by Student’s t test, or between multiple groups by one-way ANOVA followed by Dunnett’s Multiple Comparison Test. Where variances were unequal between two groups, Welch’s Correction was applied to t tests. Where not normally distributed, mean ng/mL or OD for IgE and IgA were compared between two groups by Mann Whitney test. IgE and IgA levels were displayed as mean + standard error of the mean (SEM). Peanut-specific IgG\(_1\) and IgG\(_{2a}\) were compared between two groups by Student’s t test, and displayed as mean OD + SEM. Temperature changes, FITC-dextran peritoneal influx, total IgA levels, Treg numbers, and B cell analyses were compared between two groups by Student’s t test or between multiple groups by one-way ANOVA and Bonferroni’s Multiple Comparison Test or Dunnett’s Multiple Comparison Test. Treg numbers in reconstituted Wsh mice were assessed by one-way ANOVA and Dunnett’s Multiple Comparison Test. *p<0.05, **p<0.01, ***p<0.001, n.s. denotes not significant.
Figure 2.1. Methods of ad libitum oral tolerance induction to OVA.

A, Schematic of methods for tolerance induction and antibody assessment. Mice were provided with 4mg/mL OVA in water ad libitum while control mice were provided with normal water (not depicted). On day 0, all mice were immunized by i.p. injection of OVA-alum in PBS (50μg or 10μg), then boosted by i.p. injection of OVA in PBS (10μg or 1μg). On day 21, blood was harvested from all mice and fecal samples were harvested in some studies. B, Schematic of methods for adoptive transfer and assessment of Tregs in OVA tolerance. 1x10^6 naïve CD62L^+/CD4^+ cells were purified by MACS from OT-II^+/CD45.1^+/Foxp3-GFP mice and adoptively transferred into naïve C57BL/6, Kit^W-sh/W-sh^, or TLR2^-/- recipient mice by i.v. injection. Mice were provided with 4mg/ml OVA in water ad libitum while control mice were provided with normal water (not depicted). On day 7, the PPs, MLN, and spleen were harvested and assessed for recovered OT-II^+/CD4^+/CD45.1^+ cells by flow cytometry.
Figure 2.2. Oral tolerance response to different doses of OVA in mice.

Male BALB/c mice were provided with OVA in water for 4 weeks *ad libitum* at 4mg/mL, 0.4mg/mL, or 0.04mg/mL, punctuated by untreated water on weekends. Control mice were provided with untreated water for the full time-course. On day 30, all mice were immunized i.p. with 10µg OVA-alum in PBS. On day 44, mice were boosted with 1µg OVA in PBS. Blood was harvested on day 51, and antibodies were analyzed in plasma. A, OVA-specific IgE levels were assessed by ELISA and expressed as ng/mL. B, OVA-specific plasma IgA levels were assessed by ELISA and expressed as standard-adjusted OD. C, OVA-specific plasma IgG1 levels were assessed by ELISA and analyzed by titre analysis. D, OVA-specific plasma IgG1 levels were assessed by ELISA and analyzed titre analysis. IgE and IgA levels were compared between groups by ANOVA, whereas IgG1 and IgG2a levels were compared between groups by Kruskal-Wallis test. Dunnett’s multiple comparison post-test was applied to IgE levels, comparing groups to “control sensitized” mice. Data represent one pilot experiment. *p<0.05, n.s. = not significant.
Figure 2.3. Methods of gavage oral tolerance induction to OVA.

A, Schematic of methods for tolerance induction and antibody assessment. Mice were treated for one week with OVA ad libitum and supplemented with 3 gavage treatments of OVA ± TLR activators. Control mice were provided with normal water and received gavage doses of PBS (not depicted). On day 0, all mice were immunized by i.p. injection of 10μg OVA-alum in PBS, then boosted by i.p. injection of 1μg OVA in PBS. On day 21, blood was harvested from all mice and fecal samples were harvested in some studies.

B, Schematic of methods for adoptive transfer and assessment of Tregs in OVA tolerance. Schematic of methods for adoptive transfer and assessment of Tregs in OVA tolerance. 1x10^6 naïve CD4^+ cells were purified by MACS from OT-II^+/CD45.1^+/Foxp3-GFP mice and adoptively transferred into naïve C57BL/6, Kit^W-sh/W-sh^, or TLR2^-/- recipient mice by i.v. injection. Mice were treated with OVA ad libitum and supplemented with 3 gavage treatments of OVA OVA ± TLR activators, while control mice received 3 PBS gavage treatments and normal water (not depicted). On day 7, the PPs, MLN, and spleen were harvested and assessed for recovered OT-II^+/CD4^+/CD45.1^+ cells by flow cytometry.
Male BALB/c mice were provided with milk in water for 1 or 2 weeks *ad libitum* at 36 mg/mL, 10 mg/mL, or 1 mg/mL milk protein. Control mice were provided with untreated water. On day 0, after 2 days normal water for all groups, mice were immunized i.p. with 50µg β-lactoglobulin-alum in PBS. On day 14, mice were boosted with 1µg β-lactoglobulin in PBS. Blood was harvested on day 21, and antibodies were analyzed in plasma. **A**, β-lactoglobulin-specific IgE levels were assessed by ELISA and expressed as standard-adjusted OD. **B**, β-lactoglobulin-specific plasma IgA levels were assessed by ELISA and expressed as standard-adjusted OD. **C**, β-lactoglobulin-specific plasma IgG1 levels were assessed by ELISA and analyzed by titre analysis. **D**, β-lactoglobulin-specific plasma IgG2a levels were assessed by ELISA and analyzed titre analysis. IgE and IgA levels were compared between groups by ANOVA, whereas IgG1 and IgG2a levels were compared between groups by Kruskal-Wallis test. Dunnett’s multiple comparison post-test was applied to IgE and IgA levels, comparing groups to “control sensitized” mice. Data represent one pilot experiment. *p < 0.05, **p < 0.01, n.s., not significant.
Figure 2.4
Figure 2.5 Methods of *ad libitum* and gavage oral tolerance induction to cow’s milk.

Schematic of methods for *ad libitum* tolerance induction and antibody assessment. Mice were provided with 10mg/ml cow’s milk protein in water *ad libitum* ± bacterial contamination while control mice were provided with normal water (not depicted). On day 0, all mice were immunized by i.p. injection of 50μg β-lactoglobulin-alum in PBS, then boosted by i.p. injection of 1μg β-lactoglobulin in PBS. On day 21, blood was harvested from all mice and fecal samples were harvested in some studies.
Table 2.1  Antigen consumption by mouse strain.

<table>
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<tr>
<th>Mouse Strain</th>
<th>OVA protein (mg/day)</th>
<th>Milk β-lactoglobulin protein (mg/day)</th>
<th>Peanut butter protein (mg /day)</th>
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<tr>
<td>C57BL/6</td>
<td>19.29 ± 1.18 (n=8)</td>
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<tr>
<td>KitW-sh/W-sh</td>
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<td>467 ± 26.9 (n=12)</td>
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<tr>
<td>BALB/c</td>
<td>13.75 ± 0.79 (n=8)</td>
<td>5.16 ± 0.206 (n=28)</td>
<td>448 ± 24.6 (n=10)</td>
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</tbody>
</table>

Antigen consumption data are presented by mouse strain where available. n = number of measurements from two or more cages over several days. Peanut protein calculations are based on 15g peanut butter/4g protein. Milk protein calculations are based on 25g skim milk/9g protein, then adjusted to 10% β-lactoglobulin (253).
CHAPTER 3  MAST CELLS AND IGE ACTIVATION DO NOT ALTER THE DEVELOPMENT OF ORAL TOLERANCE IN A MURINE MODEL

Chapter 3 contains material that were published online with the Journal of Allergy and Clinical Immunology, May 17, 2012. The list of contributing authors for this work includes: Matthew C. Tunis, Wojciech Dawicki, Kaitlyn R. Carson, Jun Wang and Jean S. Marshall.

3.1 Introduction

Oral immunotherapy is increasingly available as a treatment option for food allergy, with clinical trials yielding encouraging results (105, 256). Although the complex immune mechanisms of oral immunotherapy are not completely understood, they are thought to involve a variety of cell types from both the innate and adaptive arms of the immune system as well as structural cells such as the intestinal epithelium (11, 257). The functions of many immune cells can be modulated by mast cells (258-261). Since allergic disease involves mast cell activation and mast cell mediator blockade is frequently used in the context of oral immunotherapy, it is essential to understand what role mast cells play in the development of oral tolerance.

Mast cells are positioned close to the external environment and serve as effector cells in allergic disease and responses to pathogens (165). There is a growing body of work examining the interaction between mast cells, DCs, and T cells, and how these relationships might alter immunity to foreign antigens (258, 259, 261-263). Despite their strategic location in the oral mucosa and intestinal microenvironment, the role of mast cells in oral tolerance has not been directly investigated. Mast cells can activate T cells, with a bias towards expanding populations of regulatory T cells (Treg) in vitro (264). Moreover, mast cell-deficient Kit\(^{W_{sh}/W_{sh}}\) mice have shown deficient Treg activity in an experimental autoimmune encephalomyelitis model of multiple sclerosis (189). Similarly, it was recently shown in a skin transplant model that mast cells are critical in extending the maintenance of peripheral tolerance and allograft survival, most likely by
recruitment of Tregs and the action of mast cell proteases (180, 265). It has also been
demonstrated that mast cell-derived interleukin-10 can limit inflammatory infiltrate to
tissue sites, in addition to inhibiting germinal centre formation (266-268). However, the
presence of other immune cell defects in both the Kit\(^{W-sh/W-sh}\) and Kit\(^{W/W-v}\) models of mast
cell deficiency make interpretations of some of these studies problematic. This issue has
recently been highlighted by studies of alternate mast cell deficiency mouse models that
appear to have normal immune and inflammatory responses in models of multiple
sclerosis and arthritis (188, 192, 193). Given this previous body of work, Investigations
into whether mast cells regulate oral tolerance are urgently required.

IgE-mediated mast cell degranulation has been reported to break established
peripheral tolerance in a mouse tissue allograft model (182) and degranulation can
enhance antibody responses to both bacterial and viral antigens in the mast cell
microenvironment (269, 270). Furthermore, mast cell degranulation products interfere
with the suppressive activity of natural Tregs (271). Mast cells are a major tissue source
of histamine, which has multiple immunoregulatory effects (163). Histamine can
polarize the CD4\(^+\) T cell response towards a T\(_{H-2}\) IL-4 phenotype via DCs (184, 272) and
has been shown to modulate the recruitment of both the CD8\(^+\) subset of DCs and
plasmacytoid dendritic cells (pDCs) to the draining lymph node (258). Notably, the pDC
subset in the liver and other gut associated lymphoid tissues have been implicated in the
induction of immunologic tolerance by an oral route (64, 65, 273). Despite the known
effects of histamine on DCs, oral antihistamine treatment has not been directly
investigated in a model of oral tolerance. The mobilization and directed trafficking of
pDCs in response to mast cells suggests another avenue by which mast cells may
modulate oral tolerance (271).

Taken together, current evidence suggests that mast cells and their activation
products have the potential to enhance immunity to food antigens and compromise oral
tolerance. In the present study, we sought to identify the immunological outcomes of
mast cell deficiency, IgE-mediated mast cell activation, and anti-histamine treatment on
oral tolerance induction and antibody production. The models of oral tolerance induction
and mast cell activation employed here avoid the potentially confounding variables of
mast cell mediated changes in intestinal permeability and antigen access. Oral tolerance was characterized using several parameters, including antigen-specific serum IgE which is an important tool in determining food allergy potential in clinical patients (4).

To our knowledge, this report is the first examination of oral tolerance in mast cell-deficient mice and assessment of the impact of mast cell degranulation on oral tolerance induction.

### 3.2 Results

#### 3.2.1 Mast cell-deficient mice have normal IgE, IgG1, and IgG2a antibody responses after OVA-alum immunization

In order to assess whether the presence or absence of mast cells altered the normal antibody response to an i.p. immunization, mast cell containing (wild type C57BL/6) or mast cell-deficient (Kit\(^{W-sh/W-sh}\)) mice were sensitized to OVA (Figure 3.1). There were no significant differences observed between the OVA-specific IgE (p=0.16), IgG1 (p=0.0503), or IgG2a antibody responses of C57BL/6 and Kit\(^{W-sh/W-sh}\) mice (p=0.07) (Table 3.1).

#### 3.2.2 Mast cells are not required for oral tolerance induction

Wild type C57BL/6 and Kit\(^{W-sh/W-sh}\) mice were tolerized orally to OVA (Figure 3.1) or provided with normal drinking water. Following later i.p. sensitization and boost, OVA-specific IgE was significantly reduced in tolerized Kit\(^{W-sh/W-sh}\) mice (p<0.001) and in wild type animals (p<0.001) (Figure 3.2) compared with controls. Tolerized Kit\(^{W-sh/W-sh}\) mice were also found to have significantly lower OVA-specific IgG1 and IgG2a levels than Kit\(^{W-sh/W-sh}\) controls (p<0.01, p<0.01). Plasma OVA-specific IgA levels were significantly reduced following tolerance induction to OVA in both C57BL/6 wild type and Kit\(^{W-sh/W-sh}\) mice (p<0.05, p<0.05) (Figure 3.3,A). Secreted fecal OVA-specific IgA levels were also significantly reduced in tolerized Kit\(^{W-sh/W-sh}\) mice (p<0.05) (Figure 3.3,B). Oral tolerance was therefore readily induced in mast cell-deficient mice.
Using antibody responses as a readout, the degree of tolerance was compared between wild type and mast cell-deficient mice. OVA-specific IgE levels following sensitization were significantly lower in tolerized mast cell-deficient $Kit^{W-sh/W-sh}$ mice than in tolerized wild type mice (p<0.05) (Table 3.2,A). In contrast, OVA-specific IgG1, IgG2a, or IgA levels were not significantly different between tolerized mast cell containing and mast cell-deficient mice (p=0.24, p=0.55, p=0.92, respectively) (Table 3.2,A). While $Kit^{W-sh/W-sh}$ mice are understood to have few abnormalities (169), some have been described (274). In order to further evaluate the role of mast cells in oral tolerance, we therefore repeated these studies with a second strain of mast cell-deficient mice: $Kit^{W/W-v}$ and their wild type WBB6F1 controls (275). Oral tolerance was induced in both the WBB6F1 controls and the $Kit^{W/W-v}$ mice, as indicated by suppression of the IgE antibody response to OVA immunization and boost (p<0.001, p<0.01, respectively) (Figure 3.2,B). Notably, IgE levels in tolerized $Kit^{W/W-v}$ mice were elevated but not statistically different from tolerized WBB6F1 control mice (p=0.06) (Table 3.2,B). OVA-specific IgG2a was also significantly suppressed in $Kit^{W/W-v}$ mice following OVA feeding (p<0.01), but IgG1 responses were not changed in mast cell containing or deficient animals (p=0.28, p=0.46, respectively) (Figure 3.2,B). OVA-specific IgA plasma responses were significantly reduced in both $Kit^{W/W-v}$ and WBB6F1 controls when tolerized (p<0.001, p<0.01 respectively) (Figure 3.3,C).

