THE ROLE OF TOLL-LIKE RECEPTOR 2 IN MODULATION OF BREAST MILK COMPONENTS AND ORAL TOLERANCE

By

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DEDICATION

I dedicate this work to my mom and dad for all the care and love they provided for me, to my wife, Nour, and kids, Najem, Masa, and Maya, all of my life is for you, and to my brother Mayas, you have always been there for me.

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ABSTRACT

Food allergy is a common immune-mediated disease of early life that has increased in incidence over the past three decades. The immaturity of the neonatal immune system makes them prone to the development of allergy. Dietary components are essential for their immune responses to develop normal oral tolerance. A diversity of scientific opinions about breast milk's ability to protect against the development of food allergies might be related to dramatic variations in milk-associated immune mediators (IMs) between mothers. Pattern recognition receptors (PRRs) are one of the multiple components that regulate IM production. Toll-like receptor 2 (TLR2), a PRR that is highly expressed in the mammary gland and immune cells, mediates secretion of both pro and anti-inflammatory IMs, some of which are found in breast milk. The role of maternal TLR2 in regulating breast milk components and infant immune system development is still unknown. We evaluated the impact of maternal TLR2 deficiency in susceptibility to allergic diseases in infants using a cross-fostering model in mice. Milk from TLR2 deficient dams had altered IMs content compared to WT dams' milk. It failed to induce optimal gastrointestinal integrity and expansion of oral tolerance essential immune cells, including tolerogenic dendritic cells (DCs) and regulatory T cells (Tregs) in pups, thus increasing their susceptibility food sensitization. In humans, a soluble form of TLR2 (sTLR2) was detectable in breast milk, in variable amounts, generally higher than those observed in cow's milk and baby formulas. sTLR2 was undetectable in mouse milk. Human milk sTLR2 concentrations were lower in homozygous mothers with specific single nucleotide polymorphisms (SNPs) in the *TLR2* gene and higher in food allergic mothers. We found that a high level of sTLR2 in milk was associated with an increased incidence of allergies in infants if they were breastfed exclusively for six months regardless of the maternal allergic status. Collectively, our findings and experimental models demonstrate significant associations between maternal TLR2 and food allergy development in infants and the importance of considering both maternal genetic factors and feeding practices.

LIST OF ABBREVIATIONS USED

AD	Atopic dermatitis
APCs	Antigen-presenting cells
BCR	B cell receptor
BTLA	B- and T-lymphocyte attenuator
cDC	Conventional dendritic cell
CHILD	Canadian healthy infant longitudinal development
CLR	C-type lectin receptor
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
EAT	Enquiring About Tolerance
FcRn	Neonatal Fc receptor
FDR	False discovery rate
FVD	Fixable viability dye
GALT	Gut-associated lymphoid tissues
GIT	Gastrointestinal tract
GRO	Growth-regulated oncogene
HMOs	Human milk oligosaccharides
IBD	Inflammatory bowel disease
IDO	Indoleamine 2,3-dioxygenase
IEC	Intestinal epithelial cells
Ig	Immunoglobulin
IL-	Interleukin
ILC	Innate lymphoid cell
IM	Immune mediators
IP-10	Interferon gamma-induced protein 10
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
LP	Lamina propria
LPS	Lipopolysaccharides
LRR	Leucine-rich repeat
MAF	Minor allele frequency
MAMP	Microbe-associated molecular pattern
MCP-1	Monocyte chemoattractant protein-1
MDC	Macrophage-derived chemokine
MHC-II	Major histocompatibility complex type II
MLN	Mesenteric lymph nodes
NK	Natural killer
NLR	NOD-like receptor
OD	Optical density

PAF	Platelet-activating factors
pDC	Plasmacytoid dendritic cell
PG	Peptidoglycans
PGD2	Prostaglandin D_2
PP	Peyer's patches
PRR	Pattern recognition receptor
RA	Retinoic acid
RALDH2	Retinaldehyde dehydrogenase 2
RIG-1	Retinoic acid-inducible gene 1
SCFA	Short-chain fatty acid
SLIT	Sublingual immunotherapy
SNP	Single nucleotide polymorphism
TCR	T-cell receptor
TGF-β	Transforming growth factor
Th	T helper 2
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	T regulatory cell
TSLP	Thymic stromal lymphopoietin
UTRs	Untranslated regions
WHO	World Health Organization
WT	Wild type

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و الحمد لله رب العالمين أولا و آخرا

Chapter 1. INTRODUCTION

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The immune system is a complex network of cells and molecules disseminated across the body to protect the host from external or internal danger and distinguish them from innocuous challenges. Immune tolerance enables humans to ingest food antigens, interact with environmental factors, maintain organ homeostasis, and host a wide range of microbial microorganisms without any immune complications. In contrast, skewing of the immune response away from tolerance can lead to adverse reactions, such as allergic and autoimmune diseases. Educating the immune system to distinguish between harmless and harmful patterns is an essential step in immune tolerance that starts early in life. Infants are born with an immature immune system. Under the influence of genetic, nutritional, environmental, and microbial factors, the immune response can skew towards allergy development or tolerance. One of the primary factors that impact tolerance development in early life is breast milk, which is the first nutritional option for most infants after birth. Understanding the impact of milk components on tolerance vs allergy development can expand our knowledge about the mechanisms of allergic diseases and inform the development of potential allergy therapies and prevention practices.

1.1. History of food allergy and oral tolerance

Historically, before awareness of allergy emerged into the scientific field, a group of scientists, including Louis Pasteur, Paul Ehrlich, Elie Metchnikoff, Jules Bordet and Emil A. Von Behring, described a system that is responsible for protecting the host against harmful molecules [1]. Considering only the positive impact of this system for defence against infection, it was not imagined that it could have adverse effects.

With significant changes in hygiene and lifestyle during the 19th century, most notably in Europe and North America, hay fever was one of the earliest allergic reactions

described in modern history by John Bostock in 1819 [2,3]. “Starting in June every year, a sensation of heat, itchiness, and mucous discharges from the eyes, followed by swelling in the head, sneezing, and difficulty of breathing”, were the symptoms that John Bostock experienced himself and stated in his paper [3]. Similar reports increased in frequency from Germany, UK, and the USA before Blackley from the UK and Wyman from the USA investigated hay fever in response to exposure to grass pollen and ragweed pollen, respectively, in the 1870s [4–6]. With such increased knowledge about adverse reactions mediated by the immune system, it became evident that a new branch of medicine was evolving, and in 1906 the terminology “Allergy” was suggested by Von Pirquet to distinguish the difference between immunity (useful) and hypersensitivity (harmful) [7]. During this period, it became clear that allergic responses require more than one exposure to the allergen to trigger an adverse reaction [1]. After the 1960s, pediatric asthma reports increased in developed countries where it reached an epidemic scale in the 1990s [4,8,9]. Another wave of allergic reactions then started to be reported, including food allergies, most notably peanut allergy [10].

