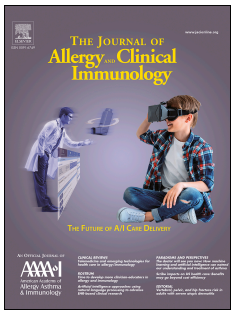
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Rima Rachid, M.D., Emmanuel Stephen-Victor, Ph.D., Talal A. Chatila, M.D., M.Sc.

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The Microbial Origins of Food Allergy

Rima Rachid, M.D.[1,2]*, Emmanuel Stephen-Victor, Ph.D.[1,2]*, Talal A. Chatila, M.D., M.Sc.[1,2,]†

Affiliations: [1]Division of Immunology, Boston Children's Hospital, Boston; [2]Department of Pediatrics, Harvard Medical School, Boston.

*These authors contributed equally to this work

†**Corresponding Author**: Talal Chatila at the Division of Immunology, Boston Children's Hospital, Karp Family Ressearch Bldg., Rm 10-214-B, 1 Blackfan Circle, Boston MA 02115. Phone: 617-9193529; Fax: 617-7300528; Email: Talal.chatila@childrens.harvard.edu.

Conflict of Interest: T.A.C. and R.R. are inventors on published US patent application, US10391131B2 that covers methods and compositions for the prevention and treatment of food allergy using microbial treatments. T.A.C., R.R. and E.S.-V. pending patent applications related to the use of probiotics in enforcing oral tolerance in food allergy (62/758,161, and, 62/823,866). T.A.C and R.R have equity in Pareto Bio.

Key words: food allergy; IgA, IgE, microbiota; microbiome; fecal microbiota transplantation; dysbiosis; regulatory T cells; RORγt+ Treg;

Abbreviations: Ag: antigen; APC: antigen presenting cells; EC: epithelial cells; EGF: epidermal growth factor; GAPs: goblet cell-associated antigen passages; GC: goblet cells; Germ free: GF; Fecal Microbiota Transplantation: FMT; Food Allergy: FA; Induced

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Treg: iTreg; Oral Immunotherapy: OIT; Specific Pathogen Free: SPF. Toll like receptors: TLRs

Abstract

Food allergy (FA) is a significant public health issue, propelled by its rapidly increasing prevalence. Its sharp rise into prominence has focused attention on causative environmental factors and their interplay with the immune system in disease pathogenesis. In that regard, there is now substantial evidence that alterations in the gut microbiome early in life shape the host gut mucosal immunity and may play a critical role in the development of FA. These changes may impact key steps in the development of the infant gut microbiome, including its shaping by maternal factors and upon the introduction of solid food (the weaning reaction). These early life changes may have long range effects on host immunity that manifest later in time as disease pathology. Experimental studies have shown that resetting the host intestinal immune responses by treatment with either a healthy fecal microbiota transplantation or defined commensal bacterial taxa can prevent or treat FA. The mechanisms by which these interventions suppress FA include restoration of gut immune regulatory checkpoints, notably the retinoic orphan receptor gamma T (RORγt)$^{+}$ regulatory T cells, the epithelial barrier and healthy immunoglobulin A responses to the gut commensals. These findings inform human studies currently in progress that evaluate the role of microbial therapies in FA.

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Introduction

Food allergy (FA) is a major public health concern, whose prevalence has grown dramatically in recent years to affect up to 8% of children and 4% of adults [1, 2]. An affluent life style common in developed countries appears to be a significant risk factor for developing FA [2], suggesting that environmental exposures play a major role in disease development. Of particular interest are factors predisposing to FA that impact the development of a healthy gut bacterial symbiosis with the host (commensalism) [3]. As detailed in this review, perturbations in gut bacterial population (termed dysbiosis) are at the foundation of a microbial hypothesis of FA predisposition.