Oral tolerance to peanut was assessed as a model of a complex, relevant food allergen. C57BL/6 and $Kit^{W-sh/W-sh}$ mice were fed peanut butter ad libitum for 1 week, while control mice from both strains were fed regular chow. Mice were immunized and boosted with CPE-Alum and CPE, respectively (Figure 3.1). Peanut-specific IgE levels were significantly reduced in both peanut butter-fed mouse groups (p<0.001, p<0.05 respectively) (Figure 3.2,C). Peanut-specific IgG1 and IgG2a were significantly reduced in C57BL/6 mice tolerized with peanut butter compared to untreated controls (p<0.01, p<0.05 respectively), but significant suppression of the IgG2a antibody response was not observed in $Kit^{W-sh/W-sh}$ mice (p=0.11) (Figure 3.2,C). IgG1 responses to peanut were too low to compare in $Kit^{W-sh/W-sh}$ mice. Peanut-specific IgA responses were significantly reduced in peanut butter fed compared to control $Kit^{W-sh/W-sh}$ mice (p<0.05) (Figure 3.3,D).
Taken together, these results demonstrate that oral tolerance can be reliably induced in the absence of mast cells, as confirmed in two different mast cell-deficient strains of mice and in two different allergenic food models. Oral tolerance induction to OVA was enhanced in one strain of mast cell-deficient mice ($Kit^{W-sh/W-sh}$), but not in another ($Kit^{W/W-v}$).

3.2.3 Physiologically relevant oral tolerance is induced by OVA or peanut butter feeding

In mice IgG antibodies can be a major contributor to anaphylaxis (148, 276). To assess the protective role of oral tolerance in our model, we employed the FcγRIII-deficient mice to isolate the IgE-dependent anaphylactic response to OVA and better model anaphylaxis in human disease. Following oral tolerance induction to OVA (Figure 3.1), mice were challenged i.p. with soluble OVA. The OVA-tolerized FcγRIII−/− mice were protected from temperature loss 30 and 50 min post-challenge compared to the FcγRIII−/− sensitized mice (p<0.01 and p<0.01, respectively), indicating a physiologically relevant degree of tolerance induction using this protocol (Figure 3.4,A). Tolerized mice from both the FcγRIII−/− and C57BL/6 strains had similar OVA-specific IgE levels (C57BL/6 4.8 ± 1.1 ng/ml, FcγRIII−/− 3.5 ± 1.0 ng/ml), as did sensitized mice (C57BL/6 20.1 ± 3.4 ng/ml, FcγRIII−/− 19.1 ± 3.9 ng/ml), n=15-20. To assess protective tolerance to peanut, wild type C57BL/6 mice were challenged i.p. with soluble CPE following tolerance induction to peanut butter (Figure 1) and anaphylaxis responses were compared to control sensitized C57BL/6 mice. The peanut-tolerized mice were protected from temperature loss 10, 30, and 50 min post-challenge (p<0.05, p<0.001, p<0.001, respectively), indicating physiologically relevant tolerance to peanut butter (Figure 3.4,B). To confirm the involvement of mast cells in peanut-induced anaphylaxis, mMCP-1 was measured in plasma 60 min after peanut challenge. Peanut butter tolerized mice had significantly lower levels of circulating mMCP-1 following peanut challenge (1.53 ± 1.17 ng/ml SEM) than control peanut-sensitized mice (9.17 ± 2.22 ng/ml SEM) (p<0.001, n=9-10).
3.2.4 Antigen-specific Treg cells are enhanced in the Kit\textsuperscript{W-sh/W-sh} MLN after oral tolerance induction to OVA through a mast cell-independent mechanism

To further investigate the mechanisms by which oral tolerance to OVA was more efficiently induced in Kit\textsuperscript{W-sh/W-sh} mice, the development of antigen-specific Tregs was assessed in the PPs, MLN, and spleen. Mice received 1x10\textsuperscript{6} naïve OT-II cells by i.v. adoptive transfer and were provided with OVA-containing or control (OVA-free) drinking water for 7 days. Lymph nodes and spleen were harvested and analysed for OT-II Tregs by flow cytometry on the 7\textsuperscript{th} day of treatment through analysis of both the overall numbers of OT-II cells and the number of GFP positive Foxp3 expressing OT-II cells. Both C57BL/6 and Kit\textsuperscript{W-sh/W-sh} mice revealed significantly higher levels of OT-II Tregs as percent of total OT-II CD4\textsuperscript{+} cells in the MLN following oral OVA treatment (p<0.05, p<0.001, respectively) (Figure 3.5,A,B). The MLN of mast cell-deficient Kit\textsuperscript{W-sh/W-sh} mice contained approximately twice as many OT-II Tregs as a proportion of the total CD4\textsuperscript{+} OT-II cells when compared to control tolerized mice (p<0.001) (Figure 3.5,B). However, when a similar experiment was performed using Kit\textsuperscript{W-sh/W-sh} mice systemically reconstituted with BMMCs, the OT-II Treg response to oral tolerance induction did not return to wild type C57BL/6 levels (Figure 3.5,C). This indicates that the enhanced tolerance response by Tregs was not mast cell-dependent. Importantly, both Kit\textsuperscript{W-sh/W-sh} and C57BL/6 mice showed increased OT-II Tregs in the spleen following oral OVA treatment – demonstrating the induction of oral tolerance in both strains at the systemic level (Figure 3.5,B).

Interestingly, tolerized Kit\textsuperscript{W-sh/W-sh} mice contained on average 26\% more total CD4\textsuperscript{+} T cells in the PPs than tolerized C57BL/6 controls (p<0.05) (Figure 3.5,C). This difference in CD4\textsuperscript{+} levels was not observed in either the MLN or spleen, and was not specific to OT-II T cells (data not shown). When Kit\textsuperscript{W-sh/W-sh} mice were systemically reconstituted with BMMCs, the CD4\textsuperscript{+} compartment of the PPs returned to wild type levels (Figure 3.5,C). This highlights a previously undescribed mast cell-dependent suppression of CD4\textsuperscript{+} T cell populations in the PPs. OVA treatment did not result in selected enhancement of OT-II Tregs (Figure 3.5,D) in wild type mice at this site. Instead, the PPs in tolerized mice revealed higher total OT-II CD4\textsuperscript{+} T cell populations with Foxp3\textsuperscript{+} expression (C57BL/6 p<0.05, Kit\textsuperscript{W-sh/W-sh} p<0.001) or Foxp3\textsuperscript{-} expression
This supports a framework of tolerance induction wherein the PPs serve as an initial site of antigen recognition and T cell activation, while the MLN is a site of robust antigen-specific Treg expansion and immunologic tolerance.

3.2.5 IgE-mediated activation of mast cells does not impede the maintenance of oral tolerance to OVA

IgE-mediated mast cell activation by food antigen in a mouse model of food allergy induced the production of antibody-promoting cytokines IL-4, IL-13, IL-6, and pro-inflammatory IL-17 (277). Furthermore, it has been shown that IgE-mediated mast cell activation can impair the maintenance of peripheral tolerance in a mouse allograft model (182). We sought to examine if a similar tolerance disruption would be observed in the case of established oral tolerance to OVA using mice passively sensitized with anti-TNP IgE antibody. In order to confirm effective IgE-mediated mast cell activation in the peritoneum, FITC-dextran influx into the peritoneum was assessed 15-20 min following activation in two separate groups of mice. IgE-mediated activation resulted in an average 31% increase of FITC-dextran influx into the peritoneum compared to unactivated controls (p<0.05, n=9-10, data not shown).

IgE-mediated mast cell activation was also confirmed by ELISA for the mast cell-specific protease mMCP-1 in blood 60 min following activation. Sensitized mice that were challenged i.p. with TNP-BSA showed average systemic mMCP-1 levels of 364.6 ± 91.09 pg/ml, significantly elevated compared to sensitized mice injected with a BSA control (147.5 ± 26.31 pg/ml SEM) (p<0.05, n=9-10). This level of systemic mast cell activation did not induce systemic anaphylaxis, assessed by rectal temperature 50 minutes after TNP-BSA delivery. To examine the outcome of IgE-mediated mast cell activation on the maintenance of oral tolerance, wild type C57BL/6 mice were sensitized i.p. with anti-TNP IgE. After one week of OVA tolerance induction treatment, TNP-BSA was delivered i.p. in order to activate mast cells concurrent with OVA-alum immunization in one group of tolerized mice. A control group of tolerized mice received BSA instead of TNP-BSA. Tolerized groups were compared to sensitized control mice injected i.p. with
BSA during the OVA-alum immunization. The OVA-specific IgE levels of both OVA
tolerized groups were significantly lower than the levels of control OVA sensitized mice
regardless of IgE-mediated activation. Therefore, the maintenance of oral tolerance was
not impaired in the IgE compartment despite mast cell activation (p<0.01) (Figure 3.6,A).
Similarly, suppression of the OVA-specific IgA response was sustained in both tolerized
groups (p<0.01, p<0.05) (Table 3.3,A). Tolerance was not induced in the IgG compartment for wild type C57BL/6 mice (p=0.07) (Table 3.3,A). Interestingly, OVA-
specific IgG2a levels were significantly lower in tolerized mice than in OVA-sensitized
mice (p<0.05), but not when IgE-mediated activation was applied to the tolerized mice
(Table 3.3,A). Levels of OVA-specific IgG2a remained significantly lower in tolerized
mast cell-deficient Kitw-sh/w-sh mice when compared to sensitized Kitw-sh/w-sh controls in
the presence of TNP-BSA activation of IgE (p<0.01) (Table 3.3,B).

3.2.6 IgE-mediated activation of mast cells does not alter antibody responses
during oral tolerance induction to OVA

The impact of allergic activation of mast cells on oral tolerance induction to OVA was
also assessed. Wild type C57BL/6 mice were passively sensitized i.p. with anti-TNP IgE.
In one group of such mice, TNP-BSA was delivered i.p. twice during one week of OVA
feeding, while a control group of animals received BSA i.p. in place of TNP-BSA. Mice
were immunized and boosted as described in Figure 3.1. Both tolerized groups produced
mean OVA-specific IgE levels more than 60% lower than the untreated sensitized control
mice, although differences were not significant when compared by ANOVA (p=0.056)
(Figure 3.6,B). OVA-specific IgA, IgG1, and IgG2a levels were not significantly different
between control and tolerized mice when analysed by ANOVA (IgA p=0.08) or Kruskall-
Wallis analysis (IgG1 p=0.13, IgG2a p=0.41) (Table 3.3,C).

3.2.7 Oral antihistamine treatment has no effect on oral tolerance induction

Histamine is an important product of mast cell degranulation and can contribute to
adverse anaphylactic reactions to oral immunotherapy. We investigated whether
histamine, acting via HRH1 or HRH2, might alter oral tolerance induction. Groups of wild type C57BL/6 mice were orally pre-treated with the HRH1 antagonist pyrilamine or the HRH2 antagonist ranitidine in drinking water for one day prior to and throughout a one week period of tolerance induction to OVA. Neither antihistamine treatment resulted in significant changes to OVA-specific antibody levels in tolerized wild type mice when compared to untreated tolerized mice for IgE, IgA, IgG1, and IgG2a levels (Table 3.2,C).

### 3.3 Discussion

The findings of this study demonstrate that mast cells are not required for the induction of oral tolerance in two strains of mice and in two model antigen systems. To our knowledge, this is the first study to directly and experimentally investigate the role of mast cells in oral tolerance induction. We also observed that there were no substantial effects of oral antihistamine treatment or IgE-mediated mast cell activation on oral tolerance development, as evaluated by the plasma IgE responses to OVA. In addition to these major findings, our studies revealed evidence for mast cell-mediated modulation of the IgG subclass response in the context of oral tolerance to OVA. We also observed enhanced Treg generation and oral tolerance responses in a commonly used model of mast cell-deficient mice.