According to a 2012 survey by Prescott *et al.* [11] some developed countries, like Australia, have around 10% of children under five years of age diagnosed with food allergy using oral food challenge. Although the rates differ between countries dramatically, possibly based on the diagnosis method, it is undeniable that the health and economic burdens of food allergies globally are much higher now than in previous decades [11]. During the last two centuries, industry, agriculture, hygiene, and social lifestyle changes are believed to be major drivers for the spike in allergic disease cases globally. The increase in air pollution, use of pesticides, chlorination of water, invention of antibiotics, increased

vaccination rates, and the adoption of an indoor lifestyle may also have, directly or indirectly, changed the behaviour of our immune system towards the environment.

Typically, our bodies interact with multiple dietary and environmental factors, some of which are useful and harmful. The immune system needs to discriminate between such elements, to tolerate or respond to those agents accordingly. Wells had described the concept of oral tolerance in 1911 [12] when he showed that feeding guinea pigs with egg white reduced their responsiveness to sensitization after systemic challenge. By definition, oral tolerance is the state of immune non-responsiveness to innocuous antigens derived from diet, environment, or microbiota that reside in the gastrointestinal tract (GIT). Failure to develop proper oral tolerance early in life can lead to several diseases, such as food allergy, inflammatory bowel disease (IBD), and celiac disease [13]. Understanding the mechanisms underlying the development of allergy or tolerance is critical for developing prevention practices and treatment.

1.1.1. Mechanisms of food allergy

By definition, food allergy is a type I hypersensitivity immune-mediated disease characterized by an adverse reaction toward harmless food antigens [14]. The potential allergic response starts when the allergen is taken up by antigen-presenting cells (APCs) in the GIT, processed, and presented on their surface via the major histocompatibility complex type II (MHC-II) to $CD4^+$ T-cells. In susceptible individuals, in the presence of IL-4, naïve $CD4^+$ T-cells differentiate towards a T helper 2 (Th2) phenotype, which secretes more IL-4 as well as IL-13. Together with other signals, such cytokines facilitate the class switching of B cells to develop into plasma cells producing IgE antibodies circulating in several body fluids, such as the plasma, cerebrospinal fluid, saliva, and even

breast milk [15,16]. Circulating antigen-specific IgE can bind via their Fc portion to special receptors called FcεRI on the surface of mast cells and basophils that become sensitized [17]. Upon a second or subsequent antigen exposure, the allergen cross-links bound IgE on sensitized mast cells and basophils, leading to their activation and degranulation (**Figure 1-1**). The majority of acute allergic response symptoms are attributed to the release of immune mediators (IMs) from mast cells and basophils, and these can have fatal consequences, as in anaphylaxis.

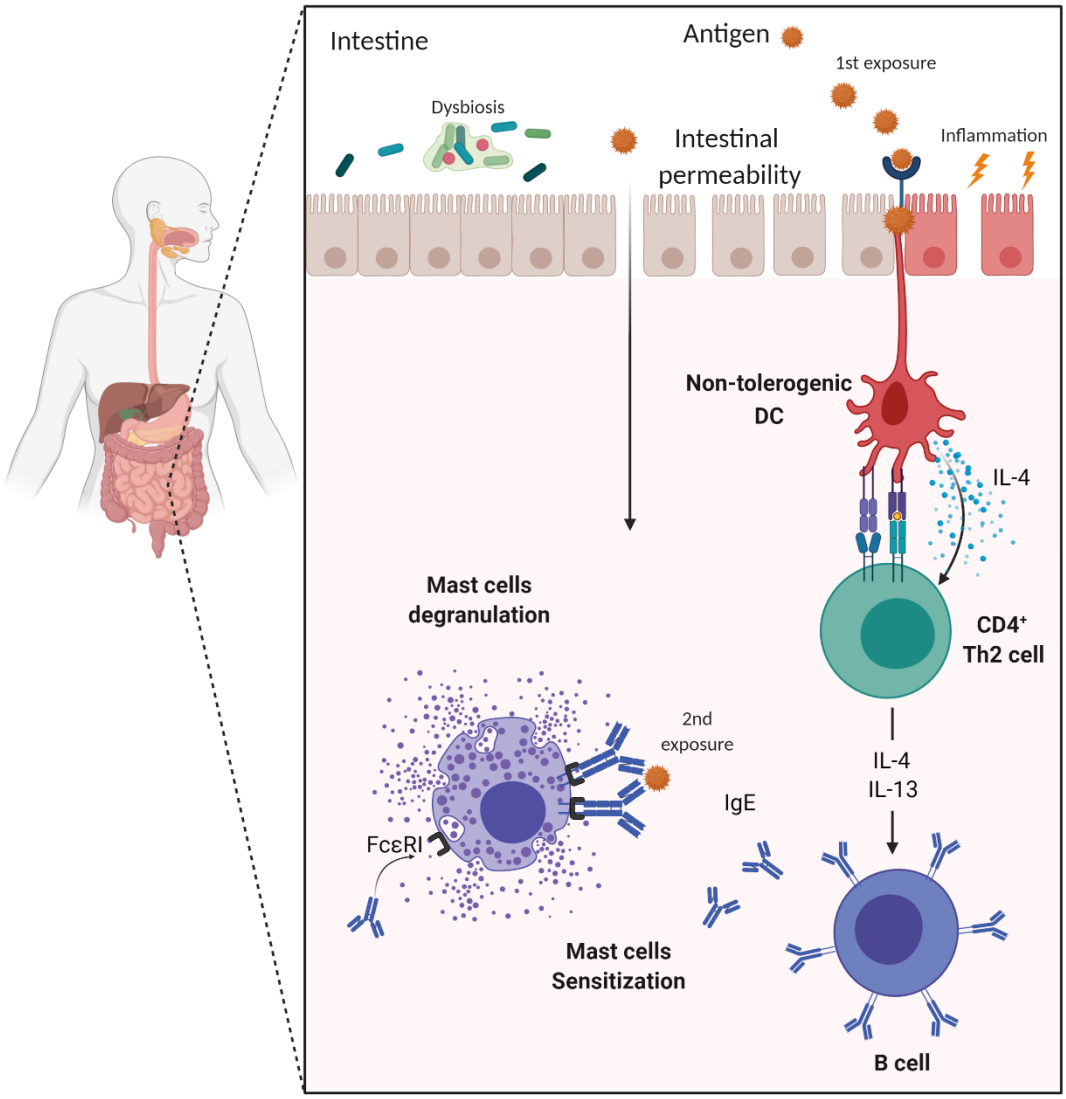

Figure 1-1: Mechanisms of food sensitization and allergy

Several intestinal factors induce the development of food allergy, including intestinal inflammation, permeability, and dysbiosis. After the first exposure to an antigen, non-tolerogenic DCs uptake allergens from the lumen and deliver processed allergen peptides to Th2 skewed T cells under the influence of IL-4. Th2 cells further secrete IL-4 and IL-13 that induces class-switching of B cells to IgE. Circulating IgE antibodies are captured by the high-affinity receptor FcεRI, on mast cells and basophils. Upon second or subsequent exposure, the antigen cross-links membrane-bound IgEs, leading to activation, degranulation, and the release of pro-inflammatory mediators from mast cells and basophils.