The role of a determining microbial influence in the development of allergic diseases was first articulated in the hygiene hypothesis, which emphasized the importance of early life microbial infections in curtailing the development of allergic disorders [4, 5]. Several studies suggested that events that affect the gut microbiota composition in early life may predispose to the development of atopic diseases [6]. Factors such as living in a farm environment and exposure to a large number of pets in the first year of life may lead to a more diverse pool of microbes and are associated with protection from allergic disease. On the other hand, cesarean section delivery, antibiotic intake prenatally or perinatally and gastric acidity inhibitors intake may adversely affect the microbiome and increase the risk of atopy [7-11].

Over the past decade, there has been mounting interest in the microbiome as a potential instigating factor for the development of FA [12-14]. More recently, a few studies evaluated this role in more depth. Here, we discuss the key role of the microbiome in FA, the function of commensals in imprinting immune tolerance to food and the

mechanisms by which aberrant gut microbiota-immune system interaction precipitates FA.

Early life immune imprinting by the gut microbiota.

The initial gut microbiota of a newborn is seeded from different sources depending on the mode of delivery: primarily from maternal vaginal and gut microbiota for vaginal delivery or otherwise from skin microbiota for cesarean delivery [15]. It is further shaped by microbial and immunological components present in the breast milk [16, 17]. The early post-natal gut microbiota is restricted in diversity and is characterized by the presence of *Bifidobacteria* and *Lactobacillus* species that are capable of degrading complex oligosaccharides present in the milk, providing energy source for the developing infant [18, 19]. Importantly, in a recent pre-print report, effective colonization with *Bifidobacteria* species early in human infancy is associated with decreased innate immune inflammatory responses and the acquisition by the gut T effector (Teff) and T regulatory (Treg) cells of markers of immunological memory, indicative of imprinting of the gut immune system by a healthy microbiota [20]. Furthermore, supplementation of full term breastfed infants with *Bifidobacterium infantis* suppressed T helper type 2 (Th2) and Th17 cell responses while inducing interferon β production, suggesting the induction of a favorable immune regulatory response [20].

The transition from an exclusive milk-based diet to a solid food one is associated with a dramatic increase in microbial diversity [15, 19, 21] (**Figure 1**). This transition is governed by cues present in the maternal milk and nutrients being supplied by solid foods. Epidermal growth factor (EGF) present in the milk is critically involved in regulating early life microbial dynamics in the gut [22]. Post-natal decline of breast milk EGF and the

89 introduction of a solid food-based diet dramatically shifts the gut microbial ecology in
90 favor of a blooming immunomodulatory *Clostridiales* and *Bacteroidetes* species, a
91 process described as the weaning reaction in mice [21]. A similar shift in microbial
92 diversity and composition is also seen in human infants upon the introduction of solid
93 foods [19] Immune exposure to microbial and dietary antigens during this period imprints
94 the immune system with long-lived tolerogenic response mediated by RORγt$^+$ Treg cell
95 population [21]. The differentiation of RORγt$^+$ Treg cell population is regulated by several
96 factors. Studies in mice have shown that the maternal IgA transferred through the
97 breast milk establishes a homeostatic set point that governs the frequencies of RORγt$^+$
98 Treg cells in the gut and which is reproduced from one generation to the other. The
99 underlying mechanism appears to involve the coating of bacteria found in the post-natal
100 intestine by the maternal IgA, which modulates their capacity to stimulate the
101 differentiation of RORγt$^+$ Treg cells [23]. Immunomodulatory components of the microbiota
102 (cell surface polysaccharide), dietary factors (Vitamin A) and secondary metabolites
103 production by the microbiota (short chain fatty acids and secondary bile acid
104 metabolites) promote the differentiation of RORγt$^+$ Treg cell population [24-26]. Additionally,
105 the weaning reaction upregulates the expression of TGF-β1 in Treg cells, which is
106 critical for the differentiation of RORγt$^+$ Treg cells (**Figure 1**) [27].

