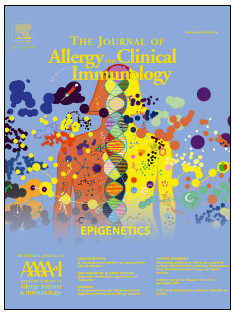
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Toll-like receptor 2 impacts the development of oral tolerance in mouse pups via a milk-dependent mechanism

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Key Messages

The presence or absence of TLR2 in a feeding mother profoundly influences populations of tolerogenic dendritic cells, regulatory T cells, intestinal permeability and the early development of oral tolerance in mice.

Milk from TLR2-deficient mice contains reduced levels of cytokines such as IGF-1 and IL-6 which enhance tolerogenic dendritic cell populations, independent of changes in intestinal permeability.

The innate immune status of a feeding mother may need to be considered when examining the role of breast feeding and early life exposures on allergic disease development.

Capsule Summary

Expression of the innate immune receptor TLR2 by mothers profoundly influences oral tolerance development in mice, through a milk-dependent mechanism. Maternal innate immunity alters the impact of breast feeding on allergy development.

Keywords

Food allergy, Breast feeding, Innate Immunity, Oral tolerance, IGF-1, IL-6, Milk, Development

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40 **Abbreviations**

41	BTLA	B- and T-lymphocyte attenuator
42	cDC	Conventional dendritic cell
43	DCs	Dendritic cells
44	ddPCR	Drop Digital PCR
45	ELISA	Enzyme-linked immunosorbent assay
46	FOXP3	Forkhead box P3
47	FVD	Fixable viability dye
48	GALT	Gut-associated lymphoid tissues
49	IDO	Indoleamine 2,3-dioxygenase
50	IFN	Interferon
51	Ig	Immunoglobulin
52	IGF-1	Insulin-Like Growth Factor 1
53	IL-	Interleukin
54	ILCs	Innate lymphoid cells
55	KO	Knockout
56	MLN	Mesenteric lymph nodes
57	OVA	Ovalbumin

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58	pDC	Plasmacytoid dendritic cell
59	PD-L	Programmed cell death ligand
60	PP	Peyer's patches
61	RALDH	Retinaldehyde dehydrogenase
62	SNP	Single nucleotide polymorphism
63	TGF	Transforming growth factor
64	Th	T-helper cells
65	TLR2	Toll-like receptor 2
66	Tregs	T regulatory cells
67	WT	Wild type

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Abstract

Background: The role of breastfeeding in the development of oral tolerance and allergic diseases is controversial, which could be related to variability in milk components. Toll-like receptor 2 (TLR2) is an innate immune receptor implicated in regulating allergic disease development.

Objectives: We examined whether deficiency of maternal TLR2 impacts the normal development of oral tolerance and related immune parameters during lactation in a mouse model.

Methods: Heterozygous $TLR2^{+/-}$ pups from WT or $TLR2^{-/-}$ dams were fed either by their biological dam or a dam of the alternate genotype. Oral tolerance development to ovalbumin (OVA), levels of tolerogenic $CD103^{+}$ DCs and Tregs and intestinal permeability were evaluated in these pups. The levels of key immune mediators in milk from $TLR2^{-/-}$ and wild type mothers were also examined.

Results: Heterozygous $TLR2^{+/-}$ pups that were born and nursed by $TLR2^{-/-}$ dams exhibited impaired oral tolerance. This was prevented by cross-fostering on to wild type ($TLR2^{+/+}$) dams. Impairments included selective elevation in anti-OVA IgE in plasma following immunization, reduced tolerogenic DCs and Tregs in the intestinal tract, and increased intestinal permeability. TLR2 deficiency also impacted milk content of IGF-1, IFN-γ, IL-6, and IL-13.

Conclusion: Our results underline a critical role for TLR2 in regulating milk components that are essential for oral tolerance development in early life and demonstrate the importance of considering the immune status of nursing mothers in studies of immune development and responses.

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Introduction

Food allergies commonly affect infants and young children, especially in Western countries (1). According to the "window of opportunity" concept, exposure to dietary and environmental factors in early life, during early postnatal immune and gastrointestinal tract development, can impact susceptibility to allergic diseases (2,3). The major source of nutrients in this period is usually breast milk, which is recommended as an exclusive form of nutrition for infants for up to 6 months of age, followed by a gradual introduction of solid foods, up to around 1 year (4,5). While there is evidence supporting a protective role for breast milk in preventing food allergies, there remains substantial controversy (6). Genetic factors also play a key role in regulating susceptibility to allergic disease (7), but the interactions between genetics and early-life feeding remain poorly defined. Breast milk is a dynamic fluid that changes dramatically from birth until weaning under the influence of multiple factors including maternal genetics, environment, and diet. Several mechanisms have been suggested through which breast milk can promote oral tolerance (8). Milk transfers nutrients, including food antigens in tolerogenic forms, alongside immune-modulatory factors to the infant. These factors include cytokines, growth factors, soluble receptors, vitamins, oligosaccharides, and microbiota. The intestinal immune cells within the gut-associated lymphoid tissues (GALT) are arranged in organized compartments, such as Peyer's patches (PP) and the mesenteric lymph nodes (MLN), or disseminated in the lamina propria (9). The GALT encompasses immune cells such as, dendritic cells (DCs), T regulatory cells (Tregs), T-helper cells (T_h), innate lymphoid cells (ILCs), macrophages, and B cells, subsets of which are critical for tolerance development (10–12). Breast milk factors have the capacity to induce tolerogenic properties in DC subsets, which sample food antigens from the intestinal lumen and introduce them to Tregs in the GALT (13). Tolerogenic DCs express a

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number of immunomodulatory molecules, such as CD103, indoleamine 2,3-dioxygenase (IDO), transforming growth factor (TGF)-β, retinaldehyde dehydrogenase 2 (RALDH2), CD80/CD86, programmed cell death-1 ligand (PD-L1), and B- and T-lymphocyte attenuator (BTLA) (11). $CD103^+$ DCs continuously sample antigens from the intestinal lumen, migrate to local lymph nodes, induce the gut homing receptors CCR9 on T cells(14) and promote $FOXP3^+$ Treg differentiation via retinoic acid and TGF-β1 as well as PD1-PDL1 signaling (15–17). Antigen-specific Tregs enhance the development of oral tolerance and subsequently reduce class switching of B cells toward IgE secretion (18). Milk-derived factors also enhance the maturation of the intestinal barrier (19). Increased intestinal permeability is considered a potential risk factor for food allergy development (20). Genetic variations including single nucleotide polymorphisms (SNPs) in immunoregulatory genes can affect the composition of human and dairy milk (21–24). However, the impact of genetically-mediated changes in milk composition on the establishment of oral tolerance has not been previously reported.

The pattern recognition receptor, Toll-like receptor 2 (TLR2), regulates the secretion of immune mediators in response to microbial or endogenous ligands. TLR2-mediated responses can impact multiple immune processes (25). It is found in both soluble and cell-bound forms in serum and breast milk (26). TLR2 deficiency in adult mice does not impair oral tolerance development, although activation of this receptor promotes the development of IgE and IgA responses to food antigens (27). SNPs in *TLR2* in human subjects have been associated with increased susceptibility to diseases, including atopic dermatitis (28,29), asthma (30), inflammatory bowel diseases (31), tuberculosis infection (32) and reactive arthritis (33). Peripheral blood monocytes from humans with common SNPs in TLR2 have a decreased capacity to respond to some TLR2 stimuli (34). Such monocytes are also found in human milk and act as a potent source of

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cytokines (35). In dairy cattle, SNPs in *TLR2* have been associated with changes in milk components (36). We hypothesized that milk from TLR2-deficient or control dams may differ in their ability to enhance oral tolerance development in TLR2-expressing pups. Our data demonstrate a profound impact of milk from TLR2-deficient versus TLR2-expressing feeding dams in regulating oral tolerance development and intestinal permeability. This was associated with substantial differences in milk cytokine and growth factor composition between TLR2-expressing and TLR2-deficient mice.

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Methods

Mice

BALB/c mice, wild type (WT) or TLR2$^{-/-}$, were bred within the Carleton Animal Care Facility at Dalhousie University (Halifax, Canada). TLR2KO mice on a BALB/c background were produced by successive backcrosses, beginning with B6.129-*Tlr2*^{tm1Kir}/J (*Tlr2*$^{-/-}$) from The Jackson Laboratory (Bar Harbor, ME) and BALB/c (Charles River, Saint-Constant, Canada). Progeny were screened with Charles River's Mouse 384 SNP Panel throughout the breeding process, resulting in mice having greater than 99% BALB/c-derived genomic DNA, with only the TLR2 locus remaining non-BALB/c. The breeding pairs were derived from combinations of homozygous and heterozygous breeders, to minimize the variability of microbiota, and genotyped before mating. All the animals ate the same diet and were housed in a pathogen□free facility on a 12□hrs light/dark cycle. Animal experiments were performed under approved protocols by the University Committee on Laboratory Animals (protocol numbers 16-098, 18-072) in accordance with the Canadian Council for Animal Care guidelines.

Mating and cross-fostering

Cross-fostering experiments required the synchronization of female estrus cycles, which was achieved by co-housing the females in groups for 5 days with the addition of male urine to stimulate the estrus cycles through its content of pheromones (37). Two types of breeding cages of BALB/c mice (6-8 weeks old) were established; the first type contained a WT male and a TLR2$^{-/-}$ female and the second type contained a TLR2$^{-/-}$ male and a WT female, thus, the offspring from both types of breeders were heterozygous (TLR2$^{+/-}$), genetically and phenotypically normal. Pups from each breeder were divided into two groups within 1-3 days

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after birth, such that half of each litter remained with its biological mother while the other half was cross-fostered by a mother of the alternate genotype.

Assessment of food sensitization towards OVA

Using cross-fostering experiments, we tested the ability of newborn $TLR2^{+/-}$ mice nursed by WT or $TLR2^{-/-}$ dams to develop immunological tolerance to ovalbumin (OVA) during the lactation period. This was achieved in two separate experiments by either feeding the pups or feeding the mothers with OVA. Between day 10 and day 17 after birth, cross-fostered and non-cross-fostered litters received OVA either directly (20 µg per gram weight) dropwise into their mouths every second day by pipette, or indirectly via their mothers that ingested OVA by daily gavage (100 µl of 4 mg/ml OVA). Pups born to WT and $TLR2^{-/-}$ dams and not exposed to OVA by either route were used as controls. All of the pups were immunized on day 19 with 10 µg of OVA precipitated to alum, weaned on day 21, and boosted by intraperitoneal injection of 1 µg of soluble OVA in PBS on day 33. Blood samples were collected in heparinized tubes from the mice at day 40.

