

# Maternal Milk, but Not Formula, Regulates the Immune Response to $\beta$ -Lactoglobulin in Allergy-Prone Rat Pups<sup>1–3</sup>

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## Abstract

Controversy exists regarding the timing of the introduction of allergic foods into the diet. We investigated the immune response of rat pups exposed to  $\beta$ -lactoglobulin (BLG), one of the main allergenic proteins in cow milk. Brown Norway allergy-prone rats were allocated into groups: dam-reared and unchallenged (DR), DR challenged with BLG via gavage (11 mg/d), or rats fed via gastric cannula a formula containing BLG (11 mg/d). BLG was given from d 4 of life. Rats were killed at d 10, 14, or 21. Sera were assayed for total IgE, BLG-specific IgG1, and rat mucosal mast cell protease II (RMCP II; indicator of mucosal mast cell degranulation). Ileum was assessed for cytokine mRNA. Mesenteric lymph nodes (MLN) were assessed for forkhead boxP3 (Foxp3) and chemokine (C-C motif) receptor 7 (CCR7) expression by real-time PCR and immunostained for Foxp3<sup>+</sup> CD4<sup>+</sup> regulatory cells. Formula feeding compared with dam-rearing with or without oral BLG challenge resulted in significantly greater serum IgE, BLG-specific IgG1, RMCP II, and intestinal mast cells but reduced MLN Foxp3<sup>+</sup> cells, Foxp3, and CCR7 expression and ileal cytokines, interleukin (IL)-4, IL-10, and interferon- $\gamma$  ( $P < 0.05$ ). Importantly, giving BLG in the presence of maternal milk resulted in an immune response profile similar to that of unchallenged DR rats but with greater Foxp3 and CCR7 mRNA expression and CD4<sup>+</sup> Foxp3<sup>+</sup> cells ( $P < 0.05$ ). We conclude that introducing an allergenic food with breast milk reduces immunological indicators of an allergic response, whereas introduction during formula feeding generates an allergic response. J. Nutr. doi: 10.3945/jn.109.108845.

## Introduction

Breast-feeding and delayed complementary feeding are nutritional regimens that have been used to prevent development of allergy. Whereas asthma and respiratory allergic diseases are reaching a plateau in many developing countries, the incidence of food allergy is continuing to rise (1). It has also been common practice to recommend avoidance of more allergenic foods (i.e. egg, peanuts, and nuts) up to 3–4 y of age where there is a strong family history of allergy. However, intervention studies assessing the effectiveness of elimination diets have failed to reduce IgE-mediated food allergies (2–4). Conversely, there are preclinical

data and some clinical data to suggest that early oral exposure to antigens induces tolerance and prevents allergy (5–7).

Oral antigens are normally processed in a manner that results in a regulated immune response that maintains oral tolerance (8,9). In the neonatal period, the infant immune system is limited in the ability to mount an efficient immune response. This impairment is present in a number of functions in the immune system associated with mounting effective immune responses to pathogens or allergens. The neonatal immune system also has a preference for T helper 2 (Th2),<sup>9</sup> as opposed to T helper 1 (Th1) cytokines (10). Th2 cells promote humoral immunity and allergic responses by secreting interleukin (IL)-4, IL-5, and IL-13. The infant immune system requires stimulation from external factors, such as diet and the environment, to promote

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<sup>3</sup> Supplemental Table 1 and Supplemental Figure 1 are available with the online posting of this paper at [jn.nutrition.org](http://jn.nutrition.org).

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<sup>9</sup> Abbreviations used: BLG,  $\beta$ -lactoglobulin; BN, Brown Norway; CCR7, chemokine (C-C motif) receptor 7; DR, dam-reared; DR+BLG, dam-reared gavigated with oral  $\beta$ -lactoglobulin; FF+BLG, formula fed; Foxp3<sup>+</sup>, forkhead box P3<sup>+</sup> cells; GATA-3, GATA-binding protein-3; IFN $\gamma$ , interferon- $\gamma$ ; IL, interleukin; MLN, mesenteric lymph node; RMCP II, rat mucosal mast cell protease II; T-bet, T-box expressed in T-cell; Th1, T helper 1; Th2, T helper 2; Treg cell, regulatory T-cell.

a Th1 immune response as well as to develop oral tolerance (11). Maternal milk and/or formula provide nutrition for newborn infants. Maternal milk is not only a source of nutrition for the infant but also contains antigens for the neonatal intestinal immune system to learn appropriate immune regulatory mechanisms, which are critical for normal mucosal immune function (12). Maternal milk, unlike formula, also provides bioactive factors that modulate immune development and promote colonization by commensal bacteria (13).