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1.1.1.1. Anaphylaxis

A severe form of the acute, systemic allergic reaction is called anaphylaxis. It is life-threatening if not treated immediately and has been well studied over the last three decades [18]. Food allergy is one of the most common causes of anaphylaxis, accounting for 30%-50% of the surveyed cases in developed countries [19–22]. Up to 80% of reported cases are in children [23]. Anaphylaxis can affect any organ in the body leading to pulmonary, gastrointestinal, neurological, cutaneous and circulatory symptoms [18]. The hallmark symptoms of anaphylaxis include swelling, bronchospasm, hypotension, syncope, nausea, vomiting, cramping, urticaria, and rash [24]. A sudden and massive release of inflammatory mediators, including histamine, prostaglandin D_2 (PGD_2), tryptase, carboxypeptidase A, platelet-activating factors (PAF), and tumour necrosis factor (TNF), from mast cells and basophils, are thought to be mainly responsible for the anaphylactic symptoms [25]. Histamine stimulates vasodilation, capillary permeability, oedema, glandular secretion, tachycardia and contractility [26]. PGD_2, PAF, and leukotrienes such as LTC_4, induce bronchoconstriction, which, along with edema, exacerbates pulmonary symptoms [18]. TNF can activate granulocytes, such as neutrophils, stimulate chemokine synthesis and lead to other immune cell recruitment, thereby worsening the inflammatory response [27]. The synergistic effects of released mediators contribute to the overall pathophysiology and symptoms of anaphylaxis.

Two primary mechanisms for anaphylaxis have been reported in mice, either via IgE and the release of histamine and PAF from mast cells or via IgG binds to FcγRIII and the release of PAF but not histamine, as the primary mediator [28–30]. In humans, anaphylactoid reactions independent of IgE have been reported, including complement

anaphylatoxin activation, immune-complex generation, cytotoxicity, and T-cell activation [31]. Understanding the mechanisms of food allergy and anaphylaxis provides essential tools for prevention of this life-threatening reaction, and potential prophylactic or emergency treatment.

1.1.2. Mechanisms of oral tolerance

Oral tolerance is the active suppression of the immune response towards innocuous food and microbial antigens that pass through the digestive system. Failure to maintain a balance between reaction or non-responsiveness can lead to several immune diseases, including food allergy [32,33]. Two general mechanisms for the development of oral tolerance have been suggested. Feeding multiple low doses of an antigen induces the generation of antigen-specific immune suppressor cells represented by T regulatory cells (Tregs) [34,35]. In contrast, the ingestion of high doses of an antigen, even autoantigens, can lead to clonal deletion or anergy of reactive T cells in the Peyer's patches (PP) [36]. Both of these mechanisms lead to suppression of systemic immune responses to the fed antigen.

Immune tolerance to orally ingested antigens is characterized by reduced delayed-type hypersensitivity, T-cell proliferation, cytokine production, and serum immunoglobulin levels, including IgE [13]. The establishment of such tolerance is achieved by interaction among immune cells with regulatory function, such as Tregs, tolerogenic dendritic cells (DCs), epithelial cells and the microbiota discussed in more detail below (**Figure 1-2**) [13]. Several sites along the GIT are vital for the induction of oral tolerance, including the intestinal epithelium, PP, lamina propria (LP) and mesenteric lymph nodes (MLN). The spleen, liver and other sites also contribute to this process. The proper

interaction between immune cells, microbiota, and ingested food antigens in these sites are vital for homeostasis of the GIT.

1.1.2.1. Induction of regulatory T cells

Several types of immune cells are reported to support oral tolerance development; however, Tregs deserve to be on top of the list. Dysfunction in Tregs can lead to several autoimmune and allergic diseases, as observed in patients with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) [37]. Multiple groups have provided evidence of Tregs importance in inhibiting Th2 responses and the adverse effects of Treg depletion in allergic diseases [38–41]. Therefore, understanding the factors that enhance Treg expansion in healthy individuals might help in developing prophylactic treatments for allergic diseases. With the ingestion of low doses of antigens, digested peptides can be displayed by tolerogenic $CD103^+$ DCs in the LP and PPs and then migrate to the MLN via the lymphatics [42]. Migratory $CD103^+$ DCs induce naïve T cells' skewing into Tregs in the MLN using multiple mechanisms (discussed in chapter 1.2.2). Primed antigen-specific Tregs can migrate to PP and LP to inhibit effector T cells from expansion and induce secretion of IgA from B cells, thus, enhancing tolerance (**Figure 1-2**) [43,44].