107 The weaning reaction coincides with a time window at which the immune system
108 is malleable to tolerance induction to different solid foods introduced during that period.
109 Notably, early introduction of allergenic foods in infants with high risk of developing FA
110 was associated with the reduced incidence in the development of FA later in life [28, 29].
111 Thus, tolerance imprinted at this stage may have long-range effects lasting into

adulthood. Dysbiosis at the time of the weaning reaction may thus give rise to ineffective induction of RORγt$^+$ Treg cell differentiation that may have long-term implications in terms of susceptibility to gut pathologies including FA. Such long terms effects were demonstrated in the Consortium of Food Allergy (CoFAR) longitudinal cohort study, which analyzed the microbiome composition of milk allergic children in infancy whose allergy persisted or resolved by the age of 8 years [30]. In subjects who later became milk tolerant, there was enrichment of *Firmicutes* phylum and *Clostridia* class taxa in their stool microbiome at 3-6 months of age, while *Bacteroidetes* and *Enterobacter* were more abundant in the stools of those infants whose milk allergy was still ongoing at age 8 years [30]. It is plausible that the same mechanisms involved in long-range tolerance imprinting by the microbiota early in life is shared between mice and humans, although this hypothesis remains to be fully investigated in human subjects.

Several studies have documented the importance of the gut microbiome in regulating FA, however, few of them have investigated the dynamics of early life microbiome in FA. Abdel-Gadir *et al* analyzed the longitudinal development of the gut microbiota in 154 babies, with or without FA, who were enrolled between the ages of 1-15 months [31]. FA was diagnosed by a history of allergic reaction to food combined to skin and or positive food-specific IgE. 16S rRNA gene phylotyping and clinical progression of FA or tolerant state were followed every 6 months through 30 months of age. Results from this study revealed that FA infants manifested an early onset gut dysbiosis that was associated by the loss of *Subdoligranulum variabile* around the age of weaning and persisted into early childhood. Furthermore, *Clostridiales* species belonging to clusters I, IV, XI and XIVa, which are implicated in regulating tolerogenic

responses in the gut showed significant differences in specific age groups. The functional consequence of dysbiosis and the role of *Clostridiales* species in regulating RORγt$^+$ Treg cell responses and FA is discussed below.

Immunological and Microbial Mechanisms of FA.

The hallmark of FA is the generation of a pathogenic Th2 cell response directed at food allergens that is associated with the generation of FA-allergen specific high affinity IgE antibodies. Below we discuss three pertinent issues relevant to the mechanisms of FA: 1) what are the immunological mechanisms that are involved in the development of FA, including immune tolerance breakdown and barrier dysfunction, that allow for the evolution and persistence of FA. 2) What is the role of dysbiosis in initiating and sustaining these changes and 3) the capacity to reprogram immune tolerance in FA by immunomodulatory commensals.

Immunological Mechanisms of tolerance breakdown in FA. A central feature in the pathogenesis of FA is the breakdown of oral tolerance to food allergens, which is normally dependent for its maintenance on Foxp3$^+$ Treg cells. This failure reflects a broader breakdown of immunoregulation at the mucosal surface is suggested by the observations that patients with FA have multiple sensitization (IgE responses) to different food allergens and that many of them have clinical allergy to more than one food [1, 32]. It is also suggested by the fact that in both human FA subjects and mouse models of FA there existed an IgE response not only against the food allergens but also to the microbiota [31]. In mice with FA, therapy with allergen-specific Treg cells completely suppresses disease, indicating a pivotal role for these cells in disease pathogenesis [33, 34].