The cytokine milieu in which an antigen is processed is critical to the maintenance of normal homeostasis in the intestine and development of oral tolerance (14). Oral tolerance induction is thought to occur via a number of possible mechanisms, which include anergy and active cellular suppression by T cells secreting cytokines and it is widely thought that a deviation from this process results in food-allergic diseases (14–17). Cytokines, such as IL-10 and transforming growth factor- $\beta$ 2, the major cytokine present in breast milk, have a role in promoting the development of oral tolerance (18,19). The alternative to breast-feeding is formula feeding; however, whereas milk formulations contain all the necessary nutritional requirements for growth, they do not contain bioactive cytokines that help promote immune regulation (15,20). Some formulas have added bioactives such as docosa-hexaenoic acid, prebiotics, and probiotics as well as Ig, but immunoregulatory cytokines are usually destroyed by the manufacturing process (21–23).

Although there is controversy related to prolonged exclusive breast-feeding and delayed introduction of foods in providing allergy prevention, breast-feeding is still the optimum nutrition for term infants and should be promoted for its many other benefits. In this study, we address the role of breast milk during the introduction of food antigens into the infant diet. The mechanism(s) by which the normal intestinal immune system responds to food and the factors involved in subsequent development of food-related enteropathies remain unclear. A direct analysis of the local gut immune response during oral antigen introduction is not ethically feasible in human infants. We therefore aimed to determine the effect of maternal milk on the infant immune response profile in early life during oral introduction of the cow milk antigen bovine  $\beta$ -lactoglobulin (BLG) in neonatal rat pups with a genetic predisposition toward allergy. BLG was used as the target antigen to assess antigen-specific responses, as it is one of the major allergens present in formula.

## Materials and Methods

**Animals.** All rats were housed in the Animal Facility of the Child, Youth and Women's Health Services, Adelaide. Brown Norway (BN) rats were bred in the Animal Facility. The BN rat has a naturally occurring genetic predisposition toward allergy development (24). All rat experimentation was carried out with approval from the Child, Youth and Women's Health Services Animal Ethics Committee.

**Cannulation and maintenance.** The details of the formula composition (Supplemental Table 1) and the procedure for artificial feeding were as previously described (25,26). At d 4 of age, rat pups were lightly anesthetized using forthane (Isoflurathane) and surgically implanted with a flexible i.g. cannula. The cannula was connected to a polyethylene line, through which artificial rat milk (Wombaroo; Supplemental Table 1) was delivered for 20 min/h at a flow rate of 0.48 mL/h (d 4), increasing to 0.94 mL/h at d 18 by a multi-syringe infusion pump (KDS220 multi-syringe infusion pump; KD Scientific). We previously demonstrated that variations in IgE, IgG1, mast cell activation, and number as well as cytokine profiles are directly attributed to the formula and not the surgical procedure (26).

**Experimental design.** Rat dams were fed a standard nonpurified diet (Ridley Agriproducts; Supplemental Table 1) containing no added BLG. Rat pups from BN litters (16 litters were used) were randomly allocated to groups of 8. Three feeding groups were used: dam-reared (DR), DR challenged daily by a 0.1-mL oral gavage with 11 mg bovine BLG (DR+BLG), or formula fed (FF+BLG; containing ~11 mg/d of the major cow milk antigen, BLG). Formula-fed rats provided a positive control for assessing allergy markers. Because it was not possible to make formula free of BLG for use as a diet for the cannulated rat pups, we were therefore unable to include this group in the study. The dose of BLG for the DR+BLG group is equivalent to the amount of BLG in the total rat milk replacer fed at d 4–5. Rat pups were killed at d 10, 14 (prior to weaning), or 21 d of age (postweaning). Rats studied until 21 d were weaned at d 18 and given access to the nonpurified standard diet made into a slurry with the formula, as well as free access to water.

Blood was collected by cardiac puncture and sera stored at  $-80^{\circ}\text{C}$ . The thymus and spleen were removed, snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . The gastrointestinal tract was excised and tissue from the duodenum, jejunum, and ileum were isolated and were: 1) embedded and snap-frozen with liquid nitrogen for cryostat sectioning (using OCT; TissueTek); 2), weighed and snap-frozen with liquid nitrogen for later protein, cytokine, and RNA analysis; or 3) fixed in 10% neutral buffered formaldehyde for 24 h and transferred to 70% (v:v) ethanol. Mesenteric lymph nodes (MLN) were dissected out and the surrounding connective tissue removed. The MLN from each rat were excised, one-half of the nodes mounted in OCT, and the other one-half pooled and snap-frozen in liquid nitrogen for later RNA isolation.