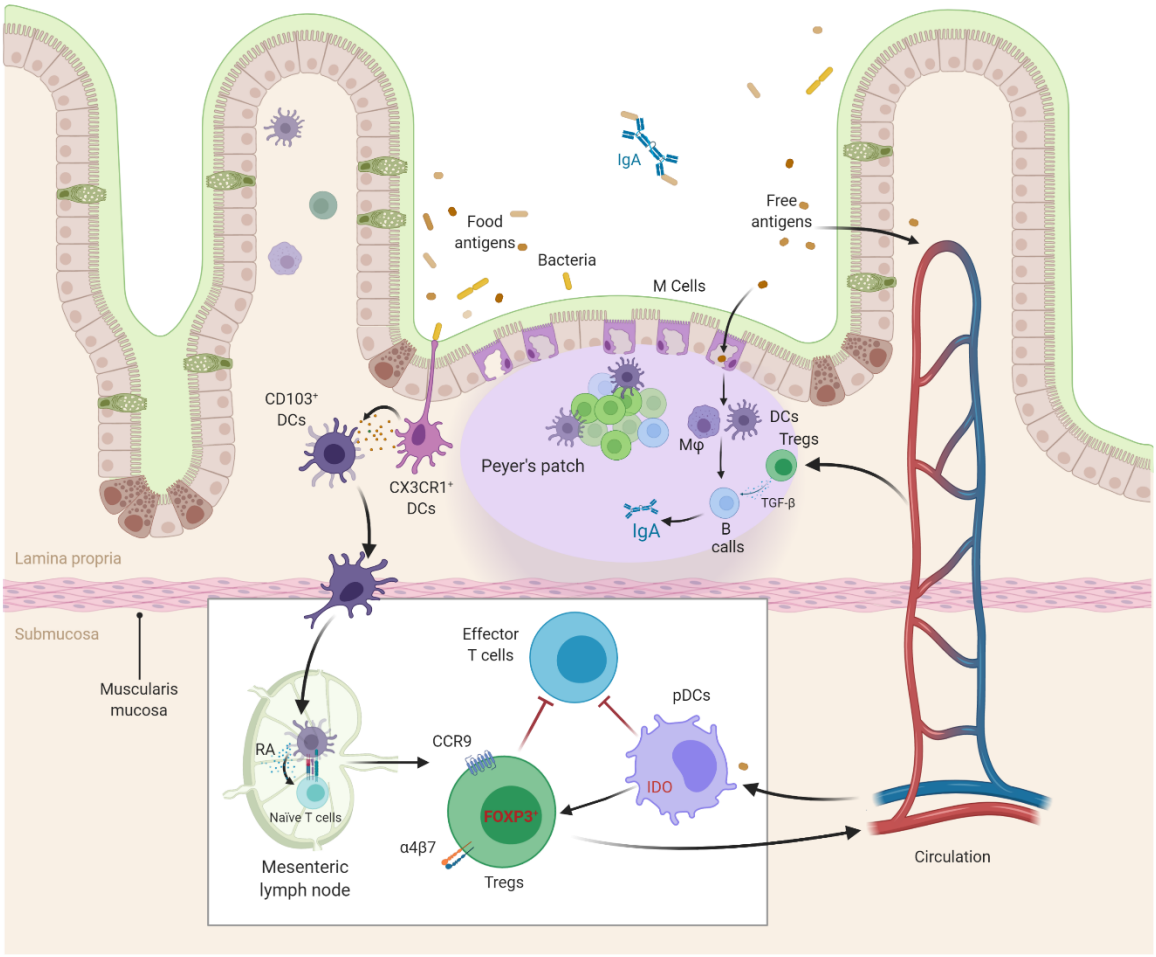

Figure 1-2: Mechanisms of oral tolerance

Different types of cells can sample food and bacterial antigens in the intestinal lumen. Resident DCs, such as CX3CR1$^+$ positive populations, capture antigens via their dendrites that reach the intestinal lumen and can be delivered to migratory CD103$^+$ DCs. Free antigens can be transported by M cells in the PPs and by transepithelial dendrites of APCs, such as CX3CR1$^+$ macrophages and CD103$^+$ DCs, which reside in PPs and LP. Free antigens that reach the blood and lymphatic circulation can be captured by pDCs in the liver and MLN; these pDCs can induce clonal anergy of effector T cells and Tregs' expansion. CD103$^+$ DCs migrate to MLN where they prime naïve T cells to differentiate into antigen-specific Tregs. These Tregs acquire homing molecules, α4β7 and CCR9, that allows them to migrate back to PPs and LP and exert their effect in enhancing oral tolerance via reducing inflammation and allergic responses. Tregs prevent allergy development by inhibiting IL-4 production from Th2 cells and induce IgA class switching by B cells. IgA-antigen complexes prevent microbial translocation into the intestine.

Mφ: Macrophages. **DCs**: Dendritic cells.

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1.1.2.2. Clonal deletion of reactive T cells

Clonal anergy is an alternative mechanism for peripheral tolerance to food antigens. This mechanism of tolerance has been proposed based on work using animal models. Chen *et al.* [36] have shown that feeding ovalbumin (OVA) to OVA-specific T-cell receptor (TCR) transgenic mice dramatically decreased the frequency and the total number of antigen-reactive T cells in the PP. Both antigen-specific Th1 and Th2 cells were subject to deletion by apoptosis after activation, while transforming growth factor (TGF-β)-secreting cells were resistant [36]. Furthermore, feeding mice with a high dose of myelin basic protein resulted in a substantial decrease in IL-2- and IFN-γ-secreting lymphocytes in the spleen and lymph node cell cultures due to clonal deletion and a reduction in the severity of experimental autoimmune encephalomyelitis [45].

1.2. Innate and adaptive immune system and oral tolerance

Before a foreign antigen acquires a "tolerance stamp" from the immune system, it must pass through a complicated detection network that can distinguish between harmful and harmless antigens. The first line of defence against pathogen and toxins is the innate immune system, which is composed of barriers to pathogens and a wide array of cellular receptors and molecules essential to reduce the impact of harmful antigens until the more robust, long-lasting adaptive immune response develops. Although the innate immune system is sufficient to protect the GIT in most instances, the development of memory by the adaptive immune system enables the rapid elimination of pathogens in case of a second exposure [46].

1.2.1. Pattern recognition receptors

Critical for the function of the innate immune system, pattern recognition receptors (PRRs) are strongly expressed in the GIT and involved in recognition of microbe-associated molecular patterns (MAMPs) and danger-associated molecular patterns (DAMPs). The term MAMPs is favoured over the older "pathogen-associated molecular patterns" term because of the shared PRRs between the commensal bacteria and pathogens. MAMPs are usually ligands that are derived from viruses, bacteria, and fungi. They can be recognized by several categories of PRRs including, Toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-1)-like receptors, NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) [47].

1.2.1.1. Toll-like receptors

The TLR family is comprised of 10 receptors (TLR1-TLR10) in humans and 12 receptors (TLR1-TLR9 and TLR11-TLR13) in mice [48]. TLRs differ in their cellular expression, ligand specificity, signalling adaptors, and induced cellular responses [49]. TLR expression varies between tissues; they have been sometimes classified into extracellular TLRs (TLR1, 2, 4, 5, 6, and 11), and intracellular TLRs (TLR3, 7, 8, and 9) [50]. However, many within the "extracellular" group are found extensively in the endosomal compartment. TLRs recognize a wide variety of ligands, including nucleic acids, proteins, lipids, lipopolysaccharides (LPS), peptidoglycans (PG), and lipoproteins [51]. All of the TLRs are type I transmembrane proteins rich with leucine-rich repeats (LRRs) in the ectodomains involved in protein-protein interaction with the ligand [50]. Ectodomains of TLRs are connected to a single α-helix transmembrane domain followed by a conserved cytoplasmic Toll/IL-1 receptor (TIR) domain [52]. TIR domains dimerize together with

several adaptor proteins, including MyD88, TIRAP, TRIF and TRAM, and induce activation of the NF-κB, MAPK, and IRF pathways [53,54]. TLRs are expressed in the GIT on epithelial and immune cells and play a critical role in the homeostasis of the intestine [55]. In the context of oral tolerance, TLR2 is one of the most studied of this receptor family due to GIT expression, particularly on epithelial cells and ability to recognize a wide array of microbial and endogenous ligands, some of which contribute to tolerance establishment.

1.2.1.1.1. Toll-like receptor 2

TLR2 forms dimers with TLR1, TLR6, and probably TLR10 as well as homodimers, to recognize lipoteichoic acid, PG, lipopeptides, zymosan, and many more ligands (**Figure 1-3**) [56,57]. The synthetic lipopeptides, Pam_2CSK4 and Pam_3CSK4, have shown that TLR2/TLR1 dimer binds triacylated lipoproteins TLR2/TLR6 binds diacylated lipoproteins [58,59]. Upon ligand binding, TLR2 dimerization causes the TIR domain-containing adaptor molecules to get closer together and trigger intracellular signalling [53,60]. While the secretion of pro-inflammatory cytokines upon binding a TLR2 signalling complex to bacteria has been well characterized, the secretion of anti-inflammatory cytokines has only been recognized recently [61–63]. The efficient recognition of microbial products by TLR2 usually requires the presence of co-receptors, including CD14 [64] and CD36 [65], which mainly contribute to pro-inflammatory cytokine secretion but are not thought to be necessary for IL-10 induction [66].

One of the essential features of TLR2 is that it can be found in a soluble form that consists of most of the extracellular domain (**Figure 1-3**) [67], known as soluble TLR2 (sTLR2). In humans, naturally occurring sTLR2 is detected in plasma, breast milk [68], cerebrospinal fluid [69] amniotic fluid [70], and saliva [71]. According to LeBouder *et al*. [68] and

Henrick *et al*. [72], the primary sources of soluble TLR2 (sTLR2) in breast milk are monocytes and mammary epithelial cells. Primarily, sTLR2 acts as a biological decoy receptor regulating membrane TLR2 activity by binding MAMPs and DAMPs without conducting downstream signalling [73]. According to Langjahr *et al.* [74], generation of sTLR2 does not involve mRNA alternative splicing but instead involves a post-translational modification of the membrane receptor. The release of sTLR2 is augmented upon activation with pro-inflammatory cytokines and mediated by metalloproteinases [74]. In a small cohort study, asthmatic patients have exhibited lower levels of sTLR2 in their serum and sputum [75] than controls. Another study of 80 children found that children with food allergy had significantly lower gene expression of *TLR2* and *CD14* on whole blood cells compared to healthy children [76].