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158 Investigations into why food allergen-specific Treg cells are dysfunctional in FA have
159 revealed that they undergo a pathogenic Th2 cell-like reprogramming such that they
160 lose their regulatory function and instead promote disease [34, 35]. This reprogramming,
161 also common to human FA subjects and FA mice, entails the increased expression by
162 the Th2 cell-like reprogrammed Treg cells of the Th2 cell transcription factors GATA3
163 and IRF4 and, in some of their cells, the expression of the cytokines IL-4 and IL-13 [34]. In
164 mice, deletion of *Il4* and *Il13* genes in Treg cells largely rescues FA in affected mice.
165 We recently elucidated the mechanism by which Th2 cell-like reprogramming impairs
166 Treg cell function by demonstrating that signaling by IL-4 activates STAT6 to repress
167 the *Tgfb1* locus, leading to decreased TGF-β1 expression by Treg cells by about 50%
168 [27]. The functional consequences of such a decrease were investigated in mice with Treg
169 cell-specific *Tgfb1* haploinsufficieny. The Treg cells of these mice, which were highly
170 atopic and prone to the development of FA, ineffectively suppressed mast cell
171 expansion and activation and IgE production. Importantly, the circulating Treg cells of
172 human subjects with FA expressed decreased levels of the TGF-β1 precursor LAP1,
173 indicating that this mechanism is operative in the human disease as well [27].

174 *Role of dysbiosis in tolerance breakdown in FA*. Studies by different groups, including
175 ours, have identified the presence of dysbiosis in human FA subjects [7, 12, 13]. The FA
176 susceptible *Il4ra*^{F709} mice also exhibit dysbiosis that was pathogenic in nature. Early
177 studies by our group employing fecal microbiota transplantation (FMT) demonstrated
178 that transfer of microbiota of FA *Il4ra*^{F709} mice to otherwise FA-resistant GF wild-type
179 (WT) mice transmitted susceptibility to disease induction [33]. Furthermore, we also
180 demonstrated that the microbiota of human subjects with FA and of FA *Il4ra*^{F709} mice

failed to protect GF *Il4ra*^{F709} mice against the development of FA. By contrast, the microbiota of healthy human subjects and FA-resistant WT mice were protective. Studies by Feehley *et al* also demonstrated that the microbiota of human subjects with cow milk allergy failed to protect against the induction of FA responses when transferred into GF mice [36].

The mechanisms by which dysbiosis impairs immune tolerance in FA involve the loss of tolerance promoting RORγt$^+$ Treg cells. In that regard, human subjects and mice with FA display decreased RORγt$^+$ Treg cells [31]. Transfer of microbiota from healthy donors to FA-prone GF *Il4ra*^{F709} mice upregulated the frequency of RORγt$^+$ Treg cells in the gut, whereas the transfer of dysbiotic microbiota from FA subjects failed to do so. The critical role of RORγt$^+$ Treg cells in oral tolerance was revealed by the susceptibility to FA of mice with Treg cell-specific deletion of *Rorc*, encoding RORγt [31]. One mechanism by which commensals promote RORγt$^+$ Treg cell differentiation involves the upregulation of *Tgfb1* transcription in Treg cells, which in turn acts in an autocrine fashion to drive the differentiation of nascent Treg cells in the gut into RORγt$^+$ Treg cells [27]. Disruption of commensalism due to the loss of critical immunomodulatory bacteria in FA subjects may be linked to their observed deficiency of TGF-β1 and RORγt$^+$ Treg cell responses.

An important unresolved issue is whether the RORγt$^+$ Treg cells induced by the microbiota are allergen specific or are acting in a non-allergen specific manner to induce infectious tolerance. Previous studies have documented that induced Treg cells in the gut are enriched in T cell receptors specific for the microbiota [37]. On the other hand, RORγt$^+$ Treg cells specific for dietary antigens can develop under certain experimental

conditions [38]. Future studies will need to delineate the specificity of the RORγt$^+$ Treg cells mediating tolerance to food and microbial antigens.