OVA-specific immunoglobulins (IgE, IgA, IgG1, IgG2a) were measured by ELISA as described in our previous protocol (27). Briefly, ELSA plates were coated with anti-mouse IgE, IgA, IgG1, or IgG2a capture antibodies (BioLegend, San Diego, CA) in 0.2M borate-buffered saline (pH 8.3). After washing and blocking the plates in PBS with 2% BSA to avoid non-specific binding, plasma and standards were added to the plate and incubated at 4°C overnight for 16-18h Plates were washed and biotinylated-OVA was added to the plates followed by HRP-Conjugated Streptavidin (ThermoFisher, Waltham, MA) and TMB substrate (ThermoFisher) to detect the level of bound OVA. The optical density (OD) of the wells was read at 450 nm using a BioTek

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187 synergy reader (BioTek, Winooski, VT) and analyzed by Gen5 software (BioTek). OVA-specific
188 IgE was quantified in comparison to a commercial standard (Chondrex Inc.; Redmond, WA,
189 USA) and reported as ng/ml. OVA-specific IgA, IgG1, and IgG2a were standardized relative to a
190 standard serum that has a high titer of anti-OVA immunoglobulins and presented as optical
191 density at 450 nm (OD450).

192 **Assessment of DC subsets, Tregs, T-helper cells and ILCs by flow cytometry**

193 The pups from selected cross-fostering experiments were euthanized at days 15 or 21 after birth.
194 PP, MLN, and spleen were harvested from each mouse and assessed for DC subsets, Tregs, T-
195 helper cells, and ILCs. Collected tissues were minced, and single-cell suspensions were blocked
196 using 10% heat-inactivated rat serum for 20 min at 4°C. Before extracellular or intracellular
197 staining, single-cell suspensions were incubated with fixable viability dye (FVD) eFluor 450
198 (ThermoFisher) diluted 1:1000 in PBS for 20 min at 4°C. To determine the frequencies of DCs
199 (adapted from (38)) and ILCs (adapted from (39)) subsets, the cells were stained with a cocktail
200 of antibodies for extracellular markers indicated in (Table E1). To identify subsets of T-helper
201 cells, the cells were stimulated with 50 ng/ml PMA and 1 µg/ml of ionomycin in 10% FBS-
202 RPMI medium for 5 hours before adding 1x Brefeldin A for another 1 hour. In contrast, Tregs
203 were assessed without cell stimulation. The frequencies of Tregs (40) and T-helper subsets were
204 identified by staining with extracellular markers (Table E1), followed by fixation,
205 permeabilization, and staining with intracellular antibodies (Table E1). Analysis of flow
206 cytometry data was performed using Flowjo software (Ashland, OR). Viable cells (negative
207 population of FVD-eFluor 450) were gated on $CD45^+$ cells. The frequencies of DC subsets,
208 Tregs, subsets were calculated as indicated in the gating strategies in (Figure 2, Figure 3,

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respectively). The levels of DC subsets, T-helper subsets and ILCs that failed to show significant differences were summarized in Table E2.

Digital droplet PCR (ddPCR) analysis for DCs and Tregs gene expression

DCs and Tregs were sorted from MLN-derived cell suspensions using FACSAria III (BD Biosciences, San Jose, CA). Lymph nodes were pushed through a 70um strainer then stained with FVD eFluor-780 (eBioscience, San Diego, CA). The tolerogenic $CD103^{+}/CD11c^{+}$ DCs were gated on lineage -ve $CD3^{-}/CD11b^{-}/CD19^{-}$ and $CD45^{+}/MHCII^{+}$. The Tregs were gated on $CD4^{+}/CD25^{hi}$. The purity of sorted cell populations was >90% for DCs and 75% for Tregs. Both types of cells were collected directly into Trizol (Life Technologies), then mRNA was extracted using RNeasy microRNA columns (Qiagen). A standard quantity of total RNA was reverse transcribed (Qiagen Quantitect kit). The transcript levels for Treg genes (*Foxp3*, *Ccr9*, and *Cd279*) and DC genes (*Ido*, *Tgfb1*, *Raldh2*, *Cd80*, *Cd86*, *Cd274*, and *Btla*) were assessed by Evagreen ddPCR (Bio-Rad, Hercules, CA) using commercially validated primer sets (Bio-Rad and Qiagen) and normalized against *Hprt* and *Gusb* transcript levels. The results of non-significantly changed genes were summarized in Table E3.

Intestinal permeability

Permeability of the small intestine of pups fed by $TLR2^{-/-}$ or WT dams was assessed *in vivo* using oral administration of FITC-Dextran 4 kDa (Sigma-Aldrich, St. Louis, MO) at the age of weaning (day 21 after birth). Briefly, the mice ingested FITC-Dextran 4 kDa (44 mg/gr body weight) by gavage. After two hours, the mice were anesthetized by isoflurane, the blood was collected via cardiac puncture, and the mice were euthanized by cervical dislocation. Blood samples were centrifuged for 10 min at 4°C (12,000 x g) and the plasma samples were assessed

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for the concentration of FITC-Dextran 4 kDa using a fluorescent spectrophotometer at an excitation wavelength of at 490 nm and an emission wavelength of 530 nm. Serially diluted FITC-Dextran in a non-gavaged mouse serum served as a standard curve.

Milk sampling

Mouse milk was collected on days 1, 5, and 10 using a 1 mm silicone tubing attached to a dosing pump DP-385 (INTLLAB, China), which keeps constant and adjustable pressure on the mammary gland. 2 IU of Oxytocin (Sigma-Aldrich) in 100 µl PBS were injected intraperitoneally before milking. Mouse milk was collected from at least 6 mammary glands in each mother with yields usually between 150-350 µl of milk/mouse. Milk was depleted of lipid by centrifuging each sample at 10,000 x g for 10 min at 4°C. Whey fractions were stored at -20°C for further analysis.

Milk cytokines, growth factors, and soluble receptors

Milk whey content of IL-6, IFN-γ, IL-4, IL-13, IL-10, and IGF-1 was quantified using a mouse premixed multi-analyte kit (R&D, Minneapolis, MN) according to the manufacturer's recommendation and acquired by Luminex 200 (Bio-Rad), with calibration and standard controls. Samples were thawed only once on ice and assayed in duplicate. Milk content of TGF-β1 and TGF-β2 was quantified by sandwich ELISA kit (R&D). In order to measure the total TGF-β1, and TGF-β2 in mouse milk, a pre-activation step was performed. Briefly, each whey milk sample was diluted in PBS and then treated with 20µl of 1N HCl per 100µl of milk for 10 min at room temperature and neutralized with 20µL of 1.2N NaOH/0.5M HEPES. Samples were loaded into pre-coated ELISA plates according to the manufacturer's recommendation.

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Mouse soluble TLR2 and total IgA in mouse milk were assessed by using ELISA kits from Abcam (Cambridge, UK) and eBioscience, respectively, according to the manufacturers' instructions.

Supplementation of IGF-1 and IL-6

Pups born to TLR2$^{-/-}$ dams were divided into three groups and gavaged with PBS, IGF-1 (240 ng/gr) (PeproTech, Rocky Hill, NJ), or IL-6 (650 pg/gr) (PeproTech). Supplementation with IGF-1 or IL-6 started on day 4 after birth and was repeated every second day until weaning on day 21.

Statistical analysis

OVA-specific IgE, levels of tolerogenic DCs, Tregs, and intestinal permeability were compared by one□way ANOVA followed by Fisher's least significant difference (LSD) post-hoc test for comparisons between individual means. Levels of cytokines in milk were compared between WT and TLR2$^{-/-}$ dam's milk at every timepoint by Student's *t*□test.

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Results

$TLR2^{+/-}$ pups cross-fostered onto WT dams have significantly less food sensitization compared to siblings that remain with their biological $TLR2^{-/-}$ dam.

The impact of milk from WT or $TLR2^{-/-}$ dams on the development of oral tolerance in early life was assessed in genetically identical ($TLR2^{+/-}$) and phenotypically normal pups by a cross-fostering experiment (Figure 1A). OVA was introduced to the pups for one week during lactation either directly by gavage or indirectly via the dam's milk. To assess tolerance development, pups were immunized and challenged with OVA as indicated (Figure 1A). The lactation period was restricted to 21 days. Pups not exposed to OVA pre-weaning produced higher levels of anti-OVA IgE, IgA, IgG1, and IgG2a in response to subsequent immunization than those that received OVA orally (Figure 1B, 1C, and Figure E3). However, pups derived from and nursed by $TLR2^{-/-}$ dams and then exposed to OVA had significantly higher levels of anti-OVA IgE in their plasma compared to their siblings nursed by WT dams ($P < 0.05$). Indeed, pups derived from $TLR2^{-/-}$ dams and nursed by WT dams showed IgE responses to immunization following oral OVA administration comparable to pups born and nursed by WT dams (Figure 1B, 1C). However, pups derived from WT dams and nursed by $TLR2^{-/-}$ dams after the first 1-3 days of life did not show significantly different IgE responses compared to their siblings which remained with their biological dams. In contrast to IgE, anti-OVA IgA, IgG1 and IgG2a responses were not impacted by the *TLR2* status of the feeding dam. TLR2 genetic status had a long-term impact on mouse capacity for oral tolerance development, as adult mice derived and nursed by $TLR2^{-/-}$ dams also had significantly higher anti-OVA IgE responses (9.3 ± 0.92 ng/ml) compared to mice that were derived and nursed by WT dams (4.0 ± 1.3 ng/ml) ($P < 0.01$) after ingesting OVA for a week,

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followed by immunization and boosting with OVA as indicated. Exposure to milk from WT dams had a significant impact on promoting food tolerance in the IgE compartment during early life, compared with milk from TLR2$^{-/-}$ dams, regardless of the *in-utero* environment.

Milk from WT dams support the expansion of tolerogenic DCs and Tregs in the GALT

Since tolerogenic DC and Tregs can play a crucial role in immune tolerance, TLR2$^{+/-}$ pups fed by either WT and TLR2$^{-/-}$ dams were assessed for populations of both of these cell types in the GALT at weaning (21 days) as shown in Figure 2A. Tolerogenic DCs have been characterized in previous studies as MHC-II^{+}/CD11c^{+}/CD103^{+} cells (38). Feeding with milk from WT dams significantly increased the number of tolerogenic DCs in the MLN and the spleen in cross-fostered pups compared to their biological siblings fed by TLR2$^{-/-}$ dams (Figure 2C, 2D). The levels of tolerogenic DCs in the PP were not significantly different between pups nursed by WT or TLR2$^{-/-}$ dams (P= 0.834, data not shown).

DCs in the intestine are heterogeneous and can be divided into conventional DCs (cDC1 and cDC2) and plasmacytoid DCs (pDCs) (41). The impact of milk consumption from WT or TLR2$^{-/-}$ dams on these subsets was determined. The frequency of cDC1, cDC2, and pDCs was not significantly different between pups fed by WT or TLR2$^{-/-}$ dams (Table E2). However, the levels of tolerogenic CD103^{+} cDC1, characterized as CD45^{+}/MHC-II^{+}/CD11c^{+}/CD8α^{+}/CD24^{+}/CD103^{+}/ CD11b^{-} cells (Figure 2B), were significantly higher in the MLN and spleen of WT-nursed pups (Figure 2D) and such cells expressed higher levels of IDO (Figure 2E) in these mice. Furthermore, the impact of feeding WT milk on the expansion of these tolerogenic cDC1, most notably in the MLN, was observed also at day 15 during the period when antigen was introduced (Figure E4).