TLR2 has previously been implicated in the regulation of oral tolerance. Awareness of the importance of TLR2 in this process arose from reports of an association between SNPs in the *TLR2* gene with several immune-mediated diseases, including asthma [77–79], atopic dermatitis [80–82], type 1 diabetes [78], IBD [83,84], and reactive arthritis [85]. In a cohort study of 1830 participants, 16 polymorphisms in 13 genes, 2 in the TLR2 gene (rs4696480 and rs1816702), were associated with increased risk of IBD. According to Loana *et al.* [86], homozygous individuals with *TLR2* 2258G>A and 1892C>A SNPs reduce the capacity to produce specific cytokines, such as IL-6, from peripheral blood mononuclear cells in response to TLR2 ligands and microorganisms. Reports of the association between TLR2 SNPs and certain allergic and intestinal diseases might inform a potential role in establishing oral tolerance.

Intestinal permeability [87,88] and the GIT nervous system [89] are also regulated with the involvement of TLR2. Stimulation of TLR2 effectively preserves the tight junction-associated barrier assembly promoting intestinal epithelial cell survival. Mice deficient in TLR2 or MyD88, an adaptor protein associated with most of the TLRs, exhibited acceleration in tight junction-associated disruption in response to inflammatory stress [87].

Expression and stimulation of TLR2 have been linked directly to the function of Tregs. $TLR2^{-/-}$ and $MyD88^{-/-}$ mice have an impaired Treg level in the spleen and circulation [90]. However, activation via TLR2 has shown controversial results in terms of modifying the suppressive function of Tregs. Sutluller *et al.* [90] demonstrated that co-stimulation of TLR2 with Pam_3Cys and TCR with anti-CD3 induced proliferation of Tregs but caused a transient decrease in their suppressive functions. In contrast, stimulation of human Tregs with endogenous heat-shock protein 60, a TLR2 agonist, augmented their suppressive capacity [91]. These results indicate that the role of TLR2 with Tregs suppressive function can be ligand-dependent, may involve additional receptors and is more complicated than anticipated. Tunis *et al.* [92] found that stimulation of TLR2 by Pam_3CSK_4 during a period of OVA ingestion in mice was associated with increased food sensitization marked by increased OVA-specific IgE in serum. There is accumulating evidence concerning the role of TLR2 and sTLR2 in oral tolerance development, which encourages further investigation into the underlying mechanisms involved in this process.

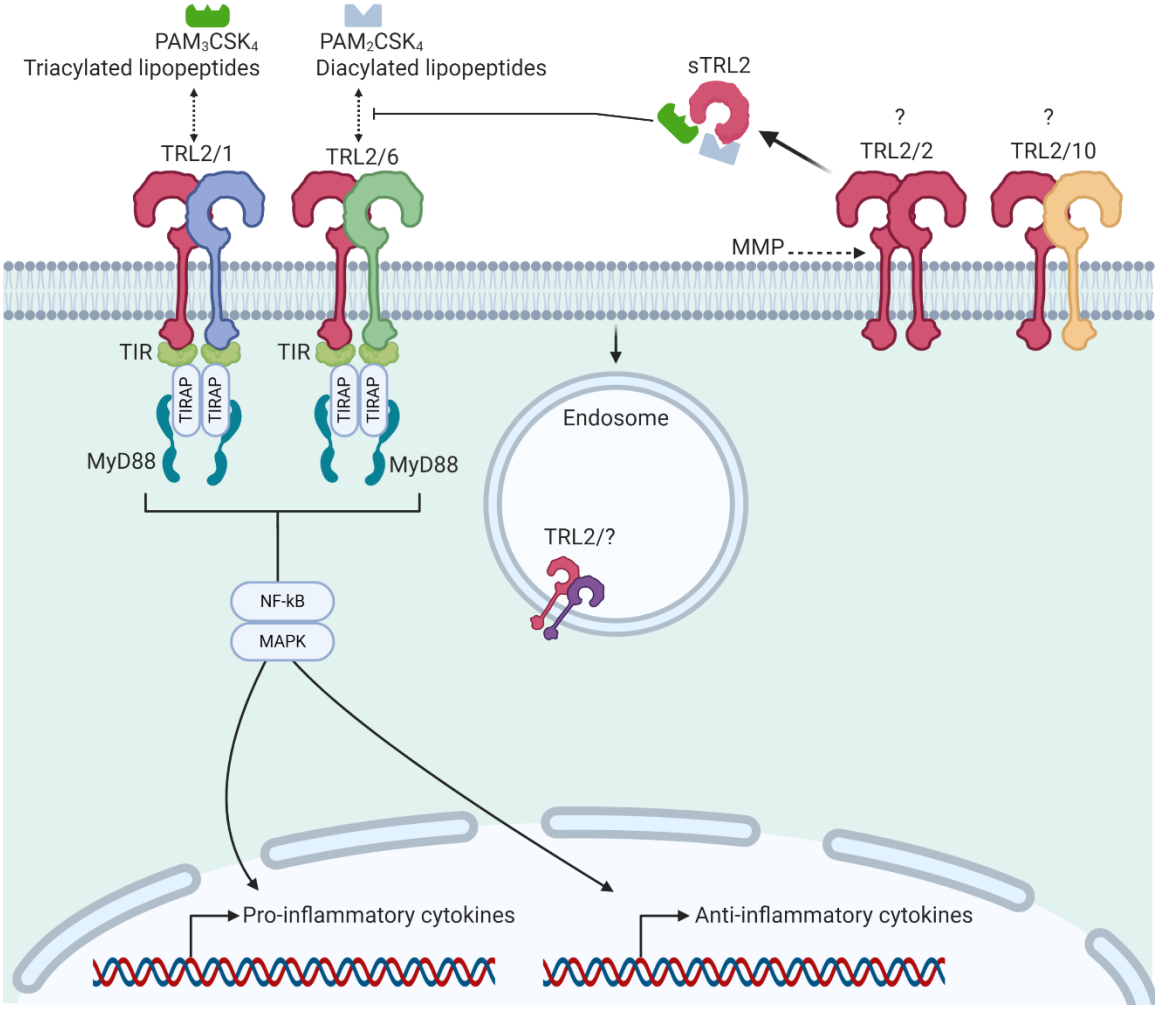

Figure 1-3: TLR2 dimerization and signalling pathway
TLR2 is an extracellular receptor that is also found in the cellular endosomes. Upon ligand recognition, TLR2 can form dimers with TLR1 or TLR6, and probably with self (TLR2/2) and TLR10, leading to the recruitment of adaptor proteins TIR, TIRAF and MyD88, and induce the secretion of pro- or anti-inflammatory cytokines through NF-κB and MAPK. The ectodomain of the receptor can shed from the cell surface under the impact of metalloproteinases (MMP). The release of sTLR2 can serve to regulate the TLR2 signalling as it can bind the ligands and reduce the innate immune activation that occurs at a cellular receptor level.
Adapted from [51]

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1.2.2. Dendritic cells (DCs)

DCs are potent APCs that act as a bridge between innate and adaptive immune responses. The localization of DCs near the epithelial layers in proximity to the environment, enables them to act as essential sentinels of the immune system [93]. DCs are equipped with abundant PRRs, such as TLRs, mannose receptors, dectin-1, and others, to sense environmental challenges and signal accordingly [94]. The behaviour of DCs changes based on the signals transduced via PRRs, which depend upon the dose and type of ligands, the involvement of multiple potential PRRs, duration of the stimuli, and DCs surrounding microenvironment [95]. For example, activation of TLR4 on DCs in the presence of IL-10 and TGF-β leads to more tolerogenic DCs. Such activation in the presence of inflammatory cytokines leads to DC promotion of a Th1 response [96]. In contrast, stimulation of DCs in the presence of histamine and thymic stromal lymphopoietin (TSLP) leads to Th2 response, while in the presence of IL-6 and TGF-β, leads to Th17 response [96,97].