Role of the microbiota in regulating the IgE response. Generation of antigen specific IgE responses depends on the clonal selection of B cell, a process requiring the help of CD4$^+$ T cells. T follicular helper (Tfh) cells that produce IL-4, a B cell growth factor influence the affinity and sequential class switching in B cells [39]. Crosslinking of high affinity IgE on mast cells upon food allergen exposure leads to mast cell degranulation and in some cases systemic anaphylaxis. Recently, Gowthaman *et al* investigated the nature of CD4$^+$ T cells that regulate high affinity IgE responses to food and environmental allergens and described a population of Tfh cells termed Tfh13. These cells produce high levels of IL-4, IL-13 and IL-21, which are necessary for generating high affinity anaphylactic IgE antibodies [40]. Interestingly, experimental studies in mice have documented a key role for the gut microbiota in regulating IgE responses. GF mice are associated with abnormal serum IgE levels that are largely directed to food antigens [41]. Furthermore, GF mice display an aberrant Tfh response that directs the IgE response to foods. Notably, GF mice exposed to conventional commensal suppressed the aberrant Tfh response generated to food antigens (**Figure 2**) [41]. These findings suggest that the gut microbiota plays an important role in regulating the Tfh responses. While the gut microbiota in general and certain commensals in particular, including some *Clostridialis* and *Bacteroidales* species, have been implicated in suppressing antigen-specific IgE responses [27, 31], their role in regulating Tfh13 responses remains to be elucidated.

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IgA and IgE response to the gut microbiota. IgA is the dominant immunoglobulin produced in healthy humans with the highest amounts being secreted in the gastrointestinal tract. IgA responses play a key role in the maintenance of gut mucosal homeostasis. Binding of IgA prevents direct sensing of commensals, promotes bacterial diversity and maintains gut mucosal homeostasis [42]. Studies evaluating the IgA responses in allergic subjects have indicated that dysregulated IgA response were associated with increased risk for allergic manifestation in early life [43, 44]. Notably, a lower IgA binding to the gut bacteria early in life was associated with an increased susceptibility to the development of asthma and allergy [45]. Accordingly, we found a lower IgA binding to gut microbiota in FA subjects and in FA-prone *Il4ra*^{F709} mice [31]. Furthermore, in both human subjects with FA and in the FA susceptible *Il4ra*^{F709} mice we detected IgE binding to the gut bacteria [31]. It is currently unknown whether the IgE bound to the bacteria is reactive to specific bacterial taxa that are actively promoting an IgE response, and the consequences that such a response may lead to. One possibility is that the IgE response directed to food antigens may be cross-reacting to bacterial epitopes presumable due to molecular mimicry. Studies in parasitic infections that are associated with the generation of an IgE response have documented cross-reacting IgE binding to food antigens [46, 47]. For example, a study investigating the cross-reactivity of IgE in children documented that infection with *Schistosoma haematobium* was associated with increased peanut-specific IgE titers that could bind to cross-reactive carbohydrate determinants [48]. Future studies will need to investigate the nature and consequences of IgE responses to the microbiota including their role in disease initiation and persistence (**Figure 2**).

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Barrier Protection by the microbiota in oral tolerance. In addition to promoting Treg cell differentiation, the microbiota may also reinforce oral tolerance by acting at other cell types, including epithelial cells, antigen presenting cells and innate lymphoid cells (ILCs). In that regard, Colonization of GF mice with Clostridial taxa was reported to protect from oral allergenic sensitization via the induction of IL-22^{+} innate lymphoid cells type 3 (ILC3) at the intestinal mucosa [49]. IL-22 reduces mucosal permeability, promotes mucus secretion by goblet cells and prevents seepage of protease-resistant allergens though the intestinal barrier [50]. Notably, ILC3 are also potent producers of IL-2 which plays a key role in maintaining gut homeostasis by supporting the generation of Treg cells in the gut. Deletion of IL-2 in ILC3 was associated with decreased frequencies of Treg cells in the gut and a failure to acquire oral tolerance to dietary antigens [51].

Resetting of the dysbiosis to promote oral tolerance in FA.