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Tregs were evaluated based on the percentage of $CD3^+/CD4^+/CD25^+/FOXP3^+$ cells out of viable $CD45^+$ leukocytes (Figure 3A). Pups derived from $TLR2^{-/-}$ dams but nursed by WT dams had substantially more Tregs in the PP and MLN compared to the siblings that were not cross-fostered at weaning (Figure 3B, 3C, 3D). Unlike $CD103^+$ cDC1, the levels of Tregs at day 15, were not influenced by milk from WT or $TLR2^{-/-}$ dams (Figure E5), which suggests that expansion of tolerogenic $CD103^+$ cDC1 in the MLN could drive the later expansion of Tregs.

We also investigated the levels of T helper cell subsets and ILCs, which could play an important role in susceptibility to food allergy. Our results have shown that frequencies of Th1, Th2, or Th17, as well as ILC2 and ILC3, were not significantly different between pups born to WT or $TLR2^{-/-}$ dams (Table E2). Collectively, this indicates that milk from WT dams promotes an increase in tolerogenic $CD103^+$ cDC1 that express high levels of IDO, which is followed by the expansion of Tregs in the GALT (42).

Pups born to $TLR2^{-/-}$ dams and nursed by WT dams have decreased intestinal permeability

Intestinal permeability is an important factor in the development of oral tolerance (20). Intestinal maturation takes place close to weaning in rodents, while in humans it normally occurs earlier (43). The passage of inappropriately digested antigens via more permeable intestinal epithelial barrier might predispose to food sensitization (44,45). We, therefore, tested intestinal permeability in pups to determine if it was impacted by the TLR2 expression of the nursing dam. By monitoring FITC-Dextran absorption from the small intestine 2 hours after ingestion, we found that the pups born and nursed by $TLR2^{-/-}$ dams had significantly higher intestinal permeability at the time of weaning compared to their siblings nursed by WT dams (Figure 4).

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This implicates early exposure to colostrum and milk from WT dams as having a different impact in promoting intestinal barrier integrity than milk from TLR2$^{-/-}$ dams.

Milk from TLR2$^{-/-}$ dams has lower levels of IL-6, IL-13, IFN-γ, and IGF-1

Given the different oral tolerance development outcomes of nursing TLR2$^{+/-}$ pups by WT and TLR2$^{-/-}$ dams, we investigated the levels of key immune modulatory factors in mouse milk, including cytokines, growth factors, sIgA, and soluble TLR2 that influence oral tolerance development. Using a multiplex assay, we observed that milk from WT dams had significantly higher levels of IFN-γ and IL-6 after day 5, as well as IL-13, and IGF-1 at day 10 compared to milk from TLR2$^{-/-}$ dams. These differences were most notable at later time points pre-weaning (Figure 5). Levels of IL-4, total IgA, TGF-β1, and TGF-β2 were not significantly different between TLR2$^{-/-}$ and WT dams (Figure 5). Levels of IL-10 and soluble TLR2 in mouse milk were below the limit of detection of our current assays. Differences in multiple regulatory factors in milk taken from WT and TLR2$^{-/-}$ dams might provide one explanation for altered tolerance induction, regulatory cell populations and intestinal permeability in pups nursed by them.

IGF-1 and IL-6 impacts the expansion of tolerogenic cDC1 and Tregs

To further investigate the mechanism whereby milk components from wild type dams enhanced tolerogenic cDC and Treg populations associated with tolerance, we supplemented the diet of neonatal mice with IGF-1 or IL-6. These cytokines have been shown to modify oral tolerance mechanisms and were reduced in the milk from TLR2$^{-/-}$ dams. Heterozygote pups born from TLR2$^{-/-}$ dams remained with their birth mother and were divided into 3 groups that were gavaged with IGF-1, IL-6, or PBS, respectively, every second day from day 4 until weaning on day 21 (Figure 6A). IGF-1 supplementation was found to increase the populations of CD103^{+} cDC1 in

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353 both the MLN and the spleen and Tregs in the MLN (Figure 6B), while IL-6 supplementation
354 had a more restricted impact in increasing $CD103^{+}$ cDC1 in the MLN only. Notably, neither
355 IGF-1 nor IL-6 supplementation had any significant impact on small intestine permeability.
356 These data suggest that IGF-1 and IL-6 are important factors in milk from wild type mice and
357 that their decreased levels in $TLR2^{-/-}$ derived milk can impact oral tolerance through the
358 expansion of tolerogenic DCs and subsequently Tregs.

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Discussion

In mice, the development of oral tolerance in genetically identical $TLR2^{+/-}$ litters was found to be highly dependent on the nursing dam's TLR2 genotype. We sought to test the impact of milk from WT or $TLR2^{-/-}$ dams on tolerance towards ingested antigens, provided either directly to pups or via the maternal milk (46). Although the antigens in maternal milk are delivered in more tolerogenic forms (43), both intact and milk-derived OVA-induced oral tolerance. However, the levels of tolerance observed depended on the nursing mother's genotype. Exposure to OVA was sufficient to protect pups cross-fostered with WT dams compared to their siblings that remain with their biological $TLR2^{-/-}$ dams against food sensitization, marked by a selective reduction of anti-OVA IgE. These findings demonstrate that the innate immune status of the feeding dam can define the extent of later immune responses in the pups, even though these pups are phenotypically normal. To our knowledge, the ability of a maternal innate immune deficiency to modify later immune responses in normal offspring, via a milk-dependent mechanism, has not been previously reported. However, profound immunoglobulin deficiency in dams has been reported to impair early immunoglobulin responses in normal pups (47).

This work has important implications for the interpretation and design of studies of mice with deficiencies in TLR2 and related immunoregulatory molecules. It has been generally assumed that the immune phenotype of mice is genetically determined and not highly dependent on the nature of the breeding pairs from which they are derived. Our studies demonstrate that the immune status of the feeding dam can be critical in defining the later immune phenotype of "control" heterozygous littermate pups. There has been considerable controversy regarding the role of TLR2 is some models, including multiple sclerosis (48), colitis-associated colorectal

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cancer (49,50) and Th1/Th2 inflammatory responses (51–53). The immune status of the feeding dam is not usually considered in such studies and may require further attention.

TLR2 can bind a broad range of microbial or endogenous ligands and induces cells to respond via secretion of pro- or anti-inflammatory cytokines (54). TLR2 is expressed in mammary epithelial cells and milk-derived cells (Figure E2) and plays an important role in the normal development of the mammary gland and the formation of the ductal tree (55). Stimulation of TLR2 can induce ectodomain shedding, which can be detected as several soluble isoforms in human fluids, such as saliva, plasma, and breast milk and act as a decoy receptor (26,56). Levels of sTLR2 in human milk have been related to infant susceptibility to viral infection (56). Notably, unlike the substantial levels of sTLR2 reported in human milk (57) levels of sTLR2 in mouse milk were below the sensitivity of current assay systems and could not be reliably evaluated using either Western blot or commercial ELISA reagents.

At birth, the digestive and immune systems are immature, with exposure to breast milk playing a significant role in their maturation (58). Critical to the development of oral tolerance are local dendritic cell populations (59). Among several DC subsets, the frequency of tolerogenic $CD103^+$ cDC1 was substantially induced by the milk from WT dams with an increase in their expression of IDO. Generally, $CD103^+$ tolerogenic DCs exhibit regulatory responses, promote the expansion of Tregs, and play a unique role in inducing homing molecules on Tregs in the MLN, which enable these cells to migrate to other sites in the gastrointestinal tract, including PP and LP to induce tolerance (14,38,60,61). According to Yamazaki et al, $CD8^+$ DCs (cDC1) are stronger inducer of Tregs than other DC subsets (62). Increased expression of IDO by the tolerogenic DCs from mice fed by WT dams, could enhance Tregs expansion. Inhibition of IDO *in vivo* significantly affects the development of antigen-specific Tregs in the gut (42).

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Consistently, we found a higher frequency of tolerogenic DCs in the MLN associated with increased levels of Tregs, within the MLN and the PP in TLR2$^{+/-}$ pups nursed by WT dams compared to those nursed by TLR2$^{-/-}$ dams. Tregs can directly inhibit class switching of B cells towards IgE and induce IgA production, which has a protective role in food allergy (63,64). The enhanced regulatory milieu, marked by higher tolerogenic DCs and Tregs levels, might contribute to the observed ability of pups fed by WT dams to develop effective oral tolerance.

The homeostasis and proper development of the intestinal barrier are critical for the establishment of oral tolerance. Although the impact of intestinal epithelial integrity on the development of food allergy is not well defined experimentally, it remains classified as a risk factor for allergy development (65). TLR2 $^{+/-}$ pups cross-fostered onto WT dams had significantly less permeable small intestines on day 21 compared to siblings that were fed by a TLR2$^{-/-}$ dam. Collectively, our data suggested that the milk from TLR2$^{-/-}$ mothers impairs appropriate immune regulation and barrier function development.

It is notable that tolerance induction and intestinal permeability in pups born to WT dams were not robustly affected by the milk from TLR2$^{-/-}$ dams, which could be attributed to the early exposure to WT milk during the 1-3 days after birth and before cross-fostering as well as *in utero* factors.

Although several cytokines and growth factors were detectable in mouse milk, only IGF-1, IL-6, IFN-γ, and IL-13 were significantly higher in the milk from WT dams compared to TLR2$^{-/-}$ dams, most notably at day 10 after birth. These cytokines have pleiotropic effects, which include direct impacts on immunoglobulin responses as well as Tregs, DCs and intestinal epithelial cells. Their reduced level in milk from TLR2$^{-/-}$ dams is consistent with a combined impact in

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promoting oral tolerance in nursed pups. Variation of such factors in milk might be linked to the role of TLR2 on the development of the mammary ductal tree (55).

The link between IGF-1 and TLR2 has not been well studied. However, Bohacek et al. (66) showed that TLR2 deficiency decreases the secretion of IGF-1 from activated microglia in the brains of TLR2KO mice. IGF-1 plays an important role in the normal development of the immune system and the gastrointestinal tract. Importantly, our results indicate that IGF-1 can significantly increase the levels of tolerogenic $CD103^+$ DC1 in the MLN and the spleen of the pups that are nursed by $TLR2^{-/-}$ dams compared to the control. In addition, the levels of Tregs were significantly increased in the MLN in IGF-1-fed pups. Although Tregs expansion might be induced by the high levels of tolerogenic DC1, IGF-1 is able to directly stimulate the proliferation of human and mouse Tregs both *in vitro* and *in vivo* (67). Burrin *et al*. showed that supplementation of IGF-1 in baby formula significantly enhanced small intestinal growth and maturation in neonatal pigs (68). Furthermore, IGF-1 increased intestinal epithelial and smooth muscle cell proliferation (69,70) and decreased rates of apoptosis in small intestine crypts (71,72). In contrast, our results have shown no significant impact of IGF-1 supplementation on intestinal integrity.