In contrast to these findings, experiments using skin graft models have demonstrated that mast cells are essential for peripheral tolerance to an allograft (180, 265). These studies investigated the maintenance of established graft tolerance, but did not address the role of mast cells in the induction phase of tolerance. Differences in mast cell protease expression could provide a further reason for the divergent findings of these models. Tolerance in graft models was found to be dependent on mouse mast cell protease 6 (mMCP6) (180), but mMCP6 is not produced by mucosal mast cells which are the most likely mast cell subset to be involved in regulating oral tolerance (278). Conversely, studies by Da Silva et al. demonstrate a correlation between elevated mast cell numbers at mucosal sites and resistance to oral tolerance in murine models (279, 280). Our results indicate that this correlation is not likely to be a critical factor regulating tolerance induction.
There are a number of murine models available to assess the role of mast cells in disease processes. We employed two of the best characterized and most widely studied models: Kit\(^{W/-}\) and Kit\(^{W/-}sh/W-sh\) (169, 275, 281, 282). While these mice come from distinct genetic backgrounds, the lack of mast cells did not impede oral tolerance development in either case. To the contrary, upon feeding OVA, Kit\(^{W/-}sh/W-sh\) mice exhibited enhanced tolerance in the IgE class and a higher proportion of OVA-specific Tregs in the MLN compared with wild type controls. These changes in MLN Tregs were not eliminated by systemic reconstitution of Kit\(^{W/-}sh/W-sh\) mice with wild type mast cells. Furthermore, Kit\(^{W/-}\) mast cell-deficient mice experienced successful tolerance but did not show enhanced humoral tolerance compared with matched mast cell-containing animals. These findings suggest that a mast cell independent feature of the Kit\(^{W/-}sh/W-sh\) mice enhances the induction, expansion, or migration of Tregs in the MLN, and the development of oral tolerance. It has been observed that Kit\(^{W/-}sh/W-sh\) mice have altered crypt architecture in the jejunum, resulting in reduced intestinal permeability and antigen transit compared to C57BL/6 controls (168). However, unlike the enhanced tolerance induction in Kit\(^{W/-}sh/W-sh\) mice, these changes were eliminated by mast cell reconstitution, so they are not likely responsible for the observed differences in oral tolerance. It is possible that the observed differences in oral tolerance to OVA between Kit\(^{W/-}sh/W-sh\) and C57BL/6 mice are a product of differences in intestinal motility or antigen access resulting from deficiency in the interstitial cells of Cajal (169). We observed that 5-8 week old Kit\(^{W/-}sh/W-sh\) mice consumed on average 26% more water than C57BL/6 controls (n=8 measurements from 2 cages per strain) (p<0.01, Table 2.1). However, further experiments showed that tolerance induced with 2 mg/ml, 4 mg/ml, or 8 mg/ml OVA provided ad libitum to C57BL/6 mice resulted in similar OT-II Treg levels in the MLN (6.60 ± 0.39, 8.45 ± 1.64, and 10.94 ± 0.78 percent of OT-II CD4\(^+\), respectively; n = 5, from two pooled experiments). This suggests that the dramatic differences between Kit\(^{W/-}sh/W-sh\) and C57BL/6 mice were not due to differences in antigen dosage. Excellent intestinal and immunologic comparisons were made between the Kit\(^{W/-}sh/W-sh\), Kit\(^{W/-}\), and wild type control mice early in the development of the Kit\(^{W/-}sh/W-sh\) model of mast cell deficiency (169). However, a more comprehensive comparison of the distribution and phenotype of DC and T cell subsets between these strains could yield valuable...
information on possible mechanisms of tolerance enhancement. Specifically, inter-strain comparisons of the Peyer’s patches, mesenteric lymph nodes, and lamina propria may identify important differences that could present therapeutic targets for enhancing oral tolerance in affected patients. Notably, since the initial description of these mast cell-deficient mice a number of cell subsets have been described with c-kit expression that might have immunoregulatory roles. These include several subsets of recently characterized innate lymphoid cells (283), the multipotent progenitor type 2 cells (284) and the B220$^{low}$ CD19$^{(-)}$ regulatory cell population identified in the nasal associated lymphoid tissue (285). These require further examination in the context of c-kit related mast cell deficiency models.

In our studies, oral tolerance to OVA was assessed not only by OVA-specific antibody levels and OVA-specific Tregs, but also by anaphylaxis upon OVA challenge in FcγRIII$^{-/-}$ mice. It has been well documented that basophils and/or macrophages can bind IgG antibodies and cause IgG-mediated anaphylaxis in wild type mice (146, 148). We therefore employed FcγRIII$^{-/-}$ mice in order to eliminate the contribution of IgG-mediated anaphylaxis in tolerized mice that is not known to occur in humans, allowing us to assess the physiological relevance of OVA-specific IgE levels in tolerized C57BL/6 and Kit$^{W-\text{sh}/W-\text{sh}}$ mice with comparable IgE levels. IgG-mediated anaphylaxis could occur in tolerized C57BL/6 or Kit$^{W-\text{sh}/W-\text{sh}}$ mice (147), but this would not reflect the significance of IgE levels and may have limited relevance to human allergic disease and the important role of mast cells in human anaphylaxis (13).

Individuals with food allergy often respond to multiple allergens and may have other allergic disorders. It is therefore important to understand to what extent allergic mast cell activation can modify oral tolerance development. Studies performed in a skin graft rejection model have demonstrated that mast cell degranulation is sufficient to break peripheral tolerance and accelerate graft rejection (182). Systemic IgE-mediated activation during a peritoneal allergen challenge did not break established oral tolerance to OVA in the IgE class (Figure 3.6,A). This demonstrates that oral tolerance can be resilient to the immunomodulatory events that accompany IgE-mediated activation. However, it remains possible that in a model of oral tolerance to a potent complex
allergen, such as peanut, there is the potential for IgE activation to weaken tolerance. Peanut is known to have additional immunogenic and inflammatory properties, such as spontaneous complement activation, that could amplify the specific immunologic effects of IgE-mediated activation during a peanut challenge (8, 286). Additionally, in a food allergy setting, local mast cell degranulation could increase intestinal permeability; this physiological change might interfere more directly with tolerance to food antigens (287). Although OVA-specific IgE responses were not altered in our model of IgE-mediated activation, the OVA-specific IgG2a levels were enhanced by IgE-mediated activation during immunization (Table 3.2,A). The apparent variability in these IgG2a responses may reflect the variation observed in the extent of mast cell activation evaluated by mMCP-1 levels (364.6 ± 91.09 pg/ml) in IgE/antigen activated mice. This change towards OVA-specific IgG2a suggests a Th1 polarized response and is therefore unlikely to result in allergic symptoms, but it does identify that there are significant immunomodulatory outcomes of IgE-mediated mast cell activation on the antigen response within the context of oral tolerance.

Oral antihistamines have been used clinically to control adverse reactions during rush or long-term oral immunotherapy protocols (288, 289) and have been administered extensively in other forms of allergen immunotherapy (290). Despite their frequent use, it is unknown how some antihistamines may influence tolerance induction to a new antigen or impact on oral immunotherapy effectiveness. The administration of levocetirizine to patients undergoing honeybee venom immunotherapy did not interfere with tolerance induction and resulted in significantly more IL-10 production from allergen-specific T cells (291). Similarly, a double-blind placebo controlled study demonstrated that patients receiving pretreatment with the HRH1 antagonist terfenadine during bee venom immunotherapy were significantly less likely to react to a sting challenge several years later (292). Taken together, these studies suggest that antihistamine treatment does not interfere with tolerance induction and may enhance immunotherapy to a preexisting allergic condition. This supports our finding that treatment with relatively high doses of pyrilamine (HRH1 antagonist) or ranitidine (HRH2 antagonist) did not interfere with the development of oral tolerance induction to a
food antigen. However, our model did not demonstrate enhanced tolerance upon antihistamine treatment.

Overall, our data demonstrate that mast cells do not alter the development of oral tolerance to a food antigen, as assessed by humoral IgE responses. Similarly, if mast cells are activated by IgE during allergen challenge, the maintenance of oral tolerance to the simple egg allergen OVA is unchanged in the IgE response. There is clear evidence from these and many previous studies that mast cells can play a profound immunomodulatory role in other types of tolerance and in other immune compartments, such as the IgG2a response to food antigens. These data identify complexity in the oral tolerance response to activated mast cells, but underscore the potential safety and usefulness of antihistamine treatment during oral tolerance approaches in clinical settings.
Table 3.1 IgE, IgG₁, or IgG₂a antibody responses to peritoneal immunization are unchanged in mast cell deficient mice.

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<thead>
<tr>
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<th>IgE</th>
<th>IgG₁</th>
<th>IgG₂a</th>
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<tbody>
<tr>
<td>C57BL/6 control</td>
<td>20.14 ± 3.41</td>
<td>4.81 (0.01 - 5.71)</td>
<td>3.28 (0.01 - 4.39)</td>
</tr>
<tr>
<td>Kit&lt;sup&gt;W-sh/W-sh&lt;/sup&gt; control</td>
<td>28.74 ± 4.11</td>
<td>5.53 (3.70 - 5.71)</td>
<td>3.77 (3.39 - 4.83)</td>
</tr>
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</table>

Antibody levels of OVA immunized and boosted control mice are expressed as mean ng/mL ± SEM for IgE, or median –Log titre with range (IgG₁, IgG₂a). C57BL/6 antibody responses were compared to Kit<sup>W-sh/W-sh</sup> by t test or Mann-Whitney: no significant differences were observed (n = 8-20).
Table 3.2 Comparison of oral tolerance induction between mouse strains and effect of oral antihistamine treatment.

OVA-specific antibody levels in plasma one week after boost

<table>
<thead>
<tr>
<th></th>
<th>IgE</th>
<th>IgA</th>
<th>IgG1</th>
<th>IgG2a</th>
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<tr>
<td><strong>A) Comparison between tolerized C57BL/6 and Kit&lt;sup&gt;W-sh/W-sh&lt;/sup&gt; (n = 8-20)</strong></td>
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<tr>
<td>C57BL/6 (tol)</td>
<td>4.81 ± 1.12</td>
<td>0.053 ± 0.014</td>
<td>4.77 (0.01 – 5.31)</td>
<td>2.15 (0.01 – 4.61)</td>
</tr>
<tr>
<td>Kit&lt;sup&gt;W-sh/W-sh&lt;/sup&gt; (tol)</td>
<td>1.47 ± 0.56*</td>
<td>0.105 ± 0.081</td>
<td>4.35 (2.65 – 5.30)</td>
<td>2.54 (0.01 – 4.01)</td>
</tr>
<tr>
<td><strong>B) Comparison between tolerized WBB6F1 and Kit&lt;sup&gt;B/W-v&lt;/sup&gt; (n = 7-8)</strong></td>
<td></td>
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<tr>
<td>WBB6 (tol)</td>
<td>13.79 ± 4.26</td>
<td>0.093 ± 0.057</td>
<td>4.965 (3.95 – 5.52)</td>
<td>0.01 (0.01 – 4.21)</td>
</tr>
<tr>
<td>Kit&lt;sup&gt;B/W-v&lt;/sup&gt; (tol)</td>
<td>24.07 ± 2.68</td>
<td>0.095 ± 0.027</td>
<td>5.612 (0.01 – 5.92)</td>
<td>2.00 (0.01 – 4.01)</td>
</tr>
<tr>
<td><strong>C) Comparison between tolerized and antihistamine-treated tolerized groups (n = 10-20)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6 (tol)</td>
<td>4.81 ± 1.12</td>
<td>0.053 ± 0.014</td>
<td>4.77 (0.01 – 5.31)</td>
<td>2.15 (0.01 – 4.61)</td>
</tr>
<tr>
<td>C57BL/6 (tol) pyrilamine treated</td>
<td>3.87 ± 1.12</td>
<td>0.057 ± 0.012</td>
<td>4.30 (4.01 – 5.46)</td>
<td>1.79 (0.01 – 3.27)</td>
</tr>
<tr>
<td>C57BL/6 (tol) ranitidine treated</td>
<td>2.95 ± 0.74</td>
<td>0.066 ± 0.026</td>
<td>4.33 (0.01 – 5.46)</td>
<td>1.91 (0.01 – 3.83)</td>
</tr>
</tbody>
</table>

Antibody levels are expressed as mean ng/mL ± SEM (IgE), mean OD ± SEM (IgA), or median –Log titre with range (IgG<sub>1</sub>, IgG<sub>2a</sub>). Groups were compared by t test or Mann Whitney test in A) and B). Antihistamine treated groups were compared to untreated controls by ANOVA with Dunnett’s post-test (IgE, IgA), or Kruskal-Wallis test with Dunn’s post test (IgG<sub>1</sub>, IgG<sub>2a</sub>). Grey fields indicate ≥40% antibody reduction compared to corresponding control group. *p<0.05.
Table 3.3 Passive IgE-mediated systemic activation in oral tolerance induction and maintenance.

Anti-OVA antibody levels in plasma one week after boost

<table>
<thead>
<tr>
<th></th>
<th>IgA</th>
<th>IgG&lt;sub&gt;1&lt;/sub&gt;</th>
<th>IgG&lt;sub&gt;2a&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Tolerance maintenance wild type C57BL/6 (n = 18-19)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control + IgE/control BSA during OVA-alum immunization</td>
<td>0.354 ± 0.063</td>
<td>5.08 (4.64 - 5.34)</td>
<td>3.58 (1.04 - 4.42)</td>
</tr>
<tr>
<td>Tol + IgE/control BSA during OVA-alum immunization</td>
<td>0.112 ± 0.015 **</td>
<td>4.65 (4.17 - 4.88)</td>
<td>0.56 * (0.01 - 3.45)</td>
</tr>
<tr>
<td>Tol + IgE/TNP-BSA activation during OVA-alum immunization</td>
<td>0.186 ± 0.044*</td>
<td>4.83 (4.33 - 5.11)</td>
<td>1.78 (0.01 - 3.63)</td>
</tr>
<tr>
<td><strong>B) Tolerance maintenance Kit&lt;sup&gt;W-/-&lt;/sup&gt;/W-/- (n = 9-14)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control + IgE/control BSA during OVA-alum immunization</td>
<td>0.387 ± 0.102</td>
<td>5.11 (3.62 - 5.47)</td>
<td>3.49 (1.53 - 4.34)</td>
</tr>
<tr>
<td>Tol + IgE/control BSA during OVA-alum immunization</td>
<td>0.082 ± 0.036 **</td>
<td>3.66* (0.01 - 4.63)</td>
<td>0.01 ** (0.01 - 2.94)</td>
</tr>
<tr>
<td>Tol + IgE/TNP-BSA activation during OVA-alum immunization</td>
<td>0.072 ± 0.019 *</td>
<td>3.34 (0.01 - 4.88)</td>
<td>0.01 ** (0.01 - 1.45)</td>
</tr>
<tr>
<td><strong>C) Tolerance induction wild type C57BL/6 (n = 10)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control + IgE/control BSA during OVA feeding</td>
<td>0.122 ± 0.031</td>
<td>4.66 (2.78 - 4.96)</td>
<td>1.93 (0.67 - 2.92)</td>
</tr>
<tr>
<td>Tol + IgE/control BSA during OVA feeding</td>
<td>0.020 ± 0.004</td>
<td>4.76 (4.63 - 4.96)</td>
<td>1.47 (0.01 - 2.50)</td>
</tr>
<tr>
<td>Tol + IgE/TNP-BSA activation during OVA feeding</td>
<td>0.084 ± 0.043</td>
<td>4.57 (4.46 - 4.69)</td>
<td>0.95 (0.01 - 2.40)</td>
</tr>
</tbody>
</table>

Antibody levels are expressed as mean OD mean ± SEM (IgE), or median –Log titre with range (IgG<sub>1</sub>, IgG<sub>2a</sub>). Antibody responses of tolerized (Tol) groups were compared to control sensitized mice by ANOVA with Dunnett (IgE, IgA) or or Kruskal-Wallis with Dunn’s (IgG<sub>1</sub>, IgG<sub>2a</sub>) multiple comparison. Grey fields indicate ≥40% antibody reduction compared to corresponding control group. *p<0.05, **p<0.01.
Figure 3.1 Oral tolerance protocol.