DCs are divided into myeloid/conventional DCs (mDCs/cDCs), which are derived from myeloid precursors, and plasmacytoid DCs (pDCs), which originate from lymphoid precursors [97,98]. In mice, cDCs are divided into cDC1 marked by $CD11c^{hi}CD11b^{-}CD8^{+}MHC\text{-}II^{+}$ and cDC2 marked by $CD11c^{hi}CD11b^{+}CD8^{-}MHC\text{-}II^{+}$, whereas $CD11c^{int}MHC\text{-}II^{lo}$ $CD45R^{+}Ly6c^{+}CD11b^{-}$ marks pDCs [99]. Human cDCs are defined with $CD11c^{+}HLA\text{-}DR^{+}$ and linked to mouse cDC1 and cDC2 via expression of $CD141^{+}$ and $CD1c^{+}$, respectively, whereas $CD11c^{int}$ $HLA\text{-}DR^{+}$ $CD45R^{+}CD304^{+}CD303^{+}$ marks human pDCs.

Establishment of peripheral tolerance in the intestine highly depends on tolerogenic $CD103^{+}$ cDCs (in both human and mice) that can migrate to the secondary lymph nodes,

such as MLN, to prime naïve T cells [100]. Treg induction mechanisms rely on retinoic acid (RA) production from vitamin A, and TGF-β, IL-10, and indoleamine 2,3-dioxygenase (IDO) secretion [101,102]. Furthermore, RA produced by $CD103^+$ DCs stimulates the expression of α4β7 (mucosal-homing receptor integrin) and CCR9 on Tregs (**Figure 1-2**) [102–105]. Tolerogenic pDCs also play a role in skewing naïve T cells into a regulatory phenotype via IDO expression in mice and humans (104,105) and ICOSL in humans [106]. Circulating antigens can be captured by pDCs, in the liver and the MLN, which mediate anergy/deletion of allergen-specific T cells (**Figure 1-2**) [107]. Therefore, the better we understand the cues that transform DCs into tolerogenic vs non-tolerogenic roles, the more we can resolve the mysteries regarding the development of allergic diseases.

1.2.3. Regulatory T cells

Tregs are a subset of T cells specialising in antigen-specific suppression of the immune response and help maintain tolerance against self-antigens and innocuous or commensal-derived antigens to protect against autoimmunity and allergic diseases. Tregs are commonly classified into naturally occurring Tregs (nTregs) and inducible Tregs (iTregs). Marked by $CD4^+CD25^{hi}FOXP3^+Helios^+CD304^+$ in mice, and $CD4^+CD25^{hi}FOXP3^+Helios^+CD127^{low/-}$ in humans, nTregs are derived from the thymus and are reactive to self-antigen; therefore, they are mainly involved in protection against autoimmunity [108–112]. In contrast, naïve T cells that acquire the suppressive phenotype after interaction with antigen carrying DCs in the periphery are called iTregs. With the transition from naïve state into iTregs, expression of FOXP3 increases, but they remain $Helios^-$ $CD304^-$ in mice and $Helios^-$ in humans [109,110,112]. Based on secreted cytokines, iTregs can

be subdivided into Tr1 that produces an excess amount of IL-10 [113] and Th3 that secrete TGF-β [114,115].

Expression of FOXP3 is essential for Tregs to exert their suppressive activity as noted in patients with IPEX, which causes severe autoimmune diseases in human [37]. Also, ”scurfy” mice have a disrupted *foxp3* gene and develop a lymphoproliferative disorder, dying within a month after birth [116]. The affinity between specific TCR on potential Tregs and antigen-MHC-II complexes and the expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4) are other factors to consider which regulate the function and activation of Tregs [117–119].

Mechanisms of Treg-mediated suppression depend on secreted cytokines and proximal interactions. They can be divided into those that act on T cells or APCs [120]. IL-10 is primarily an anti-inflammatory cytokine that can inhibit Th1 responses via MHC-II downregulation expressed on macrophages, thus, limiting their antigen-presenting function [121]. TGF-β is also an anti-inflammatory cytokine secreted by Tregs in a latent form and gets activated into the potent form after interaction with enzymes. Inhibiting naïve T cells from differentiation into Th1 and Th2 subtypes is one mechanism by which TGF-β suppresses Th1/Th2 responses [122,123]. Using high concentrations of anti-TGF-β has been shown to reverse the suppressive activity of resting and activated $CD25^+$ T cells [124].

Active Tregs express galectin-1 on the surface that can interact with effector T cells’ receptors leading to cell-cycle arrest [125]. Expression of CTLA-4 on Tregs inhibits the expression of costimulatory CD80/CD86 on APCs and subsequently reduces the interaction with effector T cells [126]. In contrast, $CD304^+$ (Neuropilin-1) on Tregs promotes

prolonged interaction between Tregs and immature DCs and decreases the chances of contact with effector T cells [120].

Understanding the mechanisms of suppressive functions of Tregs will help develop interventions to manipulate their activity, which could help enhance and foster oral tolerance development in the context of allergic disease and autoimmunity.

1.2.4. B cells and humoral immunity

B cells are APCs that play a central role in humoral immunity by developing to plasma cells that secrete antibodies, critical for multiple types of hypersensitivity. Subsets of B cells also have immunoregulatory roles. Immature B cells are produced in the bone marrow and pass through several maturation levels with changes in the genome of antibody loci. When B cells get activated by an antigen via their B cell receptor (BCR), they undergo clonal expansion, and some of them differentiate into plasma cells that are short-lived antibody-producing cells. Some will form long-lived memory B cells that respond quickly upon second antigen exposure.

The first antibodies produced by naïve mature B cells are IgM and IgD, bound to the membrane and called mIgM and mIgD, respectively, or BCR. Upon recognising an antigen and receptor cross-linking, the B cell gets activated, releases mIgM and mIgD, and begins class switching to generate different antibody subtypes, including IgG, IgA, or IgE. IgG can be divided into IgG1, IgG2a, IgG2b, and IgG3 in mice. In humans, IgG can be divided into IgG1, IgG2, IgG3, and IgG4 subclasses [127]. The particular classes of secreted antibodies produced are determined by the nature of B cell activation signals via CD40 and multiple cytokine receptors. For example, class switching to IgE is augmented by Th2 cytokines, such as IL-4. In contrast, IL-10 induces class switching to produce IgG3.