Similarly, supplementation of IL-6 to the pups that are nursed by $TLR2^{-/-}$ dams improved the levels of $CD103^+$ DC1 in the MLN, which could synergize with IGF-1 to boost the upregulation of these cells. *In vitro* experiments have shown that the splenocytes derived from WT but not $TLR2^{-/-}$ mice were responsive to TLR2 agonists by secretion of IL-6 (Figure E1). IL-6 can induce the generation of tolerogenic DCs *in vitro* in synergy with IL-10 (73). In contrast to our findings of the inability of IL-6 supplementation to improve intestinal permeability, Kuhn et al. (74)reported that $IL\text{-}6^{-/-}$ mice have increased intestinal permeability and a thinner mucus layer.

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IL-13 promotes the proliferation of goblet cells in the intestine and mucus production from these cells (75–77). In addition, this cytokine also promotes colonic wound healing (77,78) with potential impacts on barrier function. Although IL-13 traditionally skews the immune response towards Th2 and IgE production, it has been reported that a decrease in IL-13 producing cells in maternal milk predisposes nursed infants to atopic dermatitis, although the mechanism remains undefined (79).

IFN-γ has been less well studied in the context of oral tolerance. However, Prokesova *et al*. showed that the levels of IFN-γ in human milk are inversely correlated with allergic disease development (80). Furthermore, activation of cDC1 ($CD8^+$ DCs) by IFN-γ increases their expression of IDO and results in improved tolerogenic activity (81,82). The mechanisms of IFN-γ-mediated suppression of allergic responses might also involve inhibition of IgE class switch in B cells, suppression of Th2 recruitment add function, and regulation of allergen presentation(83).

IL-4, TGF-β1, TGF-β2, and total IgA were also detectable in mouse milk but not significantly different between $TLR2^{-/-}$ and WT mice. These findings suggest that the differences we observed in mouse milk cytokine profiles were selective and related to TLR2 status.

Collectively, our data demonstrate an important role for TLR2 in modulating milk composition that impacts the development of oral tolerance in early life. It is likely that the overall impact of TLR2 expression by dams on oral tolerance is multi-factorial. TLR2 has been shown to not substantially impact the composition of intestinal microbiota in BALB/c mice (84) and our WT and $TLR2^{-/-}$ mice were co-housed for long periods before and throughout breeding cycles, which suggests that variation in milk composition between WT and $TLR2^{-/-}$ could be independent of bacterial signaling. However, other immunomodulatory factors that are reported to be in mouse

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milk might be subject to changes under the TLR2 effect, such as oligosaccharides, vitamins, and soluble innate immune receptors and require further investigations.

In conclusion, the role of the maternal innate immune system on the development of oral tolerance in neonates during lactation is not well studied. We have demonstrated that milk from WT dams promotes the development of oral tolerance when compared with milk from TLR2-deficient dams. Our observations highlight the importance of genetic aberrations in innate immune receptors in modulating the composition of milk, which might impact susceptibility to allergic responses. Although our work and methodology focus on TLR2, it could be also applied to other immune factors and related signaling molecules. Further studies of innate immune regulation in defining breast milk composition may provide important opportunities for early intervention for disease prevention.

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Authors' contributions

BD completed all in vivo and in vitro experiments, analyzed the data, and wrote the first draft of the manuscript with some technical assistance. IH assisted in the planning and design of experiments and editing of the manuscript. MA assisted in the conceptualization and editing of the manuscript. JM initiated and directed the research and assisted in designing the *in vivo* experiments and drafting and editing the manuscript.

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Figure Legends

Figure 1. TLR2$^{+/-}$ pups cross-fostered onto WT dams have significantly less food sensitization compared to siblings that remain with their biological TLR2$^{-/-}$ dam. (A) Schematic of cross-fostering experiment to evaluate WT or TLR2KO milk effect on food sensitization. (B) Levels of anti-OVA IgE in pups that were ingesting OVA directly during lactation. Data are from four independent experiments with different dams (n=22-28 in OVA ingestion groups). (C) Levels of anti-OVA IgE in pups nursed by dams that were ingesting OVA during lactation. Data are from two independent experiments (n=4-11 in OVA ingestion groups). Statistical analysis was performed by one□way ANOVA followed by Fisher's least significant difference (LSD) post-hoc test. Bars represent the mean ± SEM IgE levels. *P < 0.05, **P < 0.01. # P < 0.05, ## P < 0.01, ### P < 0.001, #### P < 0.0001, OVA versus pooled data from no-OVA exposure (n=15).

Figure 2. Milk from WT dams supports the expansion of tolerogenic DCs in MLN and spleen

(A) Schematic of cross-fostering experiment to evaluate the role of maternal TLR2 during lactation on tolerogenic DCs, Tregs, and intestinal permeability. (B) Representative flow cytometry plots of gating on tolerogenic DCs. (C, D) Frequency of tolerogenic CD103^{+} DCs in MLN and spleen out of MHC-II^{+}/CD11c^{+} cells or within the cDC1 subset (E and F). Data are from two independent experiments (n=5-8 in each group). (G) The expression of mRNA for *Ido* was determined in purified CD103+ cDC1 cells from the MLN of WT or TLR2$^{-/-}$-nursed pups (n=4-5 and each point is derived from a pool of two MLNs). Statistical analysis was performed by one-way ANOVA followed by Fisher's LSD post-hoc test and the bars represent mean ± SEM. *P < 0.05, ***P < 0.001, ****P < 0.0001.

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Figure 3. Milk from WT dams supports the expansion of Tregs in MLN and PP

(A) Representative flow cytometry plots of gating on Tregs. (B-D) Frequency of Tregs in MLN, PP, and spleen (gated on $CD3^+$, $CD4^+$, $CD25^+$ and $FOXP3^+$ cells) out of viable $CD45^+$ cells. Data are from four independent experiments (n=6-18). Statistical analysis was performed by one-way ANOVA followed by Fisher's LSD post-hoc test. Bars represent the mean ± SEM. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001.

Figure 4. Pups born to $TLR2^{-/-}$ mothers but nursed by WT mice have decreased intestinal permeability

The level of FITC-Dextran in the serum of pups nursed by WT or $TLR2^{-/-}$ dams. was quantified Data are from two independent experiments (n=4-9). Statistical analysis was performed by one-way ANOVA followed by Fisher's LSD post-hoc test. Bars represent the mean ± SEM. **P* < 0.05, ***P* < 0.01.

Figure 5. Mouse milk from $TLR2^{-/-}$ mothers has lower levels of IL-6, IL-13, IFN-γ, and IGF-1

Mouse milk was collected at days 1, 5, and 10 after giving birth. The levels of IL-4, IFN-γ, IL-6, IL-13, IGF-1, and IL-10 were measured by a multiplex immune assay. Total IgA, TGF-β1, and TGF-β2 were measured by ELISA. Student's *t*-test was used for statistical analysis at every timepoint. The bars represent the mean ± SEM. **P* < 0.05, ***P* < 0.01.

Figure 6. Supplementation of IGF-1 and IL-6 enhances levels of tolerogenic DCs and Tregs

(A) Schematic of supplementation experiment. (B, C) Levels of $CD103^+$ cDC1 and Tregs in the MLN. (D, E) Levels of $CD103^+$ cDC1 and Tregs in the spleen. Statistical analysis was performed

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542 by one□way ANOVA followed by Fisher's LSD post-hoc test. Bars represent the mean ± SEM.

543 *P < 0.05, **P < 0.01, IGF-1 or IL-6 vs PBS.

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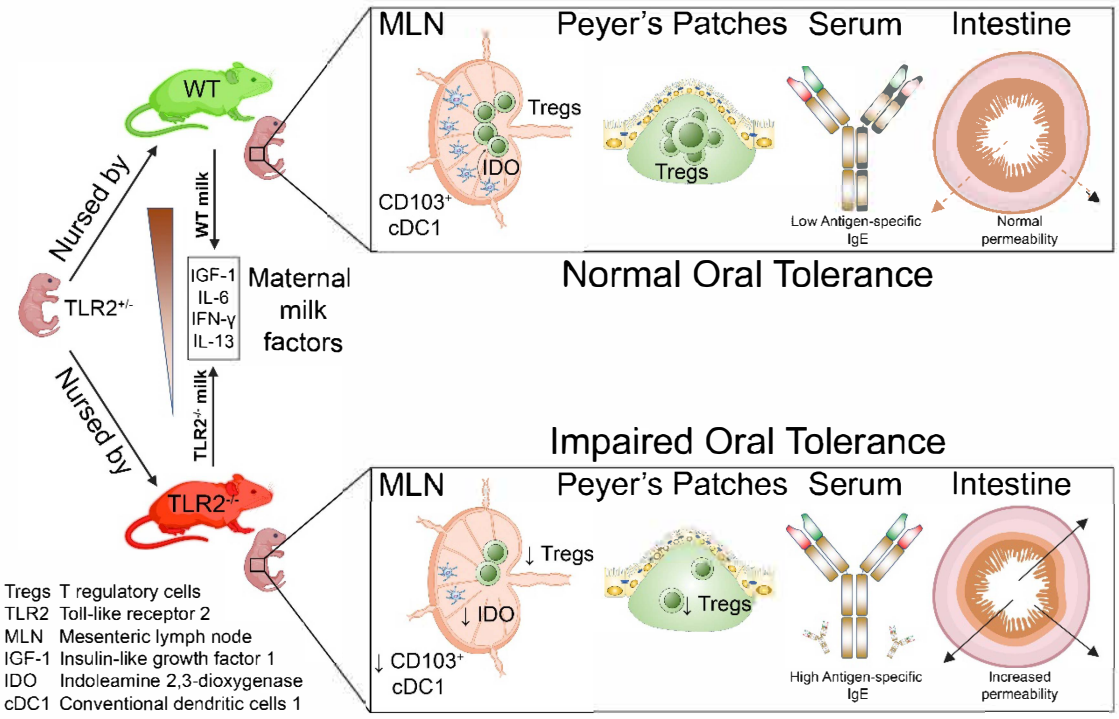
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TLR2 impacts oral tolerance via a milk-dependent mechanism



Tregs T regulatory cells
TLR2 Toll-like receptor 2
MLN Mesenteric lymph node
IGF-1 Insulin-like growth factor 1
IDO Indoleamine 2,3-dioxygenase
cDC1 Conventional dendritic cells 1

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Table 1. Antibody staining panels