Schematic outline for the feeding and immunization schedule of mice. Mice were immunized and boosted with OVA or CPE as detailed in methods. Blood was harvested for antibody analysis or mice were challenged intraperitoneally with 10 mg OVA or 1mg CPE.
Figure 3.2 Mast cells are not required for oral tolerance induction.

A, OVA-specific antibody levels were compared between OVA-fed sensitized mice (tol) and control sensitized mice (cont) in C57BL/6 or KitW-sh/W-sh mast cell deficient mice to determine relative oral tolerance induction. Bars represent mean IgE + SEM, or median IgG1 and IgG2a levels with IR. B, OVA-specific antibody levels were compared between control and tolerized KitW/W-v mice and control WBB6F1 littermates. C, Peanut-specific antibody levels were compared between peanut butter-fed sensitized mice (tol) and control sensitized mice (cont) from C57BL/6 or KitW-sh/W-sh mice. Bars represent mean IgE, IgG1, or IgG2a OD + SEM. *p<0.05, **p<0.01, ***p<0.001, n.s. denotes not significant.
Figure 3.3 IgA tolerance is intact in the absence of mast cells.

A, Mice were treated with OVA-containing drinking water (tol) or normal water (cont) for one week, then immunized and boosted with OVA as detailed in Figure 3.1A. OVA-specific IgA antibody levels in plasma were compared between “tol” and “cont” KitW-sh/W-sh or C57BL/6 mice one week after boost. Bars represent mean IgA response + SEM. B, Fecal samples were assessed for OVA-specific IgA in tolerized and control mice. C, OVA-specific IgA was compared in plasma of tolerized or control mice one week after OVA boost in KitW/W-v mice and WBB6F1 littermates. D, Mice were fed peanut butter (tol) or normal chow (cont) for one week, then immunized and boosted with CPE as detailed in Figure 3.1A. Peanut-specific IgA (mean + SEM) was compared between the plasma of tolerized or control mice one week after CPE boost in KitW-sh/W-sh mice and C57BL/6 mice. *p<0.05, **p<0.01, ***p<0.001, n.s. denotes not significant.
Figure 3.3
Figure 3.4 Physiologically relevant oral tolerance is induced by OVA or peanut butter feeding.

Mean core temperature was measured before, 10, 30, and 50 min after systemic antigen challenge to assess the impact of oral tolerance on anaphylaxis. A, FcγRIII−/- mice were challenged intraperitoneally with OVA. B, C57BL/6 mice were challenged intraperitoneally with CPE. Average temperatures were compared between groups at each time point by t test. Mean temperatures ± SEM are displayed. *p<0.05, **p<0.01, ***p<0.001, n.s. denotes not significant.
Figure 3.5 OT-II Tregs are enhanced in the KitW-sh/W-sh MLNs following oral tolerance induction to OVA through a mast cell-independent mechanism.

Naive OT-II T cells (1x10^6) were adoptively transferred to C57BL/6, KitW-sh/W-sh, or mast cell–reconstituted KitW-sh/W-sh (recon) mice. Animals were treated with OVA-containing drinking water for 7 days, and the development of Treg cells (Foxp3⁺) was compared with that seen in untreated control mice. **A**, Representative plots of CD45.1⁺/CD4⁺/Foxp3⁺ (OT-II Treg cells) cells as a percentage of OT-II CD4⁺ cells recovered from the MLNs of tolerized or untreated KitW-sh/W-sh mice. **B**, Transferred OT-II Treg cells recovered from the MLNs and spleen were compared between OVA-tolerized and untreated C57BL/6 or KitW-sh/W-sh mice. **C**, Tolerized C57BL/6, KitW-sh/W-sh, and KitW-sh/W-sh recon mice were compared by using analysis of OT-II Treg cells as a percentage of OT-II CD4⁺ T cells recovered from the MLNs or analysis of CD4⁺ T cells as a percentage of live cells recovered from the PPs. **D**, Transferred cells were compared in tolerized and untreated C57BL/6 and KitW-sh/W-sh PPs. *p< 0.05, **p < 0.01, and ***p< 0.001. n.s., not significant.
Figure 3.5

A

MLN
OT-II Treg as % of OT-II CD4⁺
(5-8 weeks old)

B

OT-II Treg as % of OT-II CD4⁺
(5-8 weeks old)

C

MLN
OT-II Treg as % of OT-II CD4⁺
(17-18 weeks old)

D

PP
OT-II Treg as % of OT-II CD4⁺
(5-8 weeks old)

PP
OT-II non-Treg as % of CD4⁺
(5-8 weeks old)

C57BL/6
KitW-sh/W-sh
Recon
C57BL/6
KitW-sh/W-sh
Recon
C57BL/6
KitW-sh/W-sh
Recon
C57BL/6
KitW-sh/W-sh
Recon

n = 5-10
n = 5-6
n = 5-10
Figure 3.6 Oral tolerance to OVA in the context of IgE-mediated mast cell activation.

A, Mice were sensitized with anti-TNP IgE prior to treatment with OVA-containing drinking water (tol) or normal water (cont). TNP-BSA or control BSA was delivered by intraperitoneal injection during OVA-Alum immunization. OVA-specific IgE levels (mean + SEM) were compared between groups by ANOVA Dunnett’s Multiple Comparison. **p<0.01. B, Mice were sensitized with anti-TNP IgE prior to treatment with OVA-containing drinking water (tol) or normal water (cont). TNP-BSA or control BSA was delivered by intraperitoneal injection during gavage treatment with OVA.
CHAPTER 4 TOLL-LIKE RECEPTOR 2 ACTIVATION SELECTIVELY ENHANCES IGA RESPONSES TO ORAL ANTIGEN BUT ABROGATES ORAL TOLERANCE IN THE FACE OF A SYSTEMIC CHALLENGE

4.1 Introduction

The mucosal immune system is a dynamic network of cells that co-ordinate to maintain a balance between immunity to pathogens and tolerance to foods and commensal microbes (27). There is growing evidence that the pattern recognition receptors used to identify pathogens, such as Toll-like receptor 2 (TLR2), may also play a role in regulating tolerance to commensals in the intestine (232, 233, 235, 236, 293). However, it is not clear what impact TLR activators within foods may have on regulating the balance between oral tolerance and sensitisation.

Food allergy has become increasingly common in the last 30 years, and it has been proposed in the “hygiene hypothesis” that dysregulated immune responses to environmental microbial stimuli may be involved in the imbalance between tolerance and sensitization (21, 294-296). Indeed, TLR2 polymorphisms have been associated with allergic asthma and atopic disease (212-214). While the function of innate immune activators in food allergy has not been fully characterized, it is believed that allergic sensitization can occur in response to a failure or breach in oral tolerance. It is also well recognized that innate immune activation is pivotal during the initial stages of generating immune responses.

Oral tolerance can be defined as antigen-specific humoral and cellular non-responsiveness (10, 11), and is readily induced in mice and humans upon oral treatment with food antigen. Oral tolerance involves several different cells, including DCs, T cells, and B cells acting in the mucosal associated lymphoid tissues (MALT) (27), and evidence suggests that regulatory T cells (Tregs) are critically involved in the induction and maintenance of oral tolerance to foods (38, 49, 77, 297). Disruption to the tolerogenic function of Tregs, DCs, and activation of B cell responses via innate signaling could alter the progression of oral tolerance to foods.
A variety of cells express TLR2 including the intestinal epithelium, DCs, T cells, and Tregs (298). TLR2 activation with Pam3CSK4 has been shown to impair the suppressive capacity of Tregs in vitro through direct actions on Tregs, T effector cells, and DCs (299-301). Within the T cell, signaling via TLR2 and MyD88 reduce the expression of Foxp3 (302). Similarly, it was found that systemic administration of the TLR2 activator Pam3CSK4 impaired the activity of adoptively transferred Tregs in vivo (299). It was also shown that elevated TLR2 stimulants in the mouse intestine are associated with the induction of colitis and ileitis (303), which suggests a deficit in regulation. Given these data, we considered the possibility that foods containing TLR2 activators will elicit impaired tolerogenic Treg responses.

Naïve and activated B cells also express a variety of TLRs, including TLR2 (304). In addition to activating epithelial cells, DCs, and T cells in the mucosal environment, TLR2 ligands can act directly on B cells. It has been recently reported that TLR2 activation of resting murine B cells in concert with CD40L stimulation can dramatically enhance proliferation, IgM and IgG antibody secretion, class switch recombination, and plasma cell differentiation (305, 306). Similarly, Pam3CSK4 treatment of naïve human peripheral B cells results in production of IL-6 and IL-13 (307), both of which can promote B cell activation and antibody production. Of particular relevance to oral tolerance, TLR2 stimulation of B cells with Pam3CSK4 results in proliferation and antibody production of mucosal Peyer’s patch (PP)-resident mouse B cells (304), while TLR2 stimulation of human B cells also promotes IgA production, J chain production, and expression of mucosal homing markers (217). Considering these data in the context of recent evidence that “B10” regulatory B cells are important regulators of cell-mediated and humoral immunity (308, 309), direct effects of TLR2 activators on B cells could enhance humoral responses to oral antigen and thereby prevent efficient oral tolerance.

Foods often contain TLR2 activators (310), and commercial yogurt is intentionally treated with bacterial cultures including Streptococcus and Lactobacillus species (254) that both activate TLR2 (311-313). Mono-colonization of germ-free mice by several Lactobacilli species can reduce humoral oral tolerance induction and maintenance to the milk protein β-lactoglobulin (314), but the implications of transient
exposure to killed bacteria and TLR2 activators have not been assessed with respect to oral tolerance induction in conventional colonized mice.

Although oral tolerance is the desired outcome of exposure to foods, it presents a substantial barrier to the immunogenicity of oral vaccines (315). When tolerance is induced to an oral vaccine, protective immunity may not be established and there are disappointingly few examples of robust mucosal immune responses to oral vaccines (136). Pam3CSK4 has proven to be a good adjuvant in a mouse model of intramuscular influenza vaccination, leading to increased IgG titres (316). Similarly, the use of Pam2CSK4 as an intramuscular adjuvant enhanced IgA and IgG levels specific to Chlamydia trachomatis, and resulted in protective immunity from infection in mice (317). Early studies with lipopeptides also suggested a role for Pam3CSK4 as an oral adjuvant to promote IgA responses (318). Understanding how oral treatment with Pam3CSK4 can modulate oral tolerance and immunity will improve the potential application of TLR2 agonists as oral vaccine adjuvants for mucosal pathogens.

Despite the evidence in vitro and in vivo that TLR2 activation counteracts regulatory functions of DCs, Tregs, and B cells, the only study to investigate the outcome of oral TLR2 activation on oral tolerance was performed in a sublingual model of immunotherapy for airway allergy (319). In this study, sublingual immunotherapy to OVA was improved in sensitized mice by the addition of Pam3CSK4, reducing airway hyperresponsiveness and the local T_{H2} response. This unexpected observation indicates that further work must be done in order to clarify and better characterize in vivo the cellular and humoral outcomes of oral TLR2 treatment on tolerance to food allergens, specifically the IgE and IgA responses. By directly investigating the outcome of TLR2 activation on oral tolerance induction we can better understand the role of innate activators in mechanisms initiating tolerance to foods and commensal microbes.
4.2 RESULTS

4.2.1 Transferred OVA-specific T cells are responsive to oral tolerance in TLR2\textsuperscript{−/−} recipient mice

TLR2 is important in maintaining a regulatory intestinal environment in the context of colitis (210, 211). Furthermore, TLR2 is known to regulate intestinal epithelial barrier function (209, 210). The role of TLR2 on the development of antigen-specific Tregs was investigated in a model of oral tolerance to OVA. Wild type C57BL/6 and TLR2\textsuperscript{−/−} mice received $1 \times 10^6$ naïve OT-II CD4\textsuperscript{+} T cells by intravenous injection. Tolerized groups were then provided with OVA in water \textit{ad libitum} for one week while control groups received regular water. The proportion of OVA-specific Tregs was significantly increased in the MLN of both tolerized C57BL/6 and TLR2\textsuperscript{−/−} mice when compared to untolerized controls (p<0.01, p<0.001, respectively) (Figure 4.1,A). Similarly, systemic OVA-specific Tregs in the spleen of tolerized C57BL/6 and TLR2\textsuperscript{−/−} mice were increased following OVA treatment (p<0.05, p<0.05, respectively) (Figure 4.1,B). Interestingly, the PPs of both C57BL/6 and TLR2\textsuperscript{−/−} mice were found to contain significantly lower proportions of OVA-specific Tregs following oral tolerance induction (p<0.01 and p<0.01, respectively) (Figure 4.1,C). This Treg response profile is consistent with oral tolerance induction that provides significant physiological protection from a systemic allergen challenge (Figures 3.5 and 3.4,A). In the PPs of C57BL/6 and TLR2\textsuperscript{−/−} mice, OT-II Tregs were comprised of an average $84\% \pm 4.5$ (SEM) and $81\% \pm 4.1$ divided cells containing low levels of proliferation dye, respectively. However, in the MLNs only $67\% \pm 5.9$ and $71\% \pm 2.8$ of OT-II Tregs were divided cells, while the spleen showed $77\% \pm 6.7$ and $71\% \pm 7.4$. Over a quarter of the OVA-specific Tregs in the MLN and spleen therefore underwent Treg conversion without division. Taken together, these results demonstrate that TLR2 is not required to facilitate typical oral tolerance. Levels of antigen-specific Tregs are intact systemically in the spleen and more locally in the MLN and PPs of TLR2\textsuperscript{−/−} recipient mice following tolerance induction.
4.2.2 Oral TLR2 activation prevents humoral oral tolerance to OVA upon systemic challenge