**Histological assessment.** Serial 4- $\mu\text{m}$  intestinal sections were cut and mounted on gelatin-coated slides. Sections were stained for mast cells with Leder chloroacetate esterase (Sigma-Aldrich Chemical) and counted (15). For each rat, a total of 6–8 fields were measured of 5 consecutive villi per field and were expressed as the mean number of mast cells per  $\text{mm}^2$  of lamina propria using Image Pro Plus software, version 5.1 (Media Cybernetics).

The transcription factor forkhead boxP3 (Foxp3) is expressed in  $\text{CD}25^{+}\text{CD}4^{+}$  T regulatory cells and has been shown to be necessary for their development and function (27). MLN sections (6  $\mu\text{m}$ ) were incubated with either biotinylated anti-mouse/rat Foxp3 (eBioscience) and mouse anti-rat CD4 (Chemicon) or an appropriate isotype control (28), which were detected by the secondary antibodies anti-mouse Alexa-488 (Invitrogen) and streptavidin–Cy3 (Sigma-Aldrich Chemical), respectively. Sections were viewed with a Leica Leitz DMRB microscope (Leica), F-View soft imaging system camera, and analysis software (version 3.2) and analyzed using Image Pro Plus software. Foxp3 $^{+}$ /CD4 $^{+}$  regulatory T-cell (Treg) cell counts were expressed as cells/ $\text{mm}^2$  and as the percentage of total CD4 $^{+}$  cells.

**Antibody, rat mucosal mast cell protease II, and cytokine analyses.** Serum IgE and BLG-specific IgG1 were quantified using an ELISA method as previously reported (15). Rat mucosal mast cell protease II (RMCPII) concentration was determined by ELISA according to the manufacturer's instructions (Moredun Scientific). Sample dilution varied depending on rat age and the presence of BLG in the diet. Typically, samples from rats at d 10 or 14 were diluted 1/10 and samples from older pups (21 d of age) were diluted 1/20 or 1/50. Data were expressed as  $\mu\text{g/L}$ . The limits of detection of the ELISA were as follows: RMCPII, 0.05  $\mu\text{g/L}$ ; IgE, 1.95  $\mu\text{g/L}$ ; BLG-specific IgG1, 1.43  $\mu\text{g/L}$ . IgE concentrations were not detectable before d 14 in each treatment group.

Concentrations of IL-4 (Th2), IL-10, and interferon- $\gamma$  (IFN $\gamma$ ) (Th1) in sera were below the detectable limit (data not shown) and therefore tissue concentrations from distal ileum were used for quantitation of cytokines. Sections (2–4 cm in length) from the distal ileum were collected and snap-frozen. Protein lysates were prepared by adding a cocktail of protease inhibitors (Sigma Chemical) to intestinal tissue (1 mL/100 mg tissue), which was then homogenized and centrifuged twice. Supernatants were collected, aliquoted, and stored at  $-80^{\circ}\text{C}$  until analyzed (29). Concentrations of IL-4, IL-10, and IFN $\gamma$  were determined per the manufacturer's instructions using anti-rat Opt-EIA ELISA kits (BD-Biosciences) in which the standards and samples were added in

duplicate and the cytokines were detected colorimetrically using 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich Chemical). Heat-inactivated fetal bovine serum was used as the blocking/assay diluent throughout the protocol. Cytokine ELISA data were expressed as ng/g of tissue.

**Real-time PCR.** Ileal IL-4, IL-10, IFN $\gamma$ , T-bet expressed in T-cells (T-bet), GATA-binding protein-3 (GATA-3), and MLN Foxp3 and chemokine (C-C) motif receptor 7 (CCR7) expression were quantified using real-time PCR. RNA was isolated using the QIAGEN RNeasy Plus Mini extraction kit (Qiagen) and cDNA synthesized using Superscript III RNase Reverse Transcriptase (Invitrogen) per the manufacturers' protocols. PCR primers were designed using Primer Premier 3 version 5 software (PREMIER Biosoft International): IL-4 (for 5'-gaa-caagtctgggttctcg-3', Rev 5'-ttgtgagcgtggactcattc-3'); IL-10 (for 5'-gccagccttgatcagaaatga-3', Rev 5'-tttctggccatggttctct-3'); IFN $\gamma$  (for 5'-gaactggcgaagacggtga-3', Rev 5'-ctgatggcctggtgtcttt-3'); T-bet (for 5'-tctgtctcagcgcgtttct-3', Rev 5'-cgctcactgctcggaactct-3'); GATA-3 (for 5'-ggcggcgagatggtactg-3', Rev 5'-tctgccattcattttatgtaga-3'); Foxp3 (for 5'-ccacacctctcttcttctt-3', Rev 5'-tgactagggcactgtaggc-3'); CCR7 (for 5'-ctgttcattttccaggtgtg-3', Rev 5'-agcacaccgactcatagc-3'); cyclophilin A (for 5'-ggttgatggcaagcatgtg-3', Rev 5'-tgctggtcttgcattcctg-3'). Samples were amplified using a RotorGene 3000 (Corbett Life Science). Melting curve and agarose gel analysis were used to verify single product amplification. PCR products were sequenced and confirmed by a BLAST search. Gene expression was quantified using either standard curve or  $2^{-\Delta\Delta C_t}$  real-time PCR method. All genes were normalized with respect to the cyclophilin A housekeeping gene.