<table>
<tr><th>DCs</th><th>ILCs</th><th>Tregs</th><th>T-helper</th></tr>
<tr><td rowspan="12">FITC-CD3ε[1]
PE-CD103[2]
PE-Cy7-Ly6c[1]
PerCP-Cy 5.5-Siglec-H[2]
APC-CD11c[1]
APC-eF780-CD8α[2]
AF700-MHC-II[2]
eF450-FVD[2]
BV786-B220[3]
BV510-CD45[1]
BV650-CD317[1]
PE-eF610-CD11b[2]
BV711-CD24[3]</td><td rowspan="12">FITC-
CD5[1]
CD3ε[1]
CD11c[1]
CD11b[1]
CD19[2]
Gr-1[2]
CD49b[1]

PerCP-Cy 5.5-CD45[3]
APC-IL-33Rα (ST2) [2]
PE-CD117[1]
AF700-CD90.2[1]
eF450-FVD[2]
BV786-CCR6[1]
BV510-CD4[3]
BV605-CD127 (IL-7R)[1]
PE-CD25[1]</td><td colspan="2">Extracellular</td></tr>
<tr><td>FITC-CD3ε[1]
BV510-CD4[3]
PE-CD25[1]
APC-CD45[1]
eF450-FVD[2]</td><td>FITC-CD4[2]
PE-Cy7-CD3ε[2]
PerCP-Cy 5.5-CD45[3]
eF450-FVD[2]</td></tr>
<tr><td colspan="2">Intracellular</td></tr>
<tr><td>PE-FOXP3[2]</td><td>PE-IFN-γ[1]
APC-IL-4[4]
BV510-IL-17[1]</td></tr>
<tr><td colspan="4">1. BioLegend (San Diego, CA)
2. Invitrogen (San Diego, CA)
3. BD Horizon™ (San Jose, CA)
4. BD Pharmingen (San Jose, CA)</td></tr>
</table>

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Table 2

		Th1	Th17	Th2	ILC2	ILC3	cDC1	cDC2	pDC
Organ	Nursing dam	% of $CD4^+$			% of ILCs		% of $MHC\text{-}II^+/CD11c^+$		
MLN	WT	1.320 ± 0.1270 N=4	0.2700 ± 0.03028 N=4	0.1094 ± 0.02281 N=4	10.28 ± 3.082 N=5	8.052 ± 1.690 N=5	29.22 ± 0.7455 N=5	45.40 ± 3.849 N=5	46.56 ± 6.970 N=5
	$TLR2^{-/-}$	1.605 ± 0.2380 N=4	0.3250 ± 0.06252 N=4	0.1775 ± 0.03003 N=4	13.75 ± 2.678 N=8	11.09 ± 1.370 N=8	31.62 ± 0.6650 N=8	41.78 ± 3.036 N=8	36.70 ± 3.998 N=8
P value		0.3314	0.4586	0.1209	0.4246	0.1931	0.2011	0.4749	0.2112
Spleen	WT	3.490 ± 0.3178 N=4	0.1500 ± 0.009129 N=4	0.3475 ± 0.02955 N=4	5.354 ± 0.4267 N=5	4.150 ± 1.014 N=5	5.353 ± 0.7613 N=5	12.27 ± 0.7548 N=5	17.25 ± 1.334 N=5
	$TLR2^{-/-}$	2.745 ± 0.3030 N=4	0.2050 ± 0.0250 N=4	0.4400 ± 0.04103 N=4	6.594 ± 1.245 N=8	3.021 ± 0.5031 N=8	5.707 ± 0.6101 N=8	13.40 ± 0.9242 N=8	20.17 ± 1.201 N=8
P value		0.1407	0.0843	0.1171	0.4640	0.2889	0.7247	0.4136	0.1442

Table 3

Nursing dam	Dendritic cell genes						Tre genes		
	Tgfb1	*Raldh2*	*Cd80*	*Cd86*	*Cd274*	*Btla*	*FOXP3*	*CCR9*	*Cd279*
WT	986.3 ± 110.0 N=5	10506 ± 2162 N=5	310.8 ± 66.73 N=5	264.4 ± 25.13 N=5	1898 ± 193.0 N=5	1081 ± 92.23 N=5	5683 ± 1204 N=4	574.0 ± 171.1 N=4	4650 ± 150.0 N=2
$TLR2^{-/-}$	1199 ± 182.8 N=4	7421 ± 1788 N=4	184.1 ± 38.10 N=4	307.4 ± 62.60 N=4	1445 ± 237.3 N=4	827.7 ± 238.4 N=4	5625 ± 878.4 N=7	597.9 ± 75.75 N=7	5600 ± 778.9 N=4
P value	0.3292	0.3246	0.1693	0.5103	0.1771	0.3157	0.9696	0.8852	0.4631

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Toll-like receptor 2 impacts the development of oral tolerance in mouse pups via a milk-dependent mechanism

Bassel Dawod MD, MSc [1,3], Ian D. Haidl PhD[2,3], Meghan B. Azad PhD[4], Jean S. Marshall PhD[1,2,3]

Supplementary text

Figure E1

WT but not TLR2$^{-/-}$ splenocytes are able to respond to TLR2 agonist in a dose depending manner.

The levels of IL-6 secreted from WT or TLR2$^{-/-}$ splenocytes that were cultured with Pam_3CSK_4 for 24 hours at 37° were measured by ELISA. Data are from a single experiment (n=6 per each timepoint). Statistical analysis was performed by one‐way ANOVA followed by Fisher's least significant difference (LSD) post-hoc test. Bars represent the mean ± SEM. **** $P < 0.0001$.

Figure E2

The leukocytes in the milk of TLR2$^{-/-}$ dams do not express TLR2 compared to the leukocytes in the milk of WT dams.

Milk from WT or TLR2$^{-/-}$ mice was centrifuged at 300 x g and the cell pellets were washed and stained with FITC-anti-mouse TLR2 before analysis by flow cytometry to determine TLR2 expression levels. The plots shown are representative of 1 experiment (n=3 in each group).

Figure E3

Milk from WT or TLR2$^{-/-}$ dams does not alter levels of anti-OVA IgA, IgG1, and IgG2a in the pups.

The levels of anti-OVA IgA, IgG1, and IgG2a were assessed by ELISA in pups that were exposed to OVA during lactation. Data are from four independent experiments with different dams (n=11-13 in OVA ingestion groups and n=4 in each no-OVA group). Statistical analysis was performed by one‐way ANOVA followed by Fisher's least significant difference (LSD) post-hoc test. Bars represent the mean ± SEM. *** $P < 0.001$, **** $P < 0.0001$ (IgG1, OVA

versus pooled data from no-OVA exposure). # $P < 0.05$ (IgG2a, OVA versus pooled data from no-OVA exposure). $ $P < 0.05$ (IgA, OVA versus pooled data from no-OVA exposure).

Figure E4

Milk from WT dams supports the expansion of tolerogenic CD103$^+$ cDC1 in MLN on day 15 after birth.

(A, B) Frequency of tolerogenic CD103$^+$ cDC1 in MLN and spleen out of MHC-II$^+$/CD11c$^+$ cells. Data are from two independent experiments (n=4-6 in each group). Statistical analysis was performed by one□way ANOVA followed by Fisher's LSD post-hoc test. Bars represent the mean ± SEM. *$P < 0.05$, **$P < 0.01$. BM= birth mother. NM=nursing mother.

Figure E5

Milk from WT or TLR2 dams does not exhibit a major expansion on Tregs in the GALT at day 15 after birth

(A-C) Frequency of Tregs in MLN, PP, and spleen (gated on CD3$^+$, CD4$^+$, CD25$^+$ and FOXP3$^+$ cells) out of viable CD45$^+$ cells. Data are from three independent experiments (n=4-8). Statistical analysis was performed by one□way ANOVA followed by Fisher's LSD post-hoc test. Bars represent the mean ± SEM. *$P < 0.05$, **$P < 0.01$. BM= birth mother. NM=nursing mother.

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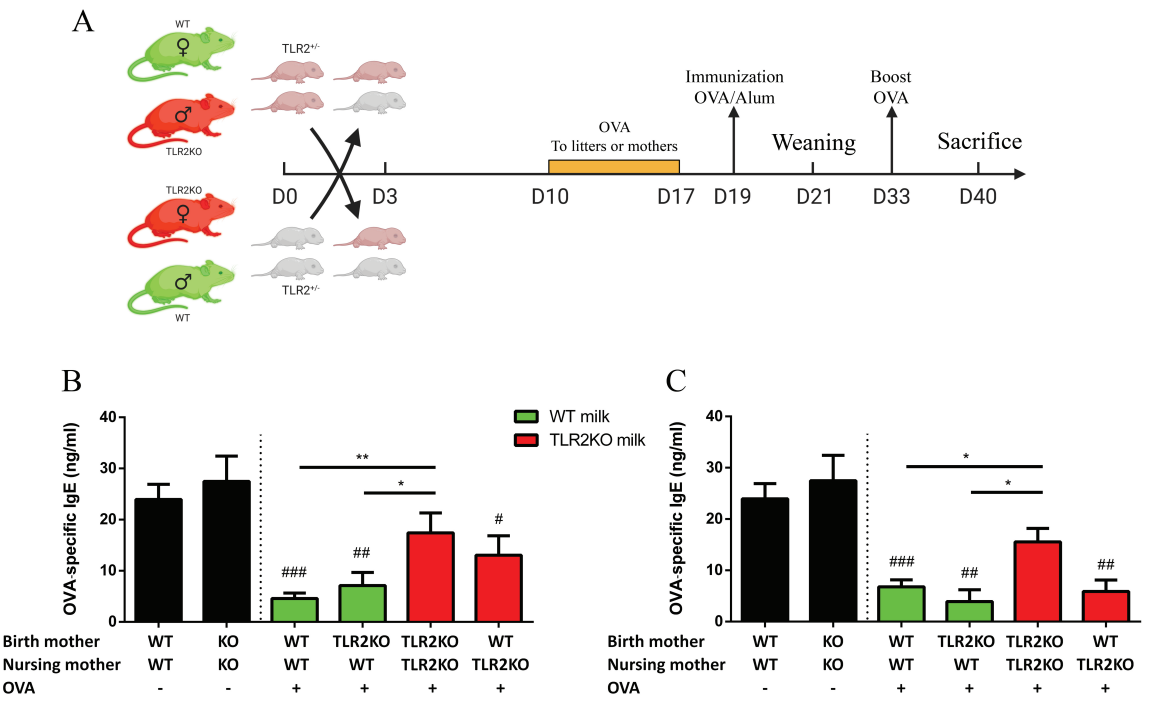
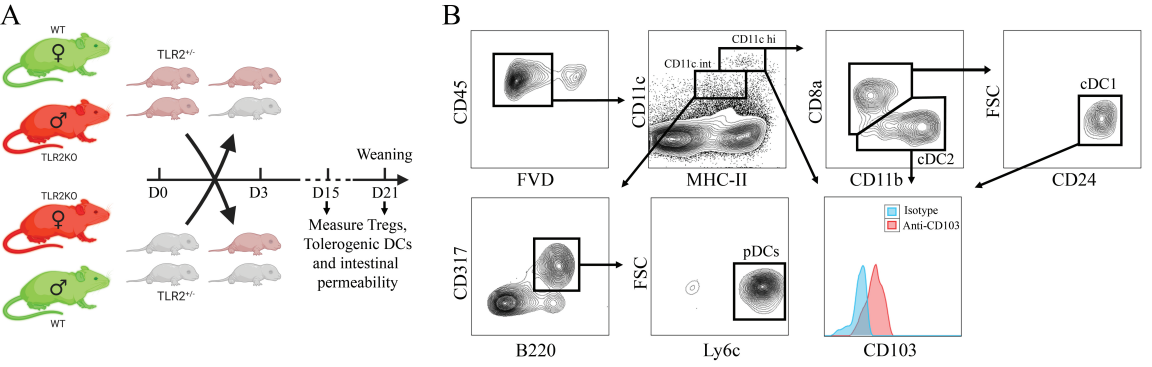