The progression of oral tolerance and the humoral response to OVA in the presence of TLR2 activation was assessed. BALB/c mice were used in these studies as an appropriate model for Th2 polarized allergic disease. Two groups of mice were fed OVA in drinking water *ad libitum* for one week. During this week, one group received 3 gavage treatments of OVA + Pam3CSK4, while the second group received gavage treatments of OVA alone on days -9, -6, and -3 (Figure 2.3,A). Antibody responses to OVA immunization and boost were compared to a control group that received PBS gavage treatments. Oral treatment with OVA resulted in suppression of the IgE, IgA, and IgG2a response to OVA compared to immunized controls (p<0.001, p<0.01, p<0.05, respectively) (Figure 4.2). Remarkably, oral treatment with the TLR2/1 heterodimer activator Pam3CSK4, concurrent with oral OVA treatment, prevented the induction of oral tolerance in these mice such that IgE, IgA, and IgG2a levels were no longer significantly suppressed after systemic challenge when compared to immunized controls (Figure 4.2). IgG1 was not significantly suppressed following OVA treatment, but oral Pam3CSK4 treatment concurrent with OVA did elevate levels compared to OVA tolerized mice (p<0.05) (Figure 4.2,C). Oral treatment with 3 separate 5 µg doses of FSL-1, a TLR2/6 activator, yielded similar results (Table 4.1,A).

Interestingly, treatment with a similar dose of a potent TLR4 activator (LPS) did not prevent oral tolerance induction of IgE compared to control groups, but did enhance plasma IgA production (p<0.05) (Table 4.1,B). The impact of TLR2 activation via Pam3CSK4 on oral tolerance induction to peanut butter was also examined. Peanut-specific antibody levels were significantly reduced in all classes examined in PB-fed mice, indicating profound tolerance to peanut (IgE p<0.05, IgA p<0.01, IgG1 p<0.001, IgG2a p<0.001) (Table 4.1,D). When mice were treated with Pam3CSK4 by gavage concurrent with peanut butter, levels of peanut-specific IgA, and IgG1 were no longer significantly reduced upon systemic peanut immunization compared to control peanut-sensitized mice (Table 4.1D). However, peanut-specific IgE and IgG2a remained significantly suppressed in the tolerized mice despite oral Pam3CSK4 treatment (p<0.01, p<0.001, respectively) (Table 4.1D). This suggests that Pam3CSK4 treatment is a potent
modulator of humoral oral tolerance across antigen systems, specifically in the IgA compartment.

4.2.3 TLR2 activation with oral Pam3CSK4 does not significantly alter antigen-specific Treg levels during oral tolerance

Having observed a potent immunomodulatory effect of oral Pam3CSK4 treatment on the induction of oral tolerance to OVA and peanut butter, the cellular mechanisms involved in tolerance induction were investigated in the context of Pam3CSK4 treatment. Evidence suggests that TLR2 activation can disrupt Treg development and function (299, 300, 319). However, the outcome of TLR2 activation at a mucosal site has not been explored in the context of antigen-specific Tregs in oral tolerance induction. C57BL/6 and TLR2−/− mice underwent adoptive transfer and OVA treatment ± Pam3CSK4 as described in (Figure 2.3,B). The PPs, MLNs, and spleens were harvested and analyzed by flow cytometry for OT-II Tregs, non-Tregs, and CD4+ T cells. No change in OVA-specific Tregs was observed in the MLN upon oral treatment with Pam3CSK4 (Figure 4.3,A). Similarly systemic OVA-specific Tregs were not significantly altered in the spleens of C57BL/6 tolerized mice upon treatment with Pam3CSK4, although a trend towards lower levels was observed in Pam3CSK4 treated mice (Figure 4.3,B). Pam3CSK4 treatment of tolerized C57BL/6 mice did not alter the proportion of OVA-specific Tregs in the PPs, but the Tregs in this group were significantly reduced compared to Pam3CSK4-treated Tregs in tolerized TLR2−/− recipient mice (p<0.05) (Figure 4.3,C).

4.2.4 Oral Pam3CSK4 treatment does not alter total B cell or plasma cell levels in the GALT and spleen

Given that antigen-specific Treg development did not appear to be modulated in vivo by Pam3CSK4 we considered B cells as a likely target for Pam3CSK4 in the context of tolerance induction to OVA. Multiple reports have indicated that B cells are responsive to TLR2 stimulation, and this can result in intestinal homing, plasma cell differentiation, and amplified antibody production (217, 305, 306). It is possible that direct modulation of the B cell response to antigen, by TLR2, amplifies antibody responses and prevents
oral tolerance to foods. Accordingly, B cell populations were directly examined in the small intestine LP, PPs, MLNs, and spleens of tolerized mice following oral OVA and Pam3CSK4 treatment. Tissues were removed following OVA treatment as described in section 2.11 (Figure 2.3,A) then assessed for the B cell marker CD19+, the B cell activation marker CD69+, and the plasma cell marker CD138+ in FSC^hi/SSC^hi and FSC^lo/SSC^lo populations. Surface IgA and IgM expression were also assessed. The FSC^hi/SSC^hi cells were predominantly observed in the LP and PPs, sites known to contain high levels of antibody-secreting plasma cells, while low levels of FSC^hi/SSC^hi cells in the MLN and spleen did not bear further analysis. Analyses are summarized in Table 4.2. Mice treated with oral Pam3CSK4 showed no changes in the number of plasma cells from any of the tissues analyzed (Table 4.2). Similarly, levels of the plasma cell marker CD138 were not altered in either the FSC^hi/SSC^hi or in the FSC^lo/SSC^lo populations upon oral Pam3CSK4 treatment (Table 4.2). Pam3CSK4 treatment did not change the proportion of IgM or IgA-expressing B cells compared to OVA treated control mice (Table 4.2). Activated cells expressing CD69 were identified in the LP, PPs, MLN, and spleen, but the proportion of CD69+ B cells was not altered following oral Pam3CSK4 treatment. Taken together, these results showed no change in the number of plasma cells or B cells in the LP, PPs, MLN, or spleen following oral Pam3CSK4 compared to OVA-tolerized mice. Furthermore, the activation state of B cells and plasma cells did not change with Pam3CSK4 treatment, while class switch recombination did not appear to be affected. These data indicate that oral TLR2 activation during tolerance induction does not enhance non-specific proliferation or migration of B cells and plasma cells.

4.2.5 Pam3CSK4 is a potent and selective oral adjuvant for IgA responses in mice

In light of the reliable capacity for oral Pam3CSK4 treatment to disrupt suppression of the IgA response to OVA and peanut, Pam3CSK4 was next assessed as an oral adjuvant to selectively promote IgA responses. Oral vaccines often face the challenge of overcoming the body’s natural tendency to induce tolerance to oral antigens, and this represents a major barrier to clinical vaccine development (315). Mice were treated by gavage with 3 weekly oral doses of either OVA, OVA + Pam3CSK4, or PBS control. One week after
the final gavage treatment, blood and fresh fecal samples were acquired and OVA-specific antibody levels were assessed by ELISA. Results were compared to mice immunized by a standard i.p. OVA-alum injection followed by an i.p. OVA boost (Figure 4.4). Surprisingly, gavage with OVA alone in the absence of i.p. injection generated detectable levels of OVA-specific IgA, IgE, IgG1, and IgG2a antibodies, although none were significantly greater than PBS treated control mice (Figure 4.4,A, B, C, D). When Pam3CSK4 was delivered as an adjuvant with OVA, OVA-specific IgA levels in plasma were dramatically enhanced (p<0.05) (Figure 4.4,D). Interestingly, levels of OVA-specific IgE, IgG1, or IgG2a were not significantly elevated in plasma from Pam3CSK4 treated mice compared to mice treated with OVA alone, but OVA-alum i.p. immunized mice did have significantly higher levels of these antibody classes (p<0.001 IgE, p<0.01 IgG1, p<0.05 IgG2a) (Figure 4.4,A, B, C). When OVA-specific secreted IgA was assessed in fecal samples, levels were significantly higher in Pam3CSK4 treated mice compared to OVA alone or OVA-alum i.p. immunized (p<0.001, p<0.001, respectively) (Figure 4.4,E). The use of Pam3CSK4 as an oral adjuvant with OVA therefore significantly improved both plasma and mucosal secreted OVA-specific IgA responses.

It has been shown that TLR2 activation of B cells can enhance non-specific IgA and J chain production (217), and TLR stimulation of the intestinal epithelium is associated with pIgR expression and subsequent IgA secretion (320). Accordingly, total levels of non-specific IgA were measured in the blood and feces of mice treated with OVA alone or with OVA + Pam3CSK4. In these mice, the use of Pam3CSK4 as an oral adjuvant with OVA did not significantly alter the production or secretion of total non-specific IgA (Figure 4.4,F). These data suggest that Pam3CSK4 treatment can selectively and specifically improve both the IgA plasma and secreted responses to an oral antigen, and therefore has potential as an effective mucosal adjuvant.

4.2.6 Bacterial cultures in yogurt do not impair oral tolerance induction to milk protein

It was recently documented that commercial yogurts contain high levels of TLR2 activators when compared to milk (310). In light of our findings that TLR2 activation
impairs oral tolerance to OVA upon systemic challenge, we hypothesized that bacterial cultures present in yogurt may impair oral tolerance to β-lactoglobulin – a common protein allergen in cow’s milk. Oral tolerance to β-lactoglobulin was successfully induced in mice treated orally with cow’s milk, evidenced by suppressed β-lactoglobulin–specific IgE, IgA, IgG1, and IgG2a antibody responses to immunization and boost (p<0.01, p<0.05, p<0.05, p<0.05, respectively) (Figure 4.5). When mice were treated orally with milk supplemented with heat-killed samples of the two most common yogurt cultures, Gram-positive L. delbrueckii and S. thermophilus, β-lactoglobulin–specific plasma IgE, IgA, and IgG2a levels remained suppressed compared to immunized controls (p<0.01 IgE, p<0.05 IgA, p<0.01 IgG2a) (Figure 4.5,A, B, D). While IgG1 levels were not significantly reduced in bacteria-treated tolerized mice compared to immunized controls, they also were not significantly greater than milk tolerized controls (Figure 4.5,C). Beyond this mild change of IgG1 levels, bacterial supplementation of milk did not disrupt humoral oral tolerance induction or reflect the results of oral Pam3CSK4 treatment.

4.3 Discussion

This study demonstrates that TLR2 is not necessary in order to establish adequate levels of food-specific Tregs in the MLN and spleen for effective oral tolerance induction. However, we also demonstrate, in two independent food tolerance models, that oral TLR2 activation reliably modulates antibody responses to oral antigen upon systemic challenge and prevents the necessary suppression of antigen-specific IgE and IgA responses to OVA tolerance. The modulation of oral tolerance by oral Pam3CSK4 treatment did not stem from changes to OVA-specific Treg numbers in the PP, MLN, or spleen; and levels of non-specific B cells or plasma cells were not changed in the GALT and spleen. An antigen-specific amplification of IgA responses to antigen upon repeated oral exposure with Pam3CSK4 was also observed. These findings have profound implications for understanding the optimal immunological environment to promote tolerance and prevent allergic disease, in addition to informing potential oral vaccination strategies.
When OVA-specific TLR2 sufficient Tregs were compared between the PPs of Pam3CSK4 treated C57BL/6 and TLR2−/− recipient mice, the proportion of OVA-specific Tregs were significantly greater in TLR2−/− recipients (Figure 4.3,C). These findings suggest that direct local TLR2 stimulation of transferred TLR2-sufficient Tregs enhanced their numbers in the PPs of tolerized TLR2−/− recipient mice; whereas TLR2 activation of other cell types such as intestinal epithelium and DCs in C57BL/6 mice may act to suppress Treg levels in the PPs of tolerized wild type mice. This highlights an interesting TLR2-dependent anti-regulatory function of the intestinal environment that warrants further investigation. Production of IL-6 by intestinal epithelial cells is a likely candidate driving this change (321-323).

Repeated local oral TLR2 activation during food exposure selectively promoted a specific IgA response, but oral TLR2 activation during tolerance induction effectively lowered the threshold for antibody responses to food antigen upon a systemic challenge. This differential effect suggests a currently undescribed potential mechanism of allergic sensitization in patients. This effect was observed with both the TLR1/2 activator Pam3CSK4 and the TLR2/6 activator FSL-1. Surprisingly, when this experiment was repeated using a second activator of the TLR2/6 heterodimer (MALP-2), tolerance was not significantly affected following a systemic OVA challenge (Table 4.1). It should be noted that MALP-2 was only administered at a 5μg dose due to commercial product availability, and MALP-2 is known to be a significantly less potent TLR2/6 activator than FSL-1 (324).