**Statistical analyses.** All data were expressed as the mean  $\pm$  SEM. Data were assessed for normality before analysis. Differences of ileal mast cell numbers and ileal cytokines were analyzed using a 2-way ANOVA with time as the first factor and BLG exposure in the diet as the second factor, followed by Bonferroni's post hoc comparisons. Differences were considered significant at  $P < 0.05$ . Differences in body weight gain, serum IgE, RMCPII, cytokine mRNA, Foxp3, and CCR7 mRNA among treatments were evaluated utilizing a 1-way ANOVA followed by a Tukey's post hoc test. BLG-specific IgG1, T-bet, GATA-3, and IL-10 mRNA data were evaluated utilizing a nonparametric 1-way ANOVA (Kruskal-Wallis) followed by a Dunn's Multiple Comparisons post hoc test. Differences were considered significant at  $P < 0.05$ . All statistical analyses and comparisons were made using GraphPad Prism software, version 3.00 for Windows (GraphPad Software).

## Results

**Bodyweight change.** Feeding formula containing BLG or feeding BLG to DR pups did not affect body weight gain at d 10 or 14 of age. FF+BLG rats at d 21 of age were lighter compared with the DR rats ( $29.9 \pm 1.5$  g compared with  $35.2 \pm 1.2$  g, respectively) ( $P < 0.001$ ). Body weights of DR+BLG and DR rats did not differ at any time.

**Serum IgE, RMCPII, and BLG-specific IgG1.** At d 14, the serum IgE concentration was greater in the FF+BLG group ( $P < 0.05$ ) than in the DR and DR+BLG groups, which did not differ (Fig. 1A). The serum RMCPII concentration was greater in FF+BLG rats ( $P < 0.05$ ) at d 14 compared with DR and DR+BLG, which did not differ from one another (Fig. 1B). Formula feeding (FF+BLG) resulted in a greater BLG-specific IgG1 response compared with DR and DR+BLG rats (Fig. 1C) at d 14 ( $P < 0.05$ ). Importantly BLG-specific IgG1 titers in the DR groups did not differ regardless of oral BLG exposure at d 14.

**Mast cell infiltration in the ileum.** Mast cell numbers in FF+BLG rats were greater at d 10, 14, and 21 compared with DR and DR+BLG rats ( $P < 0.05$ ; Fig. 2). Mast cell numbers also increased with age from d 10 to 21 in the FF+BLG groups ( $P < 0.05$ ) but not in DR or DR+BLG pups. Mast cell numbers did not differ between DR+BLG or DR groups at d 10, 14, or 21.

**Cytokines in ileal homogenates.** FF+BLG rats had a lower concentration of IL-4 at d 10 of age compared with DR and DR+BLG rats ( $P < 0.001$ , respectively; Fig. 3A), which persisted at d 14 for the FF+BLG rats (Fig. 3D). In contrast, DR+BLG did not differ from DR rats at d 10. At d 14, the intestinal IL-4 concentration was greater in the DR+BLG than in the DR pups ( $P < 0.05$ ). IFN $\gamma$  concentration in the intestine was lower in FF+BLG rats compared with DR and DR+BLG rats at d 10 and 14, respectively ( $P < 0.001$ ; Fig. 3B,E). Intestinal IFN $\gamma$  in DR+BLG rats at d 10 and 14 were also lower compared with DR rats ( $P < 0.05$ ; Fig. 3B,E). The intestinal IL-10 concentration after formula feeding (FF+BLG) was lower at d 10 and 14 compared with DR rats ( $P < 0.001$ ; Fig. 3C,F). Oral BLG exposure at d 4 in the presence of maternal milk (DR+BLG) resulted in a lower concentration of IL-10 compared with unchallenged DR rats ( $P < 0.001$ ). Cytokine concentrations in the jejunum were the same as for the ileum (data not shown).