A
WT
TLR2+/-
TLR2KO
TLR2KO
TLR2+/-
WT
Immunization
OVA/Alum
Boost
OVA
OVA
To litters or mothers
Weaning
Sacrifice
D0
D3
D10
D17
D19
D21
D33
D40
B
WT milk
TLR2KO milk
OVA-specific IgE (ng/ml)
40
30
20
10
0
**
*
###
##
#
Birth mother WT KO WT TLR2KO TLR2KO WT
Nursing mother WT KO WT WT TLR2KO TLR2KO
OVA - - + + + +
C
OVA-specific IgE (ng/ml)
40
30
20
10
0
*
*
###
##
##
Birth mother WT KO WT TLR2KO TLR2KO WT
Nursing mother WT KO WT WT TLR2KO TLR2KO
OVA - - + + + +

Figure 1


A
WT
TLR2+/-
TLR2KO
TLR2KO
TLR2+/-
WT
Weaning
D0
D3
D15
D21
Measure Tregs, Tolerogenic DCs and intestinal permeability
B
CD45
FVD
CD11c
CD11c int
CD11c hi
MHC-II
CD8a
CD11b
cDC2
FSC
cDC1
CD24
CD317
B220
FSC
pDCs
Ly6c
Isotype
Anti-CD103
CD103

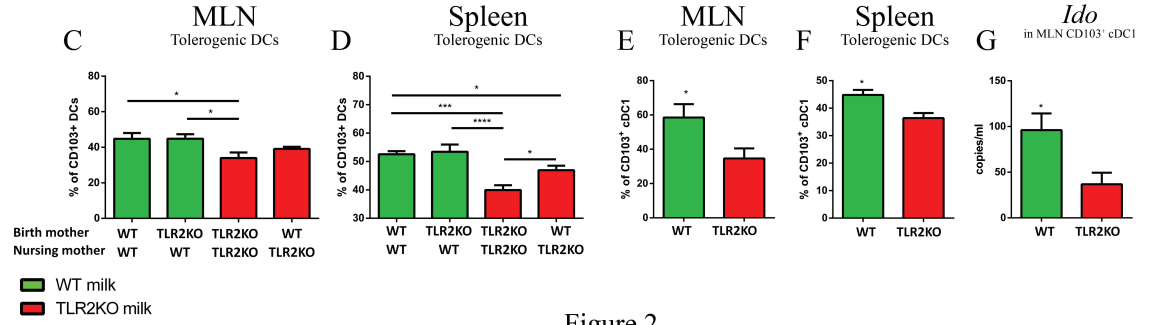

C
MLN
Tolerogenic DCs
% of CD103+ DCs
D
Spleen
Tolerogenic DCs
% of CD103+ DCs
E
MLN
Tolerogenic DCs
% of CD103+ cDC1
F
Spleen
Tolerogenic DCs
% of CD103+ cDC1
G
Ido
in MLN CD103+ cDC1
copies/ml
Birth mother
Nursing mother
WT WT
TLR2KO WT
TLR2KO TLR2KO
WT TLR2KO
WT milk
TLR2KO milk

Figure 2

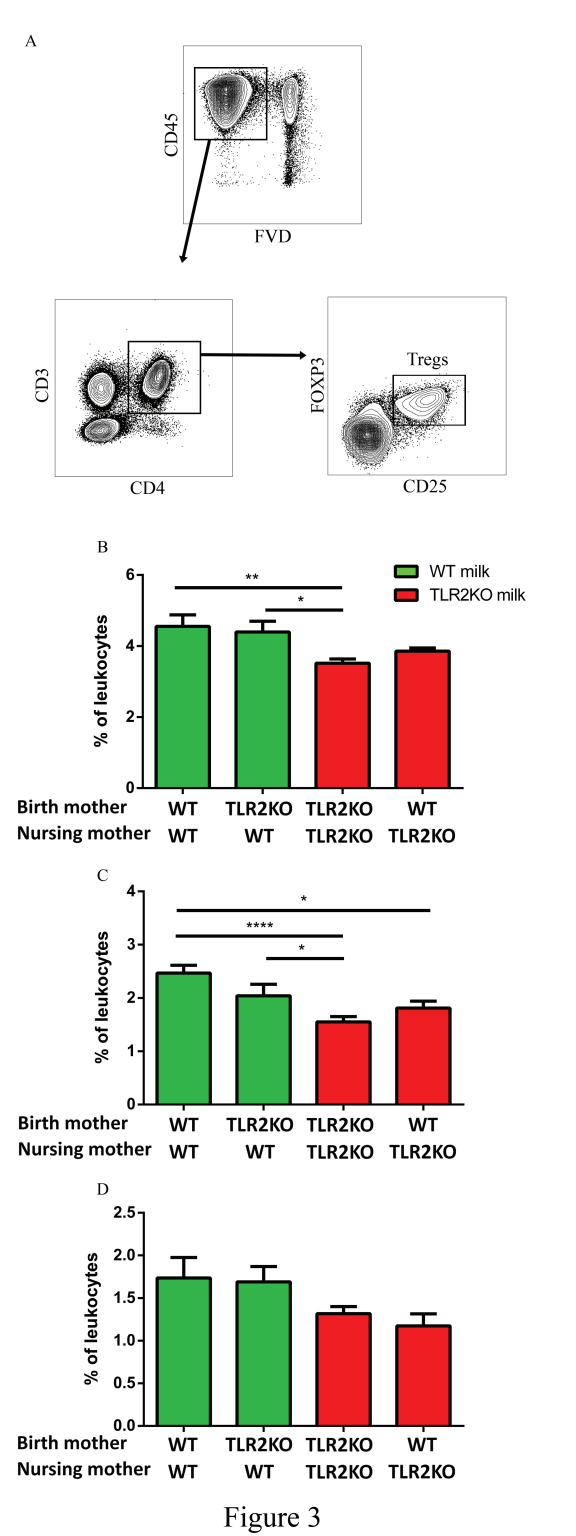

A
CD45
FVD
CD3
CD4
FOXP3
CD25
Tregs
B
WT milk
TLR2KO milk
% of leukocytes
**
*
Birth mother
Nursing mother
WT WT
TLR2KO WT
TLR2KO TLR2KO
WT TLR2KO
C
% of leukocytes
*
****
*
D
% of leukocytes

Figure 3

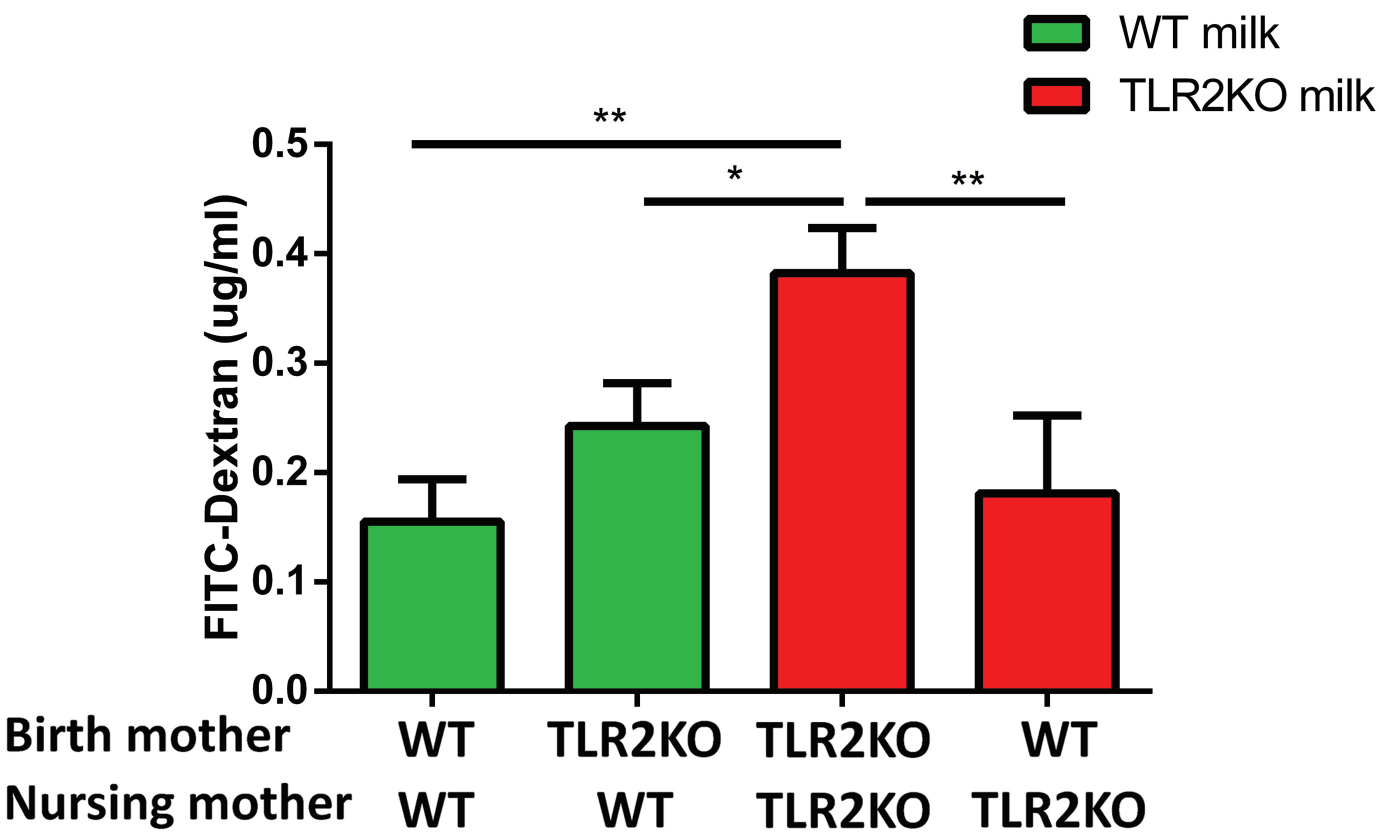

WT milk
TLR2KO milk
**
*
**
FITC-Dextran (ug/ml)
0.5
0.4
0.3
0.2
0.1
0.0
Birth mother
Nursing mother
WT
WT
TLR2KO
WT
TLR2KO
TLR2KO
WT
TLR2KO