Interestingly, our data showed that levels of total B cells and plasma cells were not altered in the GALT and spleen following Pam3CSK4 treatment that altered humoral tolerance and enhanced antibody responses. This does not rule out the possibility that B cell activation thresholds are lowered by TLR2 stimulation. Indeed, work by Jain et al. showed that TLR2 activation of B cells enhanced their ability to respond to CD40 stimulation by T cells upon antigen presentation (306). Furthermore, it is possible that the antigen-specific B cell numbers were significantly changed in vivo but these cells were too small a population to have a detectable impact on the total cell populations.
Similarly, OVA-specific T cell and Treg levels were not changed in Pam3CSK4 treated mice. TLR2 activation of Tregs can result in simultaneous expansion and loss of suppressive function (299, 301), and although Pam3CSK4 treatment did not significantly alter antigen-specific Treg levels \textit{in vivo} during OVA tolerance in our studies it is possible that their suppressive function was altered. An \textit{ex vivo} analysis of Treg suppression would be informative. Also, unchanged levels of Tregs \textit{in vivo} do not preclude the possibility that the T cell population contains a higher proportion of activated T\textsubscript{H}17 cells that may impact tolerance.

It was recently suggested that antigen-specific Tregs migrate from the MLN to the LP in order to maintain intestinal tolerance (49). We attempted to assess the proportion of antigen-specific Tregs in the LP of tolerized mice, but in our system only 10\textsuperscript{6} naïve OT-II T cells are transferred in an attempt to minimize homeostatic regulation of the transferred population or other unwanted effects that might occur to a large population of antigen-specific T cells. The recovered population of transferred cells in the LP was too small to allow a reliable analysis of Foxp3\textsuperscript{+} T cells (Figure 5.3). It is therefore possible that the levels or suppressive function of antigen-specific Tregs are altered in the LP of mice treated with Pam3CSK4, precipitating the observed defects in oral tolerance.

There is precedent for enhanced mucosal immune responses following an oral TLR activation. In a very similar oral vaccine model system, oral treatment with OVA and CPG-rich synthetic oligodeoxynucleotides has been employed to enhance the mucosal immune response to oral OVA vaccination (246). CPG motifs are known to activate TLR9, and OVA-specific secreted IgA was markedly enhanced from CPG-ODN stimulation. Similar to our observations, the \textit{E.coli}-derived heat labile enterotoxin LT-IIa-B5 was shown to be an effective TLR2-dependent mucosal adjuvant, amplifying antigen-specific CD4\textsuperscript{+} proliferation, secreted IgA, and serum IgG following intranasal delivery (325). These findings complement our observation that TLR2 activation via an oral route with Pam3CSK4 can elicit a potent serum and secreted antigen-specific IgA response.

Serum and secreted antigen-specific IgA have been shown to prevent anaphylaxis (116) and allergic diarrhea (326) in mice, suggesting that IgA responses can be protective
in the context of an oral allergen challenge. In light of this, Pam3CSK4 should be explored as an oral adjuvant for oral allergen immunotherapy. If applied exclusively in an oral context Pam3CSK4 may help promote food-specific IgA responses that could protect patients from anaphylaxis. However, the differential outcomes of Pam3CSK4 on the modulation of humoral responses to repeated oral antigen or systemic antigen challenge must first be fully characterized.

Our observations suggest that oral treatment with \textit{L. delbrueckii} and \textit{S. thermophilus} do not alter humoral responses to milk tolerance, but treatments with \textit{L. delbrueckii} or \textit{S. thermophilus} have previously been demonstrated to enhance IgA-producing plasma cells (327) and IgA secretion (328). Secreted IgA is believed to have implications for oral tolerance (111). Accordingly, we investigated whether the bacterial enrichment of milk altered secreted IgA levels in feces of tolerized mice, but \(\beta\)-lactoglobulin–specific IgA levels assessed in fecal samples were highly variable. When total fecal IgA was measured from these mice as mean \(\mu\)g/100mg fecal weight, no significant differences were observed between treatment groups [controls, 1.65 ± 0.347 (SEM); milk-treated, 3.24 ± 0.788 (SEM); milk/bacteria-treated, 1.52 ± 0.368 (SEM)]. Surprisingly, we observed that repeated oral exposure over 1 month with OVA containing killed \textit{L. delbrueckii} and \textit{S. thermophilus} at levels found in yogurt did not alter antigen-specific or total IgA in the blood (data not shown), but the presence of these killed bacteria did enhance antigen-specific IgA levels in the feces (OD 0.157 ± 0.012 (SEM) from OVA-treated mice compared to 0.323 ± 0.023 (SEM) from OVA/bacteria treated mice; \(p<0.01, n=9-10\)). This contrasts the results from isolated TLR2 ligand oral treatment with soluble Pam3CSK4 + OVA that elevated antigen-specific IgA in both the blood and feces (Figure 4.4,D,E). These findings highlight an important difference between selective TLR2 activation and whole bacteria as adjuvants in the mucosal IgA response.

Induction of a robust secreted IgA response to antigen in the context of TLR2 stimulation likely represents an adaptive mechanism to support tolerance to commensal bacteria and foods. When the antigen is reintroduced by an oral route, intestinal B cells respond by selective IgA production (114). However, if antigen is reintroduced by a
systemic antigen challenge event then the ensuing response is broad, implicating antibody production in all classes by systemic B cells. This may represent a safeguard response to systemic infection by commensal bacteria that have breached the intestinal barrier. When the same principle is applied to a food antigen, the result is a systemic failure of humoral oral tolerance to the new food. These differential local and systemic response pathways to TLR2-contaminated food suggest that we need to expand our analysis of the roles that PRRs play in oral tolerance induction and maintenance to include both local and systemic outcomes.
Figure 4.1 OVA-specific transferred T cells are responsive to oral tolerance in TLR2\(^{-/-}\) recipient mice.

Naive OT-II CD\(^{4+}\) T cells were adoptively transferred to C57BL/6 or TLR2\(^{-/-}\) mice. Animals were treated with OVA-containing drinking water for 7 days, and the development of Treg cells (Foxp3\(^{+}\)) was compared with that seen in untreated control mice. Transferred OT-II Treg cells recovered from the MLNs (A), spleens (B), and PPs (C) were compared between OVA-tolerized and untreated C57BL/6 or TLR2\(^{-/-}\) mice by t test. Analysis of flow cytometry was performed using the gating strategy outlined in Figure 3.5,A. Bars represent mean+SEM. *p < 0.05, **p < 0.01, *** p < 0.001.
Figure 4.2 Oral Pam3CSK4 treatment impairs humoral tolerance to OVA upon systemic challenge.

BALB/c mice were treated with OVA ± Pam3CSK4 by gavage treatment 3 times during a week of *ad libitum* 4mg/mL OVA-water treatment. Control groups were treated with PBS and provided with normal water. All mice were immunized with OVA-alum and boosted with OVA. Levels of OVA-specific antibodies were assessed by ELISA in plasma samples harvested one week after boost. A, OVA-specific IgE levels were assessed by ELISA and expressed as ng/mL. B, OVA-specific plasma IgA levels were assessed by ELISA and expressed as standard-adjusted OD. C, OVA-specific plasma IgG1 levels were assessed by ELISA and analyzed by titre analysis and expressed as median with IR. D, OVA-specific plasma IgG2a levels were assessed by ELISA and analyzed by titre analysis. IgE and IgA levels were compared between groups by ANOVA followed by Bonferroni's Multiple Comparison Test, whereas IgG1 and IgG2a levels were compared between groups by Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. Bars represent mean±SEM IgE and IgA levels, or median –Log titre IgG1 and IgG2a levels with IR. *p < 0.05, **p < 0.01, ***, p < 0.001, n.s., not significant.
Figure 4.3 OVA-specific Treg levels are not altered in vivo by oral Pam₃CSK₄ treatment.

Naive OT-II CD4⁺ T cells were adoptively transferred to C57BL/6 or TLR2⁻/⁻ mice. Animals were treated with OVA-containing drinking water for 7 days, and the development of Treg cells (Foxp3⁺) was compared between groups of C57BL/6 or TLR2⁻/⁻ mice treated with OVA ± Pam₃CSK₄ 3 times during the week of ad libitum 4mg/mL OVA-water treatment. Transferred OT-II Treg cells recovered from the MLNs (A), spleens (B), and PPs (C) were compared by ANOVA followed by Bonferroni's Multiple Comparison Test. Bars represent mean±SEM. *p < 0.05, n.s., not significant.
Figure 4.4 Pam3CSK4 can be used as an oral adjuvant to selectively enhance antigen-specific IgA.

Male BALB/c mice were treated by gavage 3 times over 3 weeks (days 1, 7, 14) with PBS, 1mg OVA, or 1mg OVA + 10μg Pam3CSK4. Other mice were immunized by intraperitoneal injection of 10μg OVA-alum on day 1, then boosted by intraperitoneal injection of 1μg OVA on day 14. Blood and fecal samples were harvested from all mice on day 21. OVA-specific IgE (A), IgG1 (B), IgG2a (C), and IgA (D) were measured in plasma by ELISA and compared by ANOVA with Bonferroni's Multiple Comparison Test (IgE, IgA) or by Kruskal-Wallis test with Dunn's Multiple Comparison Test (IgG1, IgG2a). OVA-specific IgE levels were expressed as mean+SEM ng/mL, while IgG1 and IgG2a levels were analyzed by titre analysis and expressed as median –Log titre with IR. E, OVA-specific IgA was measured in fecal samples by ELISA and expressed as mean+SEM standard-adjusted OD, then groups were compared by ANOVA with Bonferroni's Multiple Comparison Test. F, Total non-specific IgA was measured in plasma and fecal samples from mice treated orally 3 times with 1mg OVA or 1mg OVA + 10μg Pam3CSK4. Plasma levels were expressed as mean+SEM μg/mL and fecal levels were expressed as mean +SEM μg/100mg feces. *p < 0.05, **p < 0.01, ***p < 0.001, n.s., not significant.
Figure 4.4

1 = PBS oral treatment  
2 = OVA oral treatment  
3 = OVA + Pam3CSK4 oral treatment  
4 = OVA-alum i.p. immunization  
n = 9-10
Figure 4.5 Yogurt bacteria do not impair oral tolerance to cow’s milk.

BALB/c mice were treated with cow’s milk at 10mg/mL protein in water ± *L. delbrueckii* / *S. thermophilus* for 1 week *ad libitum*. Control groups were provided with normal water. All mice were immunized with β-lactoglobulin-alum and boosted with β-lactoglobulin. Levels of β-lactoglobulin-specific antibodies were assessed by ELISA in plasma samples harvested one week after boost. **A**, β-lactoglobulin-specific IgE levels were assessed by ELISA and expressed as mean+SEM standard-adjusted OD. **B**, β-lactoglobulin-specific plasma IgA levels were assessed by ELISA and expressed as standard-adjusted OD. **C**, β-lactoglobulin-specific plasma IgG1 levels were assessed by ELISA and analyzed by titre analysis. **D**, β-lactoglobulin-specific plasma IgG2a levels were assessed by ELISA and analyzed by titre analysis. IgE and IgA levels were compared between groups by ANOVA followed by Bonferroni's Multiple Comparison Test and are represented as mean + SEM. IgG1 and IgG2a levels were compared between groups by Kruskal-Wallis test followed by Dunn's Multiple Comparison Test and represented as median with IR. *p < 0.05, **p < 0.01, n.s., not significant.
Table 4.1 Comparison of oral tolerance induction to OVA or peanut butter with different innate activators.

OVA-specific antibody levels are expressed as mean ng/mL ± SEM (IgE), mean OD ± SEM (IgA), or median –Log titre with range (IgG\textsubscript{1}, IgG\textsubscript{2a}) (A, B, D). IgE and IgA levels were compared between groups by ANOVA followed by Bonferroni’s Multiple Comparison Test. IgG\textsubscript{1} and IgG\textsubscript{2a} levels were compared between groups by Kruskal-Wallis test followed by Dunn’s Multiple Comparison Test. C, Peanut-specific antibody levels are expressed as mean OD ± SEM. Antibody levels were compared between groups by ANOVA followed by Bonferroni’s Multiple Comparison Test. Grey fields indicate ≥40% antibody reduction compared to corresponding control group. Significant differences following post-test comparisons between tolerized groups and the corresponding control group are identified by “*”. *p<0.05, **p<0.01, ***p<0.001. Significant differences following post-test comparisons between tolerized groups and tolerized + innate activator groups are represented by “†”. †P<0.05, ††P<0.01, †††P<0.001.
Table 4.1

OVA-specific antibody levels in plasma one week after boost

<table>
<thead>
<tr>
<th></th>
<th>IgE</th>
<th>IgA</th>
<th>IgG1</th>
<th>IgG2a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Comparison</strong></td>
<td></td>
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<td></td>
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<tr>
<td>between control</td>
<td>59.23 ± 6.70</td>
<td>0.293 ± 0.067</td>
<td>5.12 (0.01 – 5.41)</td>
<td>3.30 (0.01 – 3.87)</td>
</tr>
<tr>
<td>OVA tolerized</td>
<td>11.59 ± 3.59***</td>
<td>0.186 ± 0.040</td>
<td>4.88 (1.87 – 5.43)</td>
<td>2.65 (1.32 – 3.57)</td>
</tr>
<tr>
<td>OVA tolerized + FSL-1</td>
<td>30.79 ± 6.38</td>
<td>0.453 ± 0.073 †</td>
<td>5.30 † (4.30 – 5.64)</td>
<td>3.38 (1.98 – 3.77)</td>
</tr>
<tr>
<td><strong>B) Comparison</strong></td>
<td></td>
<td></td>
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<tr>
<td>between control</td>
<td>28.87 ± 4.66</td>
<td>0.138 ± 0.045</td>
<td>4.23 (0.97 – 5.71)</td>
<td>2.94 (0.01 – 3.75)</td>
</tr>
<tr>
<td>OVA tolerized</td>
<td>9.59 ± 2.06***</td>
<td>0.038 ± 0.008</td>
<td>4.58 (2.92 – 5.37)</td>
<td>2.93 (0.01 – 3.77)</td>
</tr>
<tr>
<td>OVA tolerized + LPS</td>
<td>11.91 ± 2.60**</td>
<td>0.197 ± 0.062 †</td>
<td>4.80 (3.58 – 5.80)</td>
<td>3.15 (0.01 – 3.69)</td>
</tr>
<tr>
<td><strong>C) Comparison</strong></td>
<td></td>
<td></td>
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<tr>
<td>between control</td>
<td>39.32 ± 3.36</td>
<td>0.186 ± 0.058</td>
<td>4.84 (0.01 – 5.45)</td>
<td>2.23 (0.01 – 4.02)</td>
</tr>
<tr>
<td>OVA tolerized</td>
<td>12.41 ± 3.98***</td>
<td>0.052 ± 0.011</td>
<td>4.41 (0.01 – 5.40)</td>
<td>2.48 (0.01 – 3.41)</td>
</tr>
<tr>
<td>OVA tolerized + MALP-2</td>
<td>7.42 ± 2.55***</td>
<td>0.192 ± 0.126</td>
<td>4.58 (0.01 – 5.24)</td>
<td>3.10 (0.01 – 3.43)</td>
</tr>
</tbody>
</table>