The above reports, while emphasizing the critical role of dysbiotic microbiota in the pathogenesis of FA, also raised the prospect of restoring immune tolerance by resetting the underlying dysbiosis, whether by bacteriotherapy with immunomodulatory bacterial species or by FMT. Of particular interest is the role of Clostridial species, which have previously been implicated in the induction of Treg cells in the gut, suggesting a role for some of these bacterial species in promoting tolerance to foods. In accordance with this proposition, dysbiosis in FA was found to affect multiple Clostridial taxa, suggesting a role in the loss of these bacterial species in disease pathogenesis. Critically, bacteriotherapy with a consortium of immunomodulatory Clostridial species impacted by the dysbiosis in human FA subjects suppressed FA in *Il4ra*^{F709} mice [31]. This effect was mediated by the induction of the Clostridial consortium of RORγt^{+} Treg cells, as

271 evidenced by the resistance of *Il4ra*^{F709} mice to disease rescue by the consortium when
272 *Rorc* is deleted in Treg cells [31]. Induction of RORγt^{+} Treg cells responses was likely
273 mediated by direct sensing of the bacteria by Treg cells. This was evidenced by the
274 observation that deletion of myeloid differentiation response gene–88 (*Myd88*) in Treg
275 cells abrogated the capacity of the bacteria to induce RORγt^{+} Treg cell differentiation
276 and to protect mice from the development of FA. Therapy with a different bacterial
277 consortium composed of immunomodulatory *Bacteroidales* species also suppressed FA
278 by inducing RORγt^{+} Treg cell differentiation in a Treg cell-specific MyD88-dependent
279 manner, indicating that this pathway is canonical in mediating the protective effects of
280 commensal bacteria in oral tolerance [31].

281 In a further refinement of the bacteriotherapy approach, we tested the capacity of an
282 individual Clostridial bacterium found profoundly depleted in FA subjects age one year
283 and older to suppress FA. Monotherapy with this bacterium, *Subdoligranulum variable*,
284 ameliorated disease induction in *Il4ra*^{F709} mice in association with the increased
285 frequency of RORγt^{+} Treg cells [31]. In a separate approach, Feehley et al showed that
286 GF mice receiving FMT from healthy subjects were associated an expansion in the
287 abundance of *Anaerostipes caccae*, a Clostridial species that ameliorated allergic
288 response to β-lactoglobulin in the sensitized mice [36]. In this case, *A. caccae* was
289 proposed to act by shoring up the epithelial barrier functions.

290 The capacity of metabolites derived from immunomodulatory bacteria to enforce
291 tolerance has received special attention. Several classes of metabolites have been
292 shown to promote gut Treg cell responses and promote epithelial barrier integrity,
293 including short chain fatty acids and secondary bile acids. Short chain fatty acids have

been implicated in promoting oral tolerance in some models of FA, but not others [52]. Secondary bile acids, which are derived from primary bile acids by the action of the gut microbiota, have been shown to activate vitamin D receptor on Treg cells to induce RORγt$^+$ Treg cell differentiation and maintain their gut homeostasis [25]. Metabolomic analysis on the plasma of FA allergic subjects revealed disturbances in secondary bile acid metabolism, consistent with the dysbiosis in FA impacting secondary bile acid generation [53]. The role of these metabolites as well as other bacterial metabolites including aryl hydrocarbon receptor ligands and sphingolipids among others requires further investigation.