Figure 4

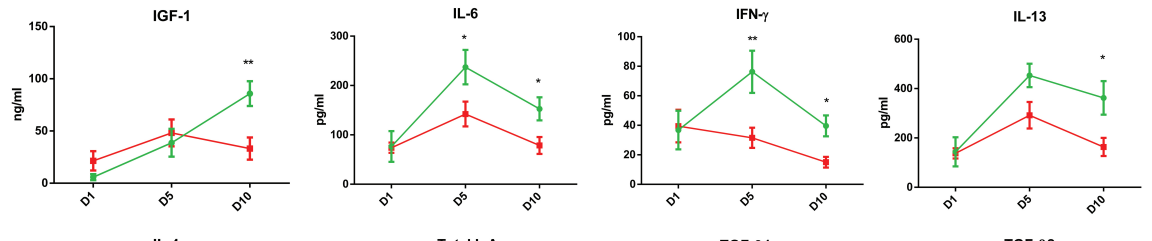

IGF-1
ng/ml
150
100
50
0
D1
D5
D10
**
IL-6
pg/ml
300
200
100
0
*
*
IFN-γ
pg/ml
100
80
60
40
20
0
**
*
IL-13
pg/ml
600
400
200
0
*
IL-4
pg/ml
250
200
150
100
50
0
Total IgA
µg/ml
400
300
200
100
0
TGF-β1
ng/ml
10
8
6
4
2
0
TGF-β2
ng/ml
80
60
40
20
0

Figure 5

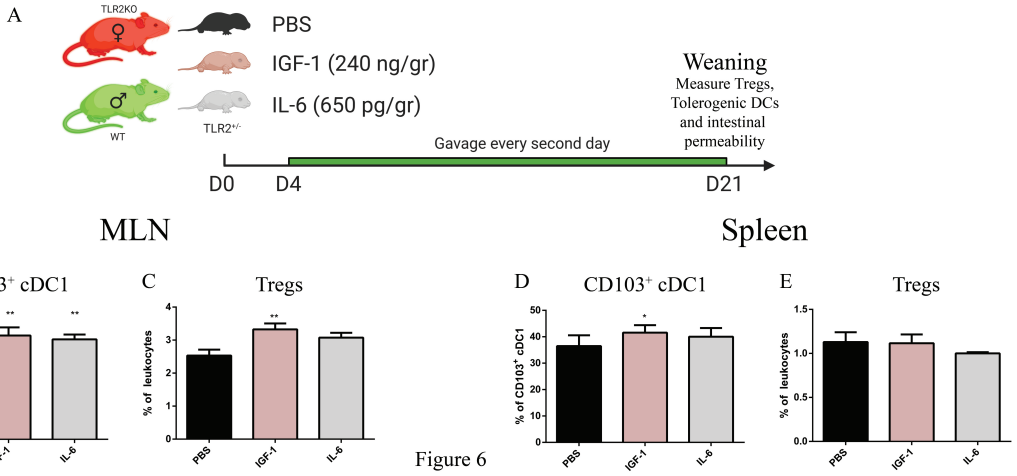

A
TLR2KO
♀
♂
WT
TLR2+/-
PBS
IGF-1 (240 ng/gr)
IL-6 (650 pg/gr)
Weaning
Measure Tregs, Tolerogenic DCs and intestinal permeability
Gavage every second day
D0
D4
D21
MLN
Spleen
B
CD103+ cDC1
% of CD103+ cDC1
C
Tregs
% of leukocytes
D
CD103+ cDC1
% of CD103+ cDC1
E
Tregs
% of leukocytes
PBS
IGF-1
IL-6