Cytokine mRNA expression at d 10 did not differ between DR, DR+BLG, and FF+BLG groups (data not shown). IL-10 mRNA expression at d 14 was greater in DR+BLG rats than in DR and FF+BLG rats ( $P < 0.01$ ; Fig. 4B). Both T-bet and GATA-3 mRNA expression were greater in the DR+BLG rats than in the DR and FF+BLG rats ( $P < 0.05$ ), indicating an increase in both Th1 and Th2 transcription after BLG exposure during maternal milk feeding.

**T regulatory cells.** The total number of MLN CD4 $^{+}$  cells did not differ among the groups (Fig. 5A). CD4 $^{+}$ /Foxp3 $^{+}$  T cells in MLN were greater in DR+BLG compared with FF+BLG rats ( $P < 0.05$ ; Fig. 5B). Importantly, a significantly greater proportion of CD4 $^{+}$  cells that were Foxp3 $^{+}$  were present in

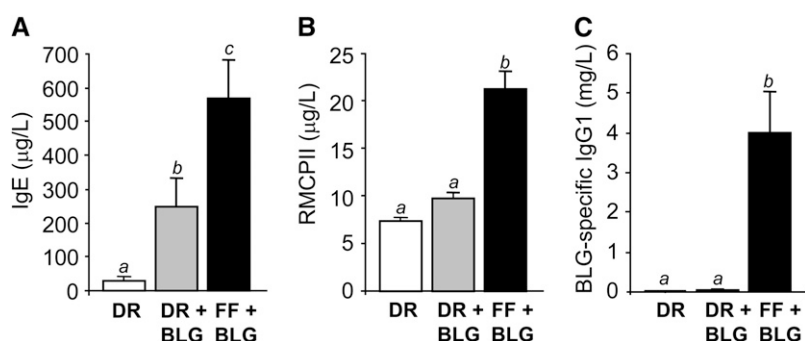


**FIGURE 1** Serum IgE (A), RMCPII (B), and BLG-specific IgG1 (C) concentrations on d 14 in DR, DR+BLG, and FF+BLG pups. Values are mean  $\pm$  SEM,  $n = 8$ . Means without a common letter differ,  $P < 0.05$ .

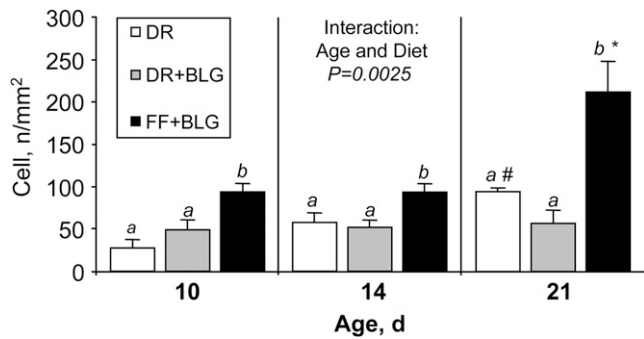


**FIGURE 2** Ileal mast cell numbers in the lamina propria at d 10, 14, and 21 of age after oral BLG commenced at d 4 in DR or FF rat pups. Values are mean  $\pm$  SEM,  $n = 8$ . Means within an age group without a common letter differ,  $P < 0.05$ . Treatment groups across time without a common symbol differ,  $P < 0.05$ . Within a group, symbols indicate different from d 10 and 14 pups,  $P < 0.05$ .

DR+BLG compared with DR rats ( $P < 0.05$ ; Fig. 5B). Further characterization of Foxp3<sup>+</sup> cells in the MLN at d 14 by quantitative real-time PCR revealed a reduction in Foxp3 mRNA expression in FF+BLG compared with DR and DR+BLG rats ( $P < 0.05$ ; Fig. 5C). CCR7 mRNA expression in the DR+BLG rats was greater than in DR and FF+BLG rats ( $P < 0.05$ ; Fig. 5D) at d 10.