The most abundant antibody in the GIT is IgA, which plays an essential role in developing oral tolerance, microbial colonization and mucosal immunity (see chapter 1.4.1). IgA can be found in the serum as monomer or dimer and can be secreted in other body fluids, including breast milk, saliva, tears, and intestinal mucus. It provides local protection by preventing the passage of foreign antigens into the host, without fixing complement. TGF-β, secreted by tolerogenic DCs, Tregs, and B cells themselves, is the primary cytokine driving class switch of B cells to produce IgA. TLRs, such as TLR2 and TLR4, can promote IgA secretion when activated by specific ligands, such as *Mycoplasma hyopneumoniae* [128]. Although IgA responses have been reported to be associated with oral tolerance development, high IgA levels are reported in animal models of oral tolerance, in sensitized animals, which could result from the adjuvants used in sensitization.

Allergic disease, which is correlated with a Th2 immune response, involves class-switching of B cells to produce IgE, primarily under the influence of IL-4. The factors that drive increased IgE responses can be complex involving local, genetic, and environmental factors. Atopic individuals usually have significantly higher than normal levels of total IgE, although they might be asymptomatic. In contrast, low or normal levels of total IgE cannot exclude the presence of IgE-mediated allergies. Therefore, the level of total or specific IgE is not a definitive diagnosis of food allergy [129]; instead, it is necessary to have antigen-specific IgE levels and a double-blind placebo-controlled oral challenge for more precise diagnosis [130]. However, having an elevation in food-specific IgE without apparent symptoms is termed food sensitization and only considered a risk factor for developing clinical food allergy [131].

The role of IgGs in food allergies has been well studied, along with IgE to understand the mechanisms of food sensitization and clinical symptoms. Healthy individuals often exhibit high levels of food-specific IgGs in their blood. The standard response to food antigens circulating in the blood often involves the formation of IgG-antigen complexes. However, these complexes get rapidly cleared by the reticuloendothelial system before eliciting any adverse reaction [132]. In contrast, food-allergic patients with increased food-specific IgE levels exhibited low levels of allergen-specific IgG1 and IgG4 antibodies [133,134]. Therefore, one of the significant aims in oral immune therapy, as a potential treatment of food allergy, is to convert the ratio between food-specific IgE/IgGs creating competition between those two classes to bind the antigen. Reports have shown that an increase in IgG4 during oral immunotherapy was associated with decreased antigen-specific IgE binding to their receptors on mast cells and basophils, thus, attenuating their activation [135].

1.3. Gastrointestinal tract and oral tolerance

The GIT is the richest organ in the body in terms of immune cells. It is lined with epithelial cells that protect the body from and interacts with, copious amounts of food and bacterial antigens. Under the epithelium is connective tissue incorporating many immune cells, specialized in maintaining intestinal homeostasis, including those involved in oral tolerance. After the ingestion of food, a series of mechanical and chemical digestions involving the oral cavity, stomach, and the small intestine, transform large particles of food into smaller molecules that are easier to tolerate and absorb via the intestine. It is usually these smaller molecules that serve as antigens for the acquired immune response.

1.3.1. Oral cavity

The first site of interaction between food and the GIT is the oral cavity mucosa. The interaction between food proteins and the immune system in the oral cavity is not well understood. It might not be as robust as the other sites of the GIT due to the intact state of food, although some enzymes are present in oral secretions. However, the role of oral cavity mucosa in tolerance development is well appreciated in sublingual immunotherapy (SLIT). Basically, in SLIT, food antigens are delivered in soluble form, held for two min in the mouth before being swallowed [136,137]. It is hypothesized that undigested food antigens will be captured by Langerhans-like DCs and migrate to the proximal lymph nodes [138]. These lymph nodes have preferential production of blocking IgG, primarily IgG4, rather than IgE, and suppressive T cells, which could be one of the successful immunotherapy mechanisms for food allergy [139].

1.3.2. Gastric acid

The majority of food antigens get chemically digested by gastric acid, pancreatic enzymes and intestinal brush border proteases that destroy the immunogenic epitopes and result in the release of smaller fractions of peptides and amino acids. In a cohort study on almost 800,000 children conducted by Mitre *et al.* [140], children exposed to antacids in the first six months of life were twice as likely to have a food allergy than the controls. Furthermore, Untersmayr *et al.* [141] have found that feeding mice with caviar extract along with antacids, ranitidine or omeprazole, resulted in increased caviar-specific IgE, reactive T-cells, and elevated the density of mast cells and eosinophils in the GIT. Less digested foods can retain some of the proteins' immunogenic properties, which can provoke the immune system in the GIT.

1.3.3. Intestinal epithelial cells (IECs)

The intestinal epithelium represents a selective barrier that regulates the absorption of molecules and microbiota into the gut's circulatory and lymphatic system. Under healthy conditions, the IECs allow for the absorption of small molecules and prevent the passage of larger ones [142]. IECs can behave as non-classical APCs with MHC-II expression on the basolateral membrane [143] capable of presenting antigens to primed T cells and inducing $CD8^+$ suppressor cells, selectively [144].

Aberrations in intestinal integrity and permeability are believed to play a role in developing several GIT diseases, including food allergy, atopic dermatitis, celiac disease, inflammatory bowel disease, and diabetes type I [142,145–147]. A study by Järvinen *et al*. [142] on 131 children with food allergies found that one-third of food-allergic infants have elevated intestinal permeability while asymptomatic. Several other reports have found a correlation between intestinal permeability and food allergy in children and adults [147–149]. However, it is still unclear whether intestinal permeability is a food allergy trait or a risk factor for developing it. Although many reports have defined intestinal permeability as a risk factor for autoimmune IBD development [150], none of them has been linked to hypersensitivity. Intestinal pathological conditions, such as infection with helminth, appears to be protective against food allergy [151]. In contrast, humans with *H.pylori* gastritis have increased susceptibility to food allergy [152]. Although intestinal integrity is not directly impacted by *H.pylori* infection *in vitro*, the passage of intact macromolecules that are less degraded surges, increasing food sensitization [153].

1.3.4. Peyer's patches (PPs)

The intestinal epithelium is backed by a complex network of immune cells disseminated in the lamina propria. Congregations of immune cells are also found organised within nodules along the small intestine known as Peyer's patches. Specialized cells overlying Peyer's patches called microfold (M) cells can endocytose antigens from the lumen and deliver them to underlying immune cells (**Figure 1-2**).

PPs are found along the small intestine, and colon with 46% concentrated at the last 25 cm of the human ileum [154]. Structurally, PPs are primarily composed of germinal centres enriched with B cells, DCs and macrophages, lined by a thin T cells layer [155]. Naïve lymphocytes enter the PPs via high endothelial venules. Within the PP, they get primed and activated before moving out through efferent lymphatic vessels that connect PPs to other lymphatic sites such as the LP and MLN [155]. The exact role of PPs in the induction of oral tolerance is not fully understood. However, PP-null mice failed to develop systemic unresponsiveness towards oral OVA but not to haptens [156]. These findings suggest that induction of tolerance to proteins is enhanced by the presence of the PPs, whereas smaller molecules like haptens, that do not require the antigen processing of complex proteins can induce tolerance by alternate mechanisms [156]. In contrast to these findings, Spahn *et al.* [157,158] found that PPs are not required for oral tolerance towards soluble antigens as long as the MLN is present.