Peanut-specific antibody levels in plasma one week after boost

<table>
<thead>
<tr>
<th></th>
<th>IgE</th>
<th>IgA</th>
<th>IgG1</th>
<th>IgG2a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D) Comparison</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>between control</td>
<td>0.241 ± 0.083</td>
<td>0.077 ± 0.023</td>
<td>0.199 ± 0.024</td>
<td>0.616 ± 0.075</td>
</tr>
<tr>
<td>Peanut butter</td>
<td>0.107 ± 0.093*</td>
<td>0.010 ± 0.003*</td>
<td>0.022 ± 0.009***</td>
<td>0.119 ± 0.049***</td>
</tr>
<tr>
<td>Peanut butter</td>
<td>0.011 ± 0.003**</td>
<td>0.038 ± 0.012</td>
<td>0.115 ± 0.037</td>
<td>0.173 ± 0.049***</td>
</tr>
</tbody>
</table>
### Table 4.2 Plasma cells and activated B cells are not changed in the GALT and spleen following oral Pam3CSK₄.

<table>
<thead>
<tr>
<th></th>
<th>LP</th>
<th>PPs</th>
<th>MLN</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19⁺ % of FSC⁺/SSC⁻</td>
<td>OVA: 0.4014 ± 0.1093</td>
<td>OVA + Pam3CSK₄: 0.4871 ± 0.1068</td>
<td>OVA: 56.72 ± 9.995</td>
<td>OVA + Pam3CSK₄: 51.55 ± 9.600</td>
</tr>
<tr>
<td>CD138⁺ % of CD19⁺</td>
<td>OVA: 5.973 ± 0.6839</td>
<td>OVA + Pam3CSK₄: 5.150 ± 0.5306</td>
<td>OVA: 14.58</td>
<td>OVA + Pam3CSK₄: 14.88</td>
</tr>
<tr>
<td>CD69⁺ % of CD19⁺</td>
<td>OVA: 15.34 ± 5.703</td>
<td>OVA + Pam3CSK₄: 12.83 ± 4.089</td>
<td>OVA: 5.778</td>
<td>OVA + Pam3CSK₄: 5.225 ± 1.524</td>
</tr>
<tr>
<td>IgA⁺ % of CD19⁺</td>
<td>OVA: 12.95 ± 2.303</td>
<td>OVA + Pam3CSK₄: 9.210 ± 1.950</td>
<td>OVA: 7.699</td>
<td>OVA + Pam3CSK₄: 7.032 ± 0.4285</td>
</tr>
<tr>
<td>FSC⁺/SSC⁻ cells</td>
<td>OVA: 6.941 ± 0.5988</td>
<td>OVA + Pam3CSK₄: 8.323 ± 1.395</td>
<td>OVA: 3.740 ± 0.1039</td>
<td>OVA + Pam3CSK₄: 4.239 ± 0.5397</td>
</tr>
<tr>
<td>CD19⁺ % of FSC⁺/SSC⁺</td>
<td>OVA: 10.68 ± 3.187</td>
<td>OVA + Pam3CSK₄: 13.06 ± 3.959</td>
<td>OVA: 48.15 ± 6.826</td>
<td>OVA + Pam3CSK₄: 47.65 ± 4.789</td>
</tr>
<tr>
<td>CD138⁺ % of FSC⁺/SSC⁺</td>
<td>OVA: 4.410 ± 0.4963</td>
<td>OVA + Pam3CSK₄: 5.723 ± 1.090</td>
<td>OVA: 38.89 ± 9.852</td>
<td>OVA + Pam3CSK₄: 33.27 ± 8.826</td>
</tr>
<tr>
<td>IgA⁺ % of CD19⁺</td>
<td>OVA: 12.95 ± 2.303</td>
<td>OVA + Pam3CSK₄: 9.210 ± 1.950</td>
<td>OVA: 20.41 ± 6.604</td>
<td>OVA + Pam3CSK₄: 15.42 ± 5.562</td>
</tr>
<tr>
<td>IgM⁺ % of CD19⁺</td>
<td>OVA: 2.947 ± 0.3294</td>
<td>OVA + Pam3CSK₄: 4.543 ± 0.860</td>
<td>OVA: 9.886 ± 1.026</td>
<td>OVA + Pam3CSK₄: 15.43 ± 2.464</td>
</tr>
</tbody>
</table>

Levels of FSC⁺/SSC⁺ or FSC⁻/SSC⁻ cells and surface expression of CD138, CD19, CD69, IgA, or IgM were assessed in the LP, PPs, MLN, and spleen then compared between mice treated with OVA or OVA + Pam3CSK₄ by t test. Groups were not significantly different upon statistical comparison.
CHAPTER 5 DISCUSSION

5.1 Summary of Major Findings
My studies have highlighted several fundamental observations about the induction and maintenance of oral tolerance. First, TLR2 is not necessary for antigen-specific Tregs to develop in the MLN and spleen during oral tolerance. However, repeated oral treatments with antigen and the TLR2 activator Pam3CSK4 selectively promote IgA responses, while oral treatment with TLR2 activator and antigen followed by a systemic antigen challenge can impair IgE, IgA, and IgG tolerance. The latter defect in humoral tolerance did not result from altered levels of antigen-specific Tregs or changes to the number and distribution of total B cells and plasma cells in the GALT.

Using two independent models of mast cell deficiency, I found that mast cells are not necessary for oral tolerance induction. IgE-mediated mast cell activation did not impair oral tolerance induction or maintenance, but it did modulate the IgG2a response following a systemic antigen challenge. These major findings are summarized in a schematic model (Figure 5.1).

5.2 Implications and Relevance of Major Findings
The observation that mast cells are not necessary for the induction of oral tolerance is significant because there is a growing body of evidence that mast cells are critically involved in the mediation of other forms of peripheral tolerance. This incongruence highlights yet another important difference between oral tolerance and other mechanisms of peripheral tolerance. Regulatory activities of mast cells in allograft tolerance have been partly attributed to expression of mMCP-6 (180), but this protease is not expressed by mucosal mast cells. It is therefore likely that there is a meaningful difference between mucosal versus connective tissue-type mast cells in respect to their interactions with Tregs and regulatory processes. These differences warrant further investigation and may significantly improve our understanding of intestinal immunity and tolerance.

The observation that systemic IgE-mediated mast cell activation does not significantly impact oral tolerance was also surprising in the context of recent literature. While I observed a modulation of the IgG2a antigen-specific response, IgE and IgA levels
were not altered suggesting that Treg function was intact. Previous studies suggest that
IgE activation of mast cells should interfere with Treg differentiation and function (182,
183, 271). My observations that oral tolerance is resistant to immunomodulation by IgE-
mediated mast cell activation further underscores the need for careful in vivo analyses of
the roles of mast cells in different disease models. Moreover, these findings suggest that
if there is a relationship between allergic responses and impaired tolerance to new foods
it would likely stem from physiological changes observed in the intestine during food
allergy reactions, not the immunomodulatory relationship between activated mast cells
and Tregs.

In this work I have also identified a previously undescribed enhancement of
antigen-specific Tregs in the MLNs of KitW-sh/W-sh mice that is not related to mast cells.
This immunological difference reinforces the necessity for mast cell reconstitution
studies when assessing the functional role of mast cells in disease models using the KitW-
sh/W-sh mice. The enhancement of oral tolerance in the KitW-sh/W-sh mice points to the
possibility that other c-kit expressing cells may serve as potential targets for enhancing
oral immunotherapies. Several recently described innate lymphoid cell subsets express c-
kit, and several members of this diverse group are implicated in disease pathologies
including allergy and inflammatory bowel disease (283). My results suggest that these
innate lymphoid cells should be investigated with respect to their potential involvement
in oral tolerance.

Antihistamines are used during OIT protocols in order to prevent allergic
responses to the antigen. Our data demonstrate that oral antihistamine treatment is not
likely to modify oral tolerance induction, and therefore is probably a safe supplemental
treatment during OIT. These results were surprising in the context of a growing body of
literature detailing profound immunomodulatory effects of histamine on APCs, T cells
and Tregs (163).

Germ-free mice have impaired oral tolerance development, suggesting an
important role for commensal bacteria and PRR signaling in regulatory processes (241,
329, 330). Furthermore, TLR2 has known effects in regulating intestinal inflammation
and immunity. Despite these known relationships, the results presented here are to my
knowledge the first direct assessment of TLR2 in oral tolerance induction in naïve mice. TLR2 activation was, however, used in one study of SLIT for asthma in sensitized mice (319). Interestingly, the authors found that sublingual Pam3CSK4 treatment concurrent with antigen significantly reduced airway disease, and they proposed that this effect was mediated by polarizing away from a Th2 antigen-specific response. Our results do not support this type of polarization, and the authors did not assess IgA levels in their system. An important difference between this study and what I have presented here lies in the fact that the authors were assessing TLR2 activation in the context of sensitized animals, whereas we have been investigating the role of TLR2 activation during the initial responses to antigen in naïve mice. The differences between our observations and their findings highlight the fact that tolerance may progress differently depending on the physiological site of induction and the preexisting state of tolerance or sensitization.

TLR2 was not necessary for the induction of antigen-specific Tregs in the PPs, MLN, and spleen in recipient mice upon oral tolerance induction. This important observation indicates that TLR2 is likely not the primary mechanism by which commensal bacteria regulate oral tolerance. However, one of the most interesting and important findings, in my studies, was the antigen-specific promotion of plasma and secreted IgA to food with Pam3CSK4 treatment. This has implications for our understanding of host responses to commensal organisms and sheds light on the systems wherein bacteria may regulate our responses to foods and immunologic tolerance.

We documented that oral treatment with Pam3CSK4, during tolerance induction, impaired oral tolerance upon a secondary systemic antigen challenge. This suggests the possibility that systemic antigen challenge through the skin or by injection could result in allergic sensitization if a food antigen was first introduced orally in the context of TLR2 activators. However, our studies with milk supplemented by yogurt bacteria showed that oral tolerance was not changed by the presence of killed *S. thermophilus* and *L. delbrueckii* when present at levels found in commercial yogurt. This finding implies that childhood exposure to yogurt as an early source of cow’s milk antigen may not result in later allergy to cow’s milk. The differential outcome of isolated oral TLR2 activation
compared to oral treatment with whole bacteria confirms that the regulation of oral
tolerance to new foods does not depend on an isolated TLR2 signal.

5.3 Limitations of the Experimental Systems
In several different mouse models I demonstrated significant changes to OVA-specific
Treg levels following oral tolerance induction. It has been well-documented that Tregs
can suppress other antigen-specific T cells, but I have not directly examined the
functionality of Tregs in my model of oral tolerance induction to OVA. TLR2 activation
of Tregs can result in simultaneous expansion and loss of suppressive function (299,
301). Although Pam3CSK4 treatment did not significantly alter antigen-specific Treg
levels in vivo during OVA tolerance in our studies, it is possible that their suppressive
function was altered. Attempts to address this in ex vivo studies with OVA323-339 peptide
treatment of MLNs and co-culture with OT-II responder T cells were not successful,
likely due to the small number of OVA323-339 –specific Tregs in the MLNs (Figure 5.2).
Future investigations of Treg suppressive functions would likely be more successful
using whole OVA protein for ex vivo treatment of tolerized MLNs prior to co-culture
with OT-II responder T cells.

The tissues and lymphoid organs of mice are not closed systems, and cells will
readily travel between many of these sites over the course of a complex immunologic
process like oral tolerance. Practical limitations prevent the assessment of all organs at
all timepoints, and we can therefore never be certain that cells were not performing
important functions in sites or at timepoints that were not assessed. For example, in my
studies I was unable to assess antigen-specific Tregs in the LP due to the low number of
recovered cells in this site (Figure 5.3). Recently, Wang et al. demonstrated that TLR2
and MyD88 are necessary for DCs to imprint T cells with intestinal homing markers α4β7
and CCR9 (216); while Hadis et al. have reported that the LP is a critical site of Treg
function in tolerance (49). In light of these reports, in future studies it may be important
to transfer a larger number of antigen-specific cells into recipient mice in order to directly
assess Tregs in the LP, particularly in the context of oral TLR2 activation.
There has been a lively controversy about the appropriate age for children to be introduced to potential food allergens (331). The timing of exposure to oral antigen is thought to be an important factor in the development of tolerance, as tolerance is established much less efficiently in older mice (31, 332). In support of this, a carefully controlled epidemiological study has suggested that early oral exposure to peanut may reduce the incidence of peanut allergy in children (333). However, it should also be noted that animal studies tracking in utero antigen exposure and oral exposure in neonatal mice show that very early responses can sensitize, with tolerance occurring only in mice fed 1 week post-birth and beyond (257). This evidence suggests that the age of tolerance induction is critical and therefore outcomes observed in one context may not apply broadly to all subjects. As young adult mice were used in our studies, the findings may not translate directly to elderly or very young subjects. Further studies exploring the impact of TLR2 activation on tolerance induction in young and old mice will be important to further characterize this effect and the implications for patients of different ages.