## Discussion

This study demonstrated that early oral BLG exposure in the presence of maternal milk maintained a similar immune response profile to DR unchallenged rats with low or undetectable concentrations of circulating BLG-specific IgG1, IgE, and RMCPII, indicating a nonallergic state. Concentrations of IL-10, IFN $\gamma$ , T-bet, and GATA-3 mRNA in the ileum, and CCR7 (a chemokine receptor involved in T cell homing) and Foxp3 mRNA in MLN, were all highly expressed in DR+BLG rat pups. This response was evident as early as d 14 of life. In contrast,

formula feeding caused an elevation in circulating BLG-specific IgG1 (antigen-specific response), IgE, and RMCPII concentrations, indicating an allergic response. Ileal IL-10 and IFN $\gamma$  and mRNA expression of the T cell transcription factors T-bet and GATA-3 were all downregulated in the local intestinal environment after formula feeding. This suggests that in early life, the mucosal immune system can respond and develop regulatory mechanisms after oral antigen challenge, particularly when the antigen is presented in the presence of maternal milk. The development of Th1 or Th2 responses is influenced by the cytokine milieu in the local environment during antigen presentation by antigen-presenting cells, including dendritic cells (30). Oral antigen exposure is important for priming the infant immune response and maternal milk may provide the regulatory environment for appropriate immune development.

Our data focuses on oral BLG given in early life. We attempted to include a formula-fed group with BLG removed from the formula; however, we were unable to do so. We acknowledge that although BLG is a major allergen in formula, there are also many other allergenic components present that can influence the overall immune response in the FF+BLG rat pups. However, when the specific response to BLG (BLG-specific IgG1) was compared with the DR+BLG rats, tolerance to BLG was evident in the DR rats but not in the formula-fed rats. Formula-fed rats were used to represent a control group mounting an active allergic response.

Mast cells play an integral role in innate and adaptive immunity (31). Mast cell numbers were significantly increased in the ileum of DR+BLG pups but not FF+BLG pups. In conjunction with an increase in mast cells, IgE is another marker of allergy development; however, not all infants (50%) have detectable circulating IgE antibodies, as most of the IgE is bound to mast cells located in tissue (32,33). We did not detect significant concentrations of BLG-specific IgE in the infant rat pups and therefore we measured total IgE and BLG-specific IgG1 (antibody associated with Th2 responses and allergy). BLG-specific IgG1 in DR-unchallenged rats and DR+BLG rats did not significantly differ when assessed at d 14 and 21 (preweaning and postweaning age, respectively). In contrast,

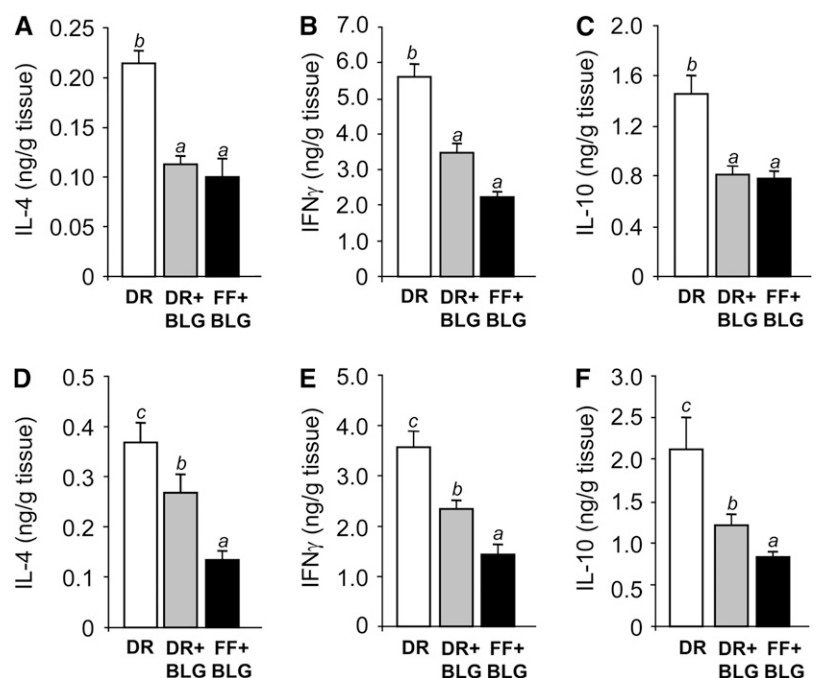


**FIGURE 3** Ileal cytokines after oral BLG commenced at d 4 in DR, DR+BLG, and FF+BLG pups. IL-4 [d 10 (A) and 14 (D)], IFN $\gamma$  [d 10 (B) and 14 (E)], IL-10 [d 10 (C) and 14 (F)]. Values are mean  $\pm$  SEM,  $n = 8$ . Means without a common letter differ,  $P < 0.05$ .