The preclinical studies discussed above provided a proof of concept for the feasibility of bacteriotherapy in reestablishing tolerance in FA, setting the stage for human interventional trials to evaluate the therapeutic and preventative effects in FA of FMT and other more targeted bacteriotherapies. A phase Ib/II study is currently ongoing to evaluate the safety and efficacy of VE416, a commensal bacterial product in patients undergoing peanut oral immunotherapy (NCT03936998). Separately, we are conducting a phase I trial to evaluate the safety and efficacy of FMT, with and without pre-treatment with antibiotics, in adults with peanut allergy (NCT02960074). Nevertheless, going forward, several issues need to be addressed, including the safety profile of such therapies, and whether such therapies act as adjuvant for oral immunotherapy (OIT) or are capable of enforcing oral tolerance independently. Also unknown at this stage is the capacity of such therapies to reset the dysbiosis in FA in favor of long-term tolerance induction. Finally, it remains unclear whether bacterial metabolites implicated in the

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316 mechanism of action of immunomodulatory bacteria could be employed therapeutically
317 in lieu of live bacteria.

318 **Conclusion**

319 There is now substantial evidence in favor of a microbial hypothesis in the pathogenesis
320 of FA. Dysbiotic perturbations early in life, including those that disrupt normative
321 microbial engraftment post birth and/or the weaning reaction, as well dysbiosis in older
322 children and adults may lead to the loss of oral tolerance and the development of FA.
323 The capacity to recapitulate early life tolerogenic imprinting of the immune system by
324 the gut microbiota especially as relates to the weaning reaction may provide a model for
325 therapeutic interventions at any age aiming to re-establish oral tolerance, correct the
326 underlying pathogenic dysbiosis and restore healthy microbiota.

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Conflict of Interest Disclosure

R.R. and T.A.C. is an inventor on published US patent application, 15/801,811, that covers methods and compositions for the prevention and treatment of FA using microbial treatments. R.R., E.S.-V. and T.A.C. have pending patent applications related to the use of probiotics in enforcing oral tolerance in FA (62/758,161, and, 62/823,866). T.A.C. is a co-founders of and has equity in ParetoBio. R.R. has equity in ParetoBio.