I determined that oral treatment with Pam3CSK4 served as a potent adjuvant to target plasma and secreted antigen-specific IgA. In these studies, the antigen-specific sIgA levels were assessed by ELISA in fecal samples at a 1/50 dilution when possible, as naïve untreated control samples had high background signals at a 1/5 dilution. Even at a 1/50 dilution, naïve untreated control samples had detectable signals for both OVA-specific and β-lactoglobulin-specific sIgA. These background signals indicate that there may be baseline levels of cross-reactive sIgA to these antigens, or that multimeric IgA-mucous complexes are interfering with the ELISA assay by non-specifically binding the biotinylated antigen, or through direct enzymatic phosphatase activity on the ELISA substrate. These possibilities indicate that fecal samples showing low levels of IgA secretion should be interpreted with caution, as these samples may not contain any actual antigen-specific IgA.

Differences can be noted between our work with selected TLR2 activation and published work examining the outcome of whole live bacteria on oral tolerance (334). Moreover, many live bacteria enhance local colonic Treg responses and some have been
shown to amplify total IgA production (114, 232, 233, 235, 335). These differences highlight the shortcomings of a reductionist approach to PRR systems, whereby dissecting the components of commensal bacteria may mislead interpretations about the function and importance of whole organisms in a complex process like oral tolerance. However, this approach will help us draw out therapeutic targets from the complex stimulation profiles of bacteria, facilitating the design of better immunotherapies and vaccination strategies. Furthermore, by comparing the differences between whole organism signaling and specific PRR activation we can identify how multiple host detection responses interact and come together to result in the final commensal tolerance response.

### 5.4 Proposed Future Directions of this Work

By isolating the immunomodulatory effects of sub-anaphylactic IgE-mediated mast cell activation on oral tolerance, I have shown that this level of allergic activation does not appear to pose a risk to tolerance development. However, the physiological implications of full-blown oral anaphylaxis must also be considered in order to fully understand the role of mast cells in oral tolerance. Mice from genetic backgrounds with low levels of intestinal mast cells, such as C57BL/6, are refractory to oral anaphylaxis (19). This made the direct study of mast cells and oral anaphylaxis challenging, but we have since begun crossing mast cell-deficient KitW-sh/W-sh mice onto the BALB/c background. With this tool, we will be able to investigate the roles of mast cells in local intestinal permeability and cytokine changes following oral anaphylaxis and examine the implications for oral tolerance to a bystander food antigen. This future study will be important in completing the analysis of mast cell activation in oral tolerance, and studies in oral anaphylaxis may uncover new clinical considerations and treatment modalities for anaphylactic patients.

TLR2 expression in recipient mice was not necessary in order to establish antigen-specific Tregs in the MLN or spleen during oral tolerance induction. However, TLR4 and TLR2 may have overlapping or redundant roles in terms of regulating intestinal responses to commensal organisms and foreign antigen. Exploring the extent of Treg induction in the GALT and spleen in MyD88−/− mice will help better characterize
the function of intestinal PRRs in oral tolerance induction to foods. Furthermore, it remains to be seen whether the antigen-specific Tregs in tolerized TLR2−/− mice will effectively suppress antibody responses to a systemic antigen challenge. It will be important to investigate the efficacy of humoral tolerance in these mice.

After delivering the TLR2 activator Pam3CSK4, during oral tolerance, we observed a failure of suppression of the humoral response upon systemic antigen challenge. This effect may not be related to antigen-specific Tregs, as their levels were not altered in the PPs, MLN, or spleen. There is evidence for intestinal microbes regulating antibody production independent of T cell helper functions (335), and several studies have shown direct effects of TLR2 agonists on B cell antibody production and activation thresholds (328) (306). Despite this evidence, total B cell and plasma cell levels were not altered in the GALT and spleen in our model of TLR2 activation. In the future, it will be important to examine Pam3CSK4-treated B cell functionality and antigen-specific responses ev vivo with proliferation assays addressing T cell activation thresholds and an enzyme-linked immunosorbent spot assay to assess antigen-specific antibody production.

Repeated oral treatment with OVA and Pam3CSK4 led to enhanced OVA-specific IgA responses. To verify that this is indeed a TLR2-dependent event, and before we can explore the mechanisms of this effect, this basic study must be repeated in TLR2−/− mice. It would then be pertinent to explore the efficacy oral Pam3CSK4 immunization in a pathogen challenge model and evaluate the protective outcomes in vivo. Candidate pathogens for this study should be vulnerable to elimination by IgA.

The promotion of antigen-specific IgA, both in plasma and secreted in the intestine, in response to repeated oral Pam3CSK4 highlights a potential therapeutic option for IgA immunodeficiencies. Some IgA deficiencies are not complete, and disease manifestations can be transient (107, 108). Oral treatment with Pam3CSK4 could be investigated as a potential agent to amplify IgA production, thereby bolstering mucosal immunity or vaccination in the context of IgA deficiency. There are several animal models of selective IgA deficiency, but it is not clear how each of these correlates to human disease (336). Careful selection of an appropriate animal model would be important in such studies.
Finally, a reductionist approach to the study of oral tolerance may not be sufficient given that we are attempting to model a complex immunologic process that explicitly relies on balancing pathogenic responses with commensal tolerance. It is critically important to dissect the interface between microbial products and the immune system, but looking to other disciplines such as ecology, systems biology, and mathematical modeling may prove informative as we attempt to understand and manipulate oral tolerance.

5.5 Conclusions
Mast cells and TLR2 are not necessary for the induction of oral tolerance. However, TLR2 activation during early exposures to oral antigen has significant immunomodulatory outcomes on oral tolerance. Notably, oral TLR2 activator administration promotes a selective IgA response to repeated oral antigen, but conversely impairs humoral tolerance in multiple antibody classes upon systemic antigen challenge. This difference underscores the complexity and compartmentalization of oral tolerance.

We have come a long way since Tregs were first identified as critical mediators of peripheral tolerance. Tolerance research is now a dynamic field exploring the complex interactions between tissue sites, host innate cells, and the myriad microbes that we harbour. A metagenomics approach to the human microbiome represents a significant milestone in this field. However, the information obtained from such studies cannot be properly applied until we better understand the fundamental relationship between innate signals and innate responder cells in the context of peripheral tolerance.
Figure 5.1 Model of oral tolerance regulation by oral TLR2 activation and mast cells.

Schematic representation of the proposed model for modulation of oral tolerance by oral TLR2 activation and the role of mast cells.  

A, Antigen is encountered in the GALT upon initial food exposure. This results in the differentiation and expansion of antigen-specific Tregs and helper T cells in the GALT, followed by systemic distribution of these cells to the spleen. B cells in the GALT will be minimally activated by antigen-specific T cells, initiating low levels of antibody production, primarily IgA.  

B, Upon secondary oral challenge or systemic antigen challenge (C), antibody levels remain suppressed by Treg activity.  

D, TLR2 is not necessary in order to establish antigen-specific Tregs in the GALT during oral tolerance. Moreover, when oral antigen is introduced in the presence of a TLR2 activator the initial antigen-specific T cell responses progress normally in the GALT and spleen, but functionality may be altered. Total B cell and plasma cell levels are not changed by oral TLR2 activation, but activation thresholds may be altered.  

E, After initial TLR2 activation, a secondary oral antigen challenge will dramatically enhance antigen-specific IgA levels but other antibody classes are unchanged.  

F, The suppression of antigen-specific antibody levels is compromised upon secondary systemic antigen challenge following oral TLR2 treatment; antigen-specific IgA, IgE, IgG1, and IgG2a are all enhanced.  

G, Mast cells are not involved in the initiation of oral tolerance, but IgE-mediated activation during secondary systemic antigen challenge modulates IgG2a responses (H).
Figure 5.1
Figure 5.2 *Ex vivo* co-culture suppression assay with OVA-treated MLN cells and OT-II CD4+ cells.

C57BL/6 mice were treated orally with water or OVA *ad libitum* for 1 week. During the week mice received gavage treatments of PBS, OVA, or OVA + Pam3CSK4. MLNs were extracted and pulsed with LPS ± OVA323-339 peptide for 3h, as indicated. Pulsed MLN cells were co-cultured for 3.5 days with naïve OT-II CD4+ T cells containing proliferation dye isolated by MACS. The % divided of total non-Treg OT-II cells in each condition was assessed at the end of the co-culture. Data represent one pilot experiment. Groups were compared by ANOVA followed by Bonferroni’s Multiple Comparison Test. ***p < 0.05, n.s., not significant.
Figure 5.3 Assessment of antigen-specific Tregs in the LP versus MLN of tolerized mice.

1x10⁶ naïve OT-II CD4⁺/CD45.1⁺/Foxp3-GFP cells were transferred into 3 male C57ML/6 mice. Mice were provided with OVA in water for 1 weeks *ad libitum* at 4mg/mL. On day 7 of OVA treatment, MLN and LP were removed from mice and the recovered transferred OT-II cells were assessed by flow cytometry. A, Flow cytometry density plots showing the number of collected events and CD45.1⁺/CD4⁺ gated events representing transferred cells in the LP and in the MLN (B) from 3 mice. Data depict 3 mice from 1 pilot study.
Figure 5.3

A  Lamina propria

B  Mesenteric lymph node

Mouse 1

Mouse 2

Mouse 3
REFERENCES


APPENDIX A. TH2 Polarization of OVA Tolerance by Peanut Butter

My data suggest that allergic mast cell activation to one food will not necessarily alter the progression of oral tolerance to a second food, but microbial contaminants such as TLR2 activators may have detrimental outcomes on tolerance induction upon systemic antigen challenge. Some food allergens, such as peanut, are known to have immunogenic properties such as complement activation (8). Furthermore, it has been shown in vitro that treatment with the allergenic peanut epitope Ara h 1 can activate DCs to promote a TH2-polarized phenotype in responder cells (7). This represents another avenue by which one food may alter the progression of oral tolerance to a second.

We investigated whether peanut could have a TH2-polarizing adjuvant effect on the induction of oral tolerance to OVA in vivo. Mice were treated with OVA in water ad libitum, with 3 additional doses of 1mg OVA by gavage on days -9, -6, and -3 (as in Figure 2.3, A). One treatment group received 1mg of peanut butter (PB) protein concurrent with the gavage treatment of OVA. A control group received PBS treatments. On day 0, all mice were immunized with OVA-alum, then boosted with OVA two weeks later. Blood was harvested one week after boost, and OVA-specific antibodies were assessed by ELISA. Oral tolerance was successfully induced in mice treated with OVA or mice treated with OVA + PB, assessed by suppression of OVA-specific IgE (p<0.001, p<0.001, respectively), IgA (p<0.001, p<0.001, respectively), IgG1 (p<0.001, p<0.01, respectively), and IgG2a (p<0.001, p<0.001, respectively) (APPENDIX B,a-d). Although tolerance was not prevented by the contamination of OVA with PB, the ratio of OVA-specific IgG1/IgG2a was dramatically enhanced in PB-treated mice (p<0.01) (APPENDIX B,e). Surprisingly, oral treatment with 1mg of CPE + OVA did not result in a significant change to the IgG1/IgG2a ratio to OVA compared to groups treated with OVA alone (APPENDIX B,f). However, a similar trend was observed as with PB treatment but the effect was more variable with CPE treatment.

This important observation suggests that PB can modulate early tolerance responses to other oral antigens. TH2-polarized responses to food are associated with the allergic phenotype, and PB should be investigated further in order to determine the
mechanism by which it is promoting a Th2-polarized response to a bystander food antigen. Possible candidate mechanisms include complement activation resulting in local production of anaphylatoxins, or direct actions of peanut on DCs thereby modifying the ensuing cytokine response. Studies employing complement anaphylatoxin receptor-deficient mice and studies with purified Ara h 1 may help elucidate these mechanisms of action in vivo.
APPENDIX B. Oral Treatment With PB Polarizes the OVA-specific Antibody Response

BALB/c mice were treated with OVA ± PB by gavage treatment 3 times during a week of ad libitum 4mg/mL OVA-water treatment. Control groups were treated with PBS and provided with normal water. All mice were immunized with OVA-alum and boosted with OVA. Levels of OVA-specific antibodies were assessed by ELISA in plasma samples harvested one week after boost.  

- **a**, OVA-specific IgE levels were assessed by ELISA and expressed as ng/mL.  
- **b**, OVA-specific plasma IgA levels were assessed by ELISA and expressed as standard-adjusted OD.  
- **c**, OVA-specific plasma IgG1 levels were assessed by ELISA and analyzed by titre analysis and expressed as median with IR.  
- **d**, OVA-specific plasma IgG2a levels were assessed by ELISA and analyzed by titre analysis.  
- **e**, OVA-specific plasma IgG1 and IgG2a levels were expressed as a ratio of IgG1 / IgG2a following oral treatment with OVA ± PB.  
- **f**, OVA-specific plasma IgG1 and IgG2a levels were expressed as a ratio of IgG1 / IgG2a following oral treatment with OVA ± CPE.  

IgE and IgA levels were compared between groups by ANOVA followed by Bonferroni’s Multiple Comparison Test, whereas IgG1 and IgG2a levels were compared between groups by Kruskal-Wallis test followed by Dunn’s Multiple Comparison Test. Bars represent mean+SEM IgE and IgA levels, or median –Log titre IgG1 and IgG2a levels with IR. *p < 0.05, **p < 0.01, ***, p < 0.001, n.s., not significant.
### APPENDIX B

<table>
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**PBS OVA OVA + PB**

**n = 9-10**

**OVA-specific IgE**

**OVA-specific IgA**

**OVA-specific IgG1 / IgG2a**

**OVA-specific IgG1 / IgG2a Ratio**
APPENDIX C. Copyright Release of Published Material

May 28, 2012

The Journal of Allergy and Clinical Immunology

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