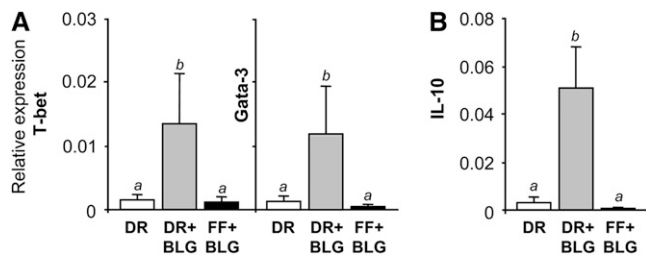


**FIGURE 4** Ileal cytokine mRNA expression after oral BLG commenced at d 4 in DR, DR+BLG, and FF+BLG pups. T-bet and GATA-3 mRNA (A) and IL-10 mRNA (B) as determined by real-time PCR. Values are mean  $\pm$  SEM,  $n = 5-8$ . Means without a common letter differ,  $P < 0.05$ .

BLG-specific IgG1 in formula-fed rats significantly increased as early as d 14 with the response persisting after weaning. IgE could not be detected at d 10 but was present at d 14 and 21. IgE and RMCPII, a marker of mucosal mast cell activation, in DR+BLG rats did not significantly differ from unchallenged DR rats.

Breast-feeding and delayed complementary feeding are nutritional regimens that have been used for allergy prevention (34). There is some evidence that continued breast-feeding during introduction of complementary foods is important for promoting tolerance (35) and there is growing support for the concept of inducing oral tolerance by early oral antigen exposure, as reviewed by Lack et al. (2). Verhasselt et al. (12) have also demonstrated that maternal milk transforming growth factor- $\beta$  and, importantly, antigen itself in breast milk were associated with prevention of asthma in recipient progeny. Formula feeding may be associated with increased risk of

inappropriate immune responses to allergens (36). We demonstrated in this study that in the local gut environment, formula feeding early in life resulted in an overall suppression of all measured ileal cytokines, both Th1 and Th2 cytokines as well as Th1 and Th2 transcription factors, T-bet, and GATA-3, respectively. Infants with a hereditary predisposition toward allergy development and who go on to develop allergic disease have been reported to show an early shift to a Th2 response after exposure to allergens in the first year of life (37). Infants who mount a higher Th1 response (with IFN $\gamma$ ) were more likely to go on to resolve their cow milk allergy compared with infants with higher Th2 responses (37). In contrast to formula feeding, early BLG exposure in the presence of maternal milk resulted in the IL-4 concentration being no different from unchallenged DR rats at d 14. IFN $\gamma$  was significantly greater in the DR+BLG rats compared with FF+BLG rats at d 14. T-bet and GATA-3 T cell transcription factors provide a surrogate marker of Th1/Th2 cytokine responses, respectively (38). Greater expression of IFN $\gamma$  and T-bet mRNA expression in the DR+BLG rats indicated an upregulation of Th1 responses. GATA-3 mRNA expression was also elevated in the DR+BLG rats, implicating activation of Th2 cytokines as well. IL-10 is a cytokine produced by Treg cells and it is associated with maintenance of gut homeostasis. IL-10 mRNA expression was greater in the ileum of DR+BLG rat pups when compared with unchallenged DR rats; however, IL-10 protein was reduced, suggesting that the protein may be utilized by responding cells. Upregulated IL-10 mRNA expression can also be explained by the concomitant increase in Treg cells that produce IL-10. The data supports a role for breast milk promoting immune regulation when food antigens are introduced (15,26).

While the Th1/Th2 balance in infants is important in immune development, the actual mechanisms driving development of food allergy remain unresolved. The finding that both Th1 and Th2 cytokines are suppressed after formula feeding was unexpected in that Th2 responses have been reported to predominate in the periphery in allergic infants (37). High-dose antigen exposure is thought to lead to anergy rather than the development of regulatory cells. Formula does not contain active cytokines that promote immune regulatory mechanisms that drive Th1 and Th2 differentiation (15). Formula provides high-dose antigen exposure in the absence of maternal milk bioactives, increasing the potential for anergy development and an aberrant Th2 response to food antigens in the periphery (39). Interestingly, our data on CCR7 mRNA expression supports a defect in Treg cell homing during formula feeding and BLG challenge.