Figure 6

Supplementary figures

Figure E1

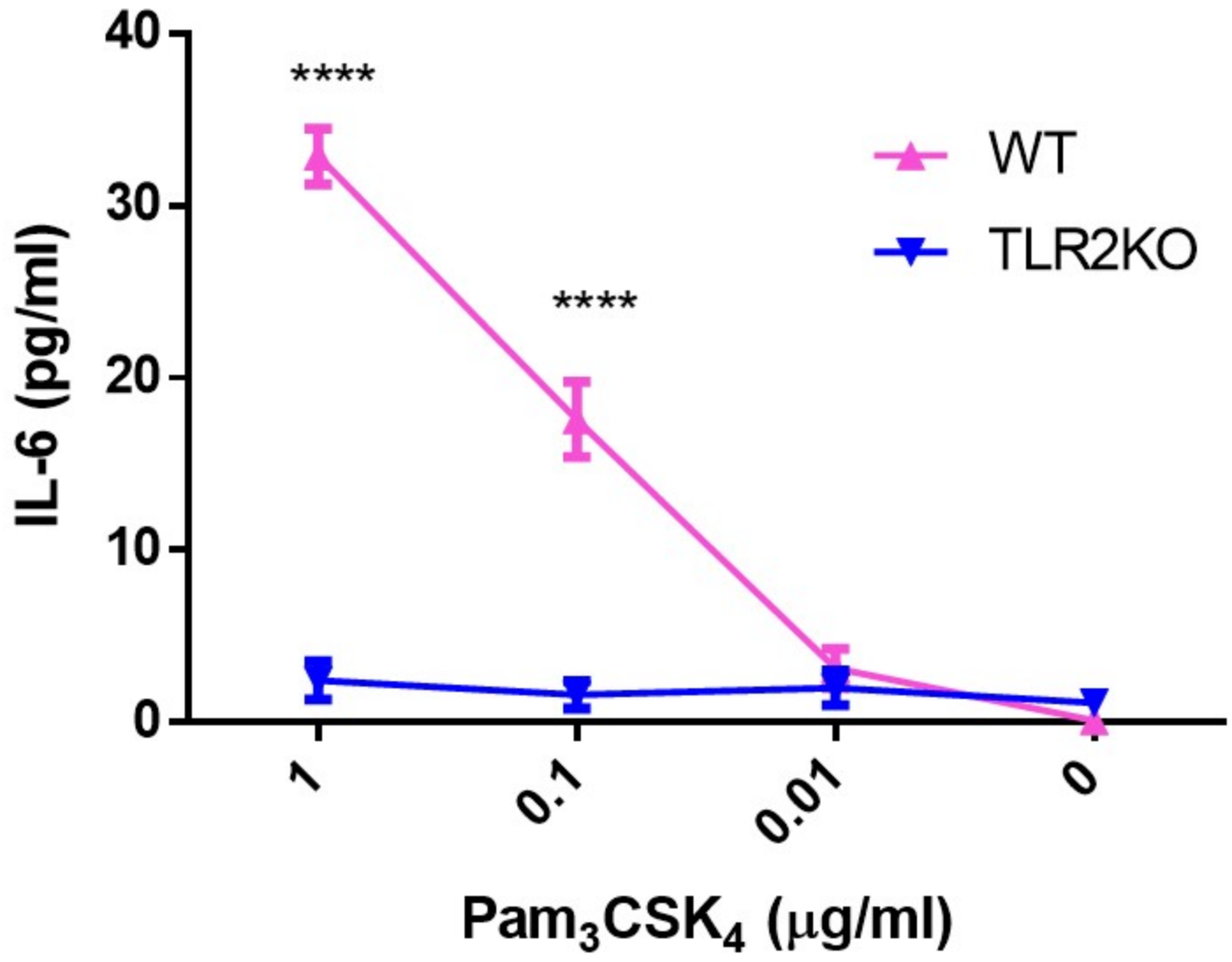


Figure E2

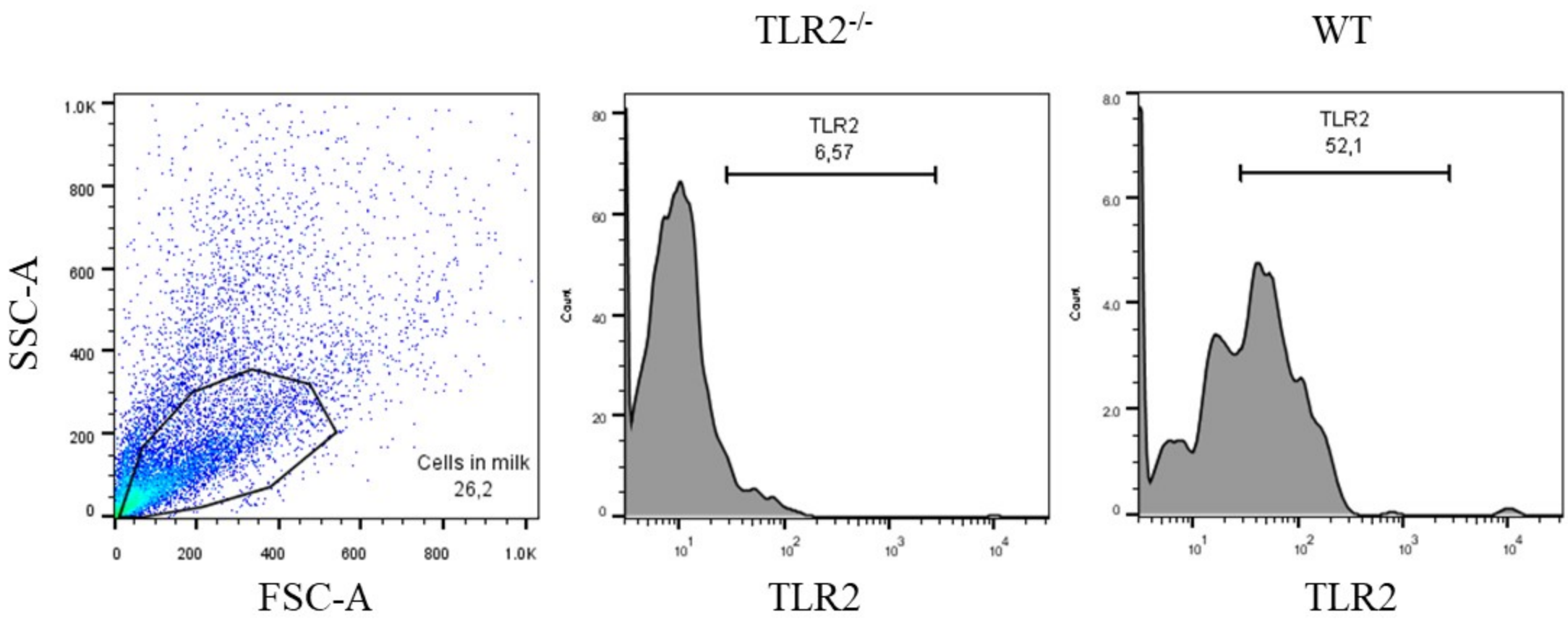

TLR2-/-
WT
SSC-A
FSC-A
Cells in milk
26,2
TLR2
6,57
TLR2
52,1
Count
TLR2
TLR2

Figure E3

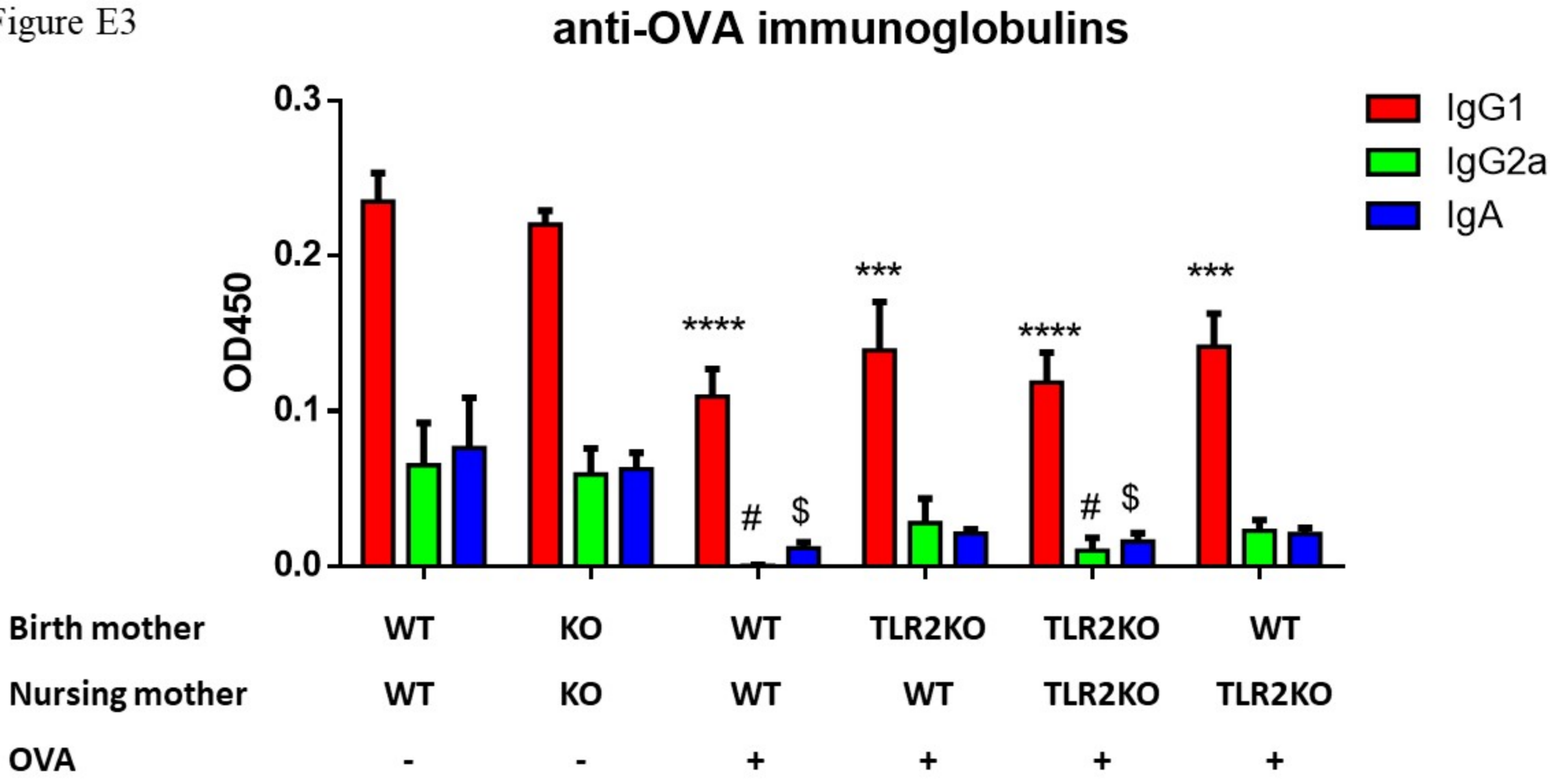

anti-OVA immunoglobulins
IgG1
IgG2a
IgA
OD450
0.3
0.2
0.1
0.0
****
***
****
***
#
$
#
$
Birth mother
WT
KO
WT
TLR2KO
TLR2KO
WT
Nursing mother
WT
KO
WT
WT
TLR2KO
TLR2KO
OVA
-
-
+
+
+
+

Figure E4

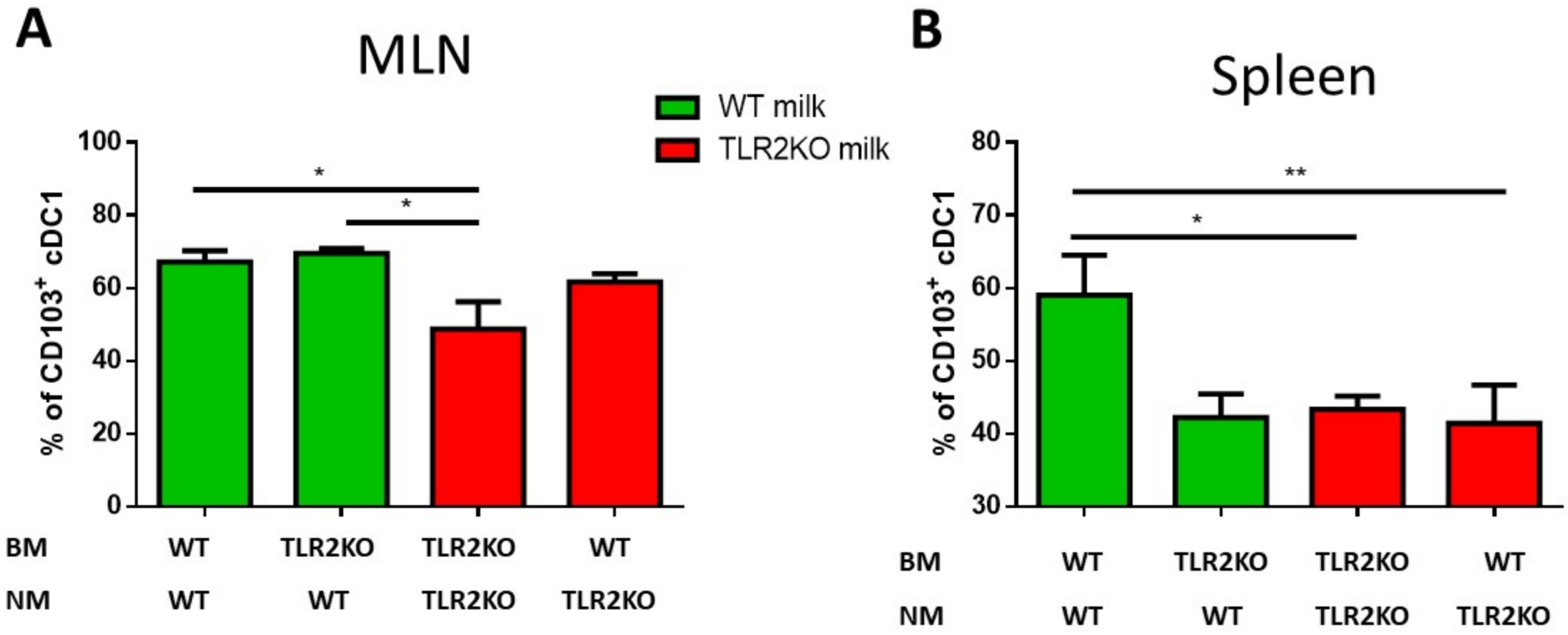
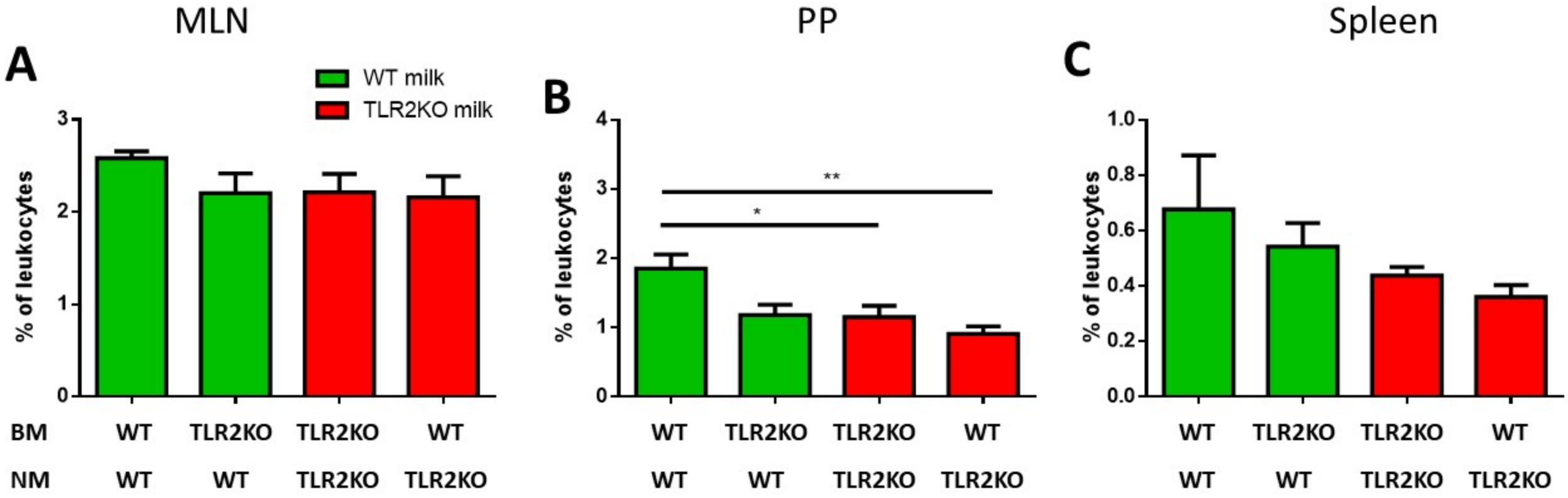

A
MLN
WT milk
TLR2KO milk
% of CD103+ cDC1
100
80
60
40
20
0
*
*
BM
NM
WT
WT
TLR2KO
WT
TLR2KO
TLR2KO
TLR2KO
WT
B
Spleen
% of CD103+ cDC1
80
70
60
50
40
30
**
*
BM
NM
WT
WT
TLR2KO
WT
TLR2KO
TLR2KO
TLR2KO
WT

Figure E5


MLN
PP
Spleen
A
B
C
WT milk
TLR2KO milk
% of leukocytes
**
*
BM WT TLR2KO TLR2KO WT WT TLR2KO TLR2KO WT WT TLR2KO TLR2KO WT
NM WT WT TLR2KO TLR2KO WT WT TLR2KO TLR2KO WT WT TLR2KO TLR2KO