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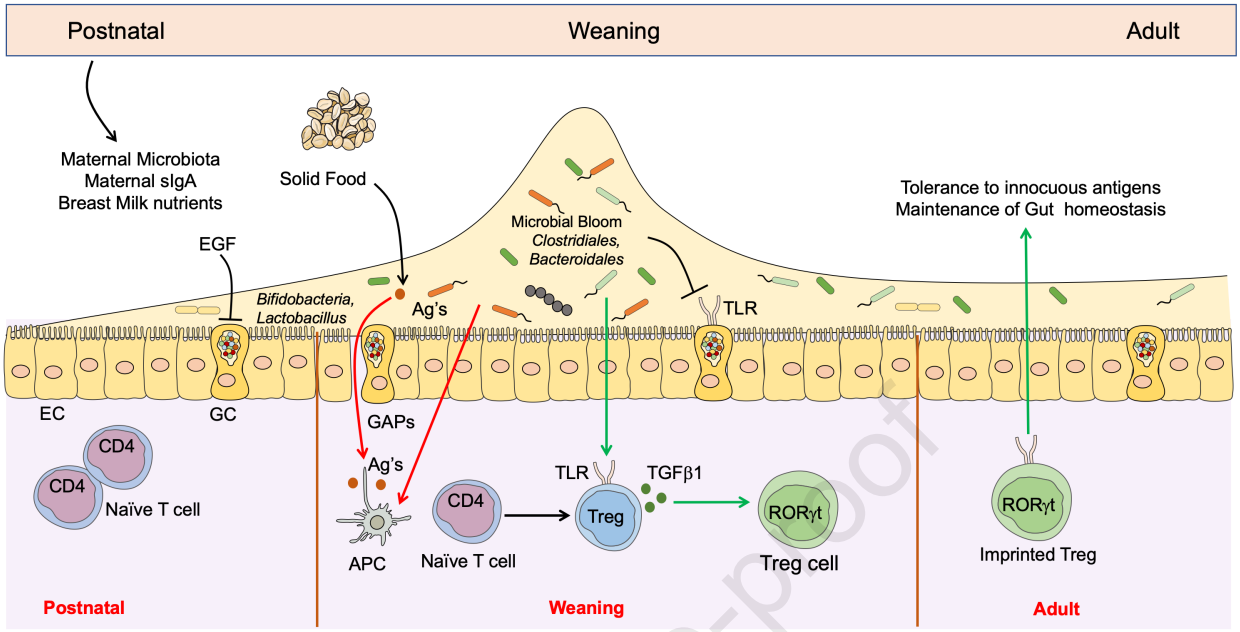
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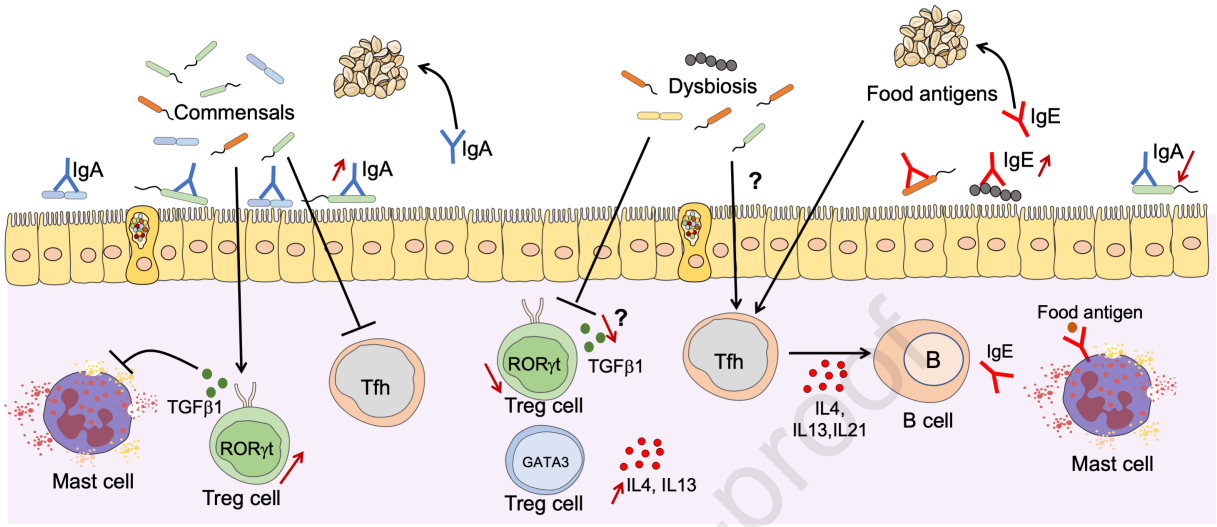
Figure Legends

Figure 1. **Maternal, Dietary, and Microbial factors shape early life oral tolerance**. Schematic representations of post-natal changes in the gut microbiome and the mucosal interface. After birth, the infant gut microbiota is shaped by Immunoglobulin A and other immunological components present in the mother's milk. Transition to a solid food diet (weaning) expands the diversity of gut microbiota composition with an expansion of *Clostridales* species. Induction of *Tgfb1* in Treg cells by commensals drives the differentiation of long-lived ROR-γt^+ Treg cells that regulate tolerance to gut content, including bacteria and dietary antigens. Ag: antigen; APC: antigen presenting cells; EC: epithelial cells; EGF: epidermal growth factor; GAPs: goblet cell-associated antigen passages; GC: goblet cells; TLR: toll-like receptors.

Figure 2. Microbial regulation of oral immune tolerance to food and its breakdown in FA. Dysbiosis caused by aberrant insults to the gut microbiota early in life impairs the differentiation of ROR-γt^+ Treg cells. Breakdown in the development of oral tolerance is associated with dysregulated anti-microbial antibody responses to gut commensals that is characterized by a decreased IgA binding and an increased IgE binding to the gut microbiota. Anti-food antigen T follicular helper cell (Tfh) responses develop that promote the generation of high affinity anaphylactic IgE.



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