The frequency of Foxp3 $^{+}$  cells in the periphery is low in food-allergic children (40). Foxp3 $^{+}$  (Foxp3 $^{+}$ /CD4 $^{+}$ /CD25 $^{+}$ ) Tregs are critical for oral tolerance development and maintaining gut immune homeostasis (41). The role of Foxp3 $^{+}$ /CD25 $^{+}$ /CD4 $^{+}$  Treg cells in regulation of immune response development to food antigens in the gut is unclear. Children with food allergies have low expression of Foxp3 and a defect in its transcription (42). In IPEX syndrome, where Treg cells are absent or functionally defective, multiple autoimmune and inflammatory disorders, together with elevated IgE, occur which we have also demonstrated. Foxp3 $^{+}$  CD4 $^{+}$  cells in the MLN of FF+BLG pups did not differ from those in DR pups; however, Foxp3 mRNA expression was significantly reduced. Importantly, Foxp3 $^{+}$  CD4 $^{+}$  cells were more abundant in DR+BLG rats than in DR rats. Tregs have been reported to actively prevent Th2 responses in healthy nonatopic individuals and their function is impaired in allergic patients (43,44).

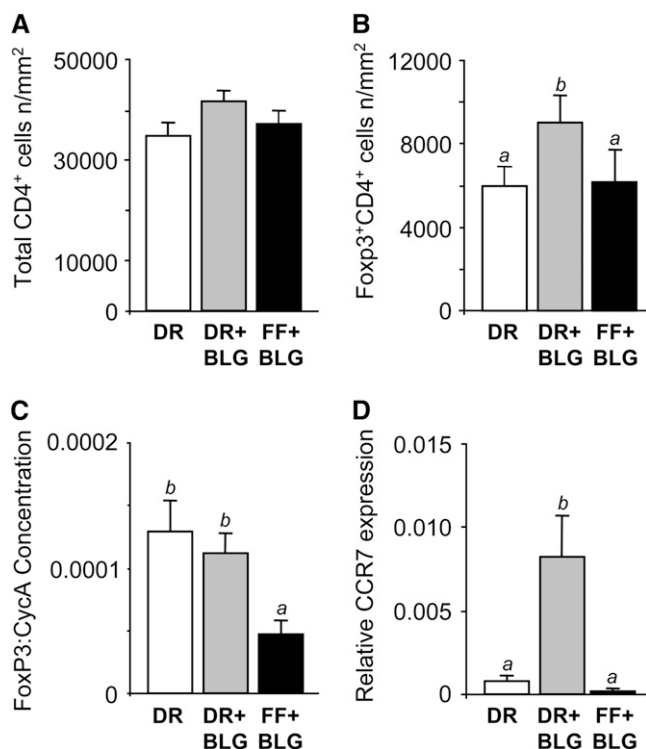


**FIGURE 5** Total CD4 $^{+}$  cells (A) and CD4 $^{+}$ /Foxp3 $^{+}$  cells (B), real-time PCR for Foxp3 expression (C), or CCR7 expression (D) from MLN after oral BLG commenced at d 4 in DR, DR+BLG, and FF+BLG pups. Values are mean  $\pm$  SEM,  $n = 7-8$ . Means without a common letter differ,  $P < 0.05$ .

Homing of Treg cells to the gut after food challenge is important in establishing a regulated immune response and maintenance of homeostasis. Menning et al. (45) have shown that the recirculation of Treg cells through lymph nodes and, to some extent, the gut is dependent on CCR7. Specifically, a lack of CCR7 almost abolishes the ability of Treg cells to control the priming phase of an immune response (45). DR+BLG rat pups had greater CCR7 mRNA expression as well as Foxp3<sup>+</sup>/CD4<sup>+</sup> cell numbers. Moreover, the expression of CCR7 and Foxp3<sup>+</sup>/CD4<sup>+</sup> Treg cells in the DR+BLG rats, along with the reduced concentration of IgG1, IgE, and RMCPII suggests that the mechanism(s) to drive a regulated immune response to early antigen challenge are present in the presence of maternal milk.

Maternal milk feeding, compared with formula feeding at the time of food antigen introduction, influences the Th1/Th2 balance in the local gut environment with greater IFN $\gamma$  production and T-bet and IL-10 mRNA expression. A recent study by Cardoso et al. (46) supports our findings demonstrating that the initiation of an allergic response involves IL-4, which directly induces the differentiation of committed effector Th2 lymphocytes in a mouse model of food allergy. They highlighted the importance of IFN $\gamma$ , decreased regulatory cytokines, and Foxp3<sup>+</sup> cells in the induction of allergic processes in the intestine.

The mucosal immune system in the suckling period may require oral antigen exposure to learn to develop immunoregulatory mechanisms. We show that in early life oral antigen exposure during maternal milk feeding helps promote appropriate immunoregulatory mechanisms toward food antigens, in contrast to oral antigen exposure during formula feeding.

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