Antigens captured by M cells are delivered to DCs in the PPs, which process the antigens further and interact with B-cells [159]. With T cell help, including secretion of TGF-β, activated B cells class switch to IgA, which is important in oral tolerance [160]. As mentioned before, migratory $CD103^+$ DCs can traffic from PPs to the MLN where they

stimulate priming of naïve T cells into antigen-specific Tregs with homing molecules that enable them to return to the intestinal mucosa to exert their regulatory effects.

1.3.5. Lamina propria (LP)

The LP represents a thin layer of connective tissue that separates the epithelium from the submucosa. This layer is rich with a heterogeneous population of immune cells, blood and lymphatic vessels, fibroblasts, and collagen. APCs in the LP can sample luminal antigens directly via their dendrites that can extend through the intestinal epithelium [161]. With such a large army of available APCs, antigen-loaded DCs are detected in LP within 15 min of antigen feeding and reach maximum levels in 1 hour with greater magnitude than antigen uptake in PPs and MLN [162]. Like the PPs, the LP contains migratory CD103+ DCs, resident CX3CR1+ DCs and macrophages capable of antigen presentation and exchange (**Figure 1-2**). The antigen is eventually presented to naïve lymphocytes, either within the MLN or locally [163]. The vast majority of antigens that drain to MLN appear to be derived from the LP rather than PPs [164,165]. Along with the PPs, LP is considered an active site for tolerance establishment via the continuous interaction between antigens and the immune system.

1.3.6. Mesenteric lymph node (MLN)

MLNs are chains of lymphatic nodes embedded in the mesentery. Efferent lymphatic vessels from PPs and LP drain towards the MLNs through which antigen-loaded DCs traffic to educate naïve T cells and prime them. According to Spahn *et al.* [157,158], mice deficient in PP could tolerate high doses of antigen as long as the MLN was present [158]. Grafting back the MLN into mice without connecting it to the PPs and LP efferent vessel fails to induce oral tolerance, as it acts as a distal lymph node, indicating the importance of

antigen-loaded immune cells trafficking through MLN. The chemokine receptor CCR7 mediates the migration of DCs to the MLN [166]. Mice deficient in CCR7 have impaired capacity for DCs to migrate to the MLN and are unable to develop oral tolerance [167]. After induction of antigen-specific Tregs in the MLN, these cells disseminate throughout the body and some home back to the intestine to exert their suppressive effects against local effector T cells.

1.3.7. Spleen

The spleen is a large lymphoid organ that is considered a part of both the circulatory and immune systems. It consists of a white pulp, enriched with lymphocytes, and a red pulp, which consists of venous sinuses filled with blood and specialized macrophages that filter out ageing red blood cells and recycle the iron [168]. There are multiple ways for antigens that enter the body to end up in the spleen, as they leave the lymph nodes into the efferent vessels that lead to the thoracic duct and eventually in the circulation [169–171]. Several studies have shown that spleen is involved in establishing central tolerance, including suppressing T cell proliferation and antibody production [172,173]. Furthermore, splenic DCs in antigen fed mice could produce suppressor cytokines like TGF-β1 and subsequently induce suppressor T cells [174,175]. Adoptive transfer of splenic DCs from OVA-fed mice could transfer acquired immune suppression, while it was absent in mice injected with splenic DCs from non-OVA fed mice [174]. However, splenectomy in mice orally administered antigens did not compromise the establishment of oral tolerance, which indicates that the MLN is still the leading site for oral tolerance induction.

1.3.8. Microbiota

Microbial communities that colonize the human mucosa after birth are not stable and fluctuate during the first three years of life [176,177]. This colonization runs in parallel with the maturation of the intestinal immune system and GIT, suggesting a crucial role of the microbiota in this process [178] and the presence of ongoing signalling between microbiota and mucosal immune cells.

An intimate relationship between the microbiota and the immune system is crucial for proper functioning and maintaining intestinal homeostasis and oral tolerance. The immature Th2-biased immune system in neonates enables the microbiota to colonize [179]; in contrast, a lack of microbiota in GF mice has been shown to result in less mature PPs, MLNs, low amounts of secretory (s)IgA and fewer lymphoid follicles [180,181]. These significant changes in the intestinal immune structure of GF animals are associated with reduced activation and frequency of immune cells, including T cells, B cells, and ILCs, which can be altered by microbial colonization later in life [182–184]. However, the immune development in GF-mice conventionalized in adulthood is substantially different from those conventionalized early in life [185]. These reports support the concept of a "window of opportunity" in early life during which the microbiota play a prominent role in shaping the immune system [186,187].

Microbiota can regulate the fetal immune system's development via molecules transferred from the mother across the placenta [188]. De Agüero *et al.* [188] have shown that mice born from gestationally colonized, otherwise GF, pregnant dams have higher levels of innate lymphoid cells-3 (ILC3) and $F4/80^{+}/CD11c^{+}$ mononuclear cells. Transmission of microbial molecules can be mediated by the transplacental movement of IgG, as seen in

gestationally colonized $J_H^{-/-}$ antibody-deficient dams [188]. However, the impact of microbiota found in the fetus on the early development of the immune system is not known.

Postnatally, the transmission of anti-microbiota IgA and IgG antibodies via the mother's milk can dampen the mucosal microbiota-specific T helper cell responses and subsequently B cell responses in early life, which limits immunity against newly acquired microbiota [189]. After colonization, the microbiota plays an essential role in developing B cells in PPs and IgA production [190,191]. IgA-class switching of intestinal B cells is vital for sufficient oral tolerance and highly dependent on Tregs, which are drastically reduced in the GIT of GF-mice [192,193]. In addition to effects in B cells, a product of the commensal *Bacteroides fragilis* called Polysaccharide A interacts directly with Tregs via TLR2 to promote immune regulation [194]. Furthermore, a defined mix of *Clostridium* strains has been shown to enhance Treg induction in the LP via the production of short-chain fatty acids (SCFAs) [195–197]. These SCFAs are suggested to serve as an epigenetic modulator of Treg function [198].

In humans, GIT maturation usually occurs shortly after birth, whereas it occurs around weaning in animals with short gestation periods like rodents [199,200]. Gut maturation is associated with a limitation of its permeability to macromolecules and an increase in intestinal integrity that is highly dependent on several factors, one being the gut microbiota [201]. Work in GF mice has demonstrated a structural change in the intestinal morphology, including decreased intestinal surface area, increased permeability, enlarged cecum, shallow intestinal crypts with decreased stem cells, reduced villous thickness, fewer goblet cells with altered mucus properties, and reduced AMPs [202–206]. It has also been noted that

GF mice have impaired oral tolerance [207] and antibiotic-treated mice are highly susceptible to immune-mediated diseases, such as asthma [208], and GIT infections [209].

Certain microbiota species, including *Bifidobacteria* and *Lactobacilli*, enriched in the gut of breastfed infants, have been shown to enhance intestinal barrier integrity, resulting in reducing epithelial permeability [210,211]. PRRs are essential in this process, as PG-mediated signalling through TLR2 was necessary for regulating tight junction-associated intestinal barrier integrity [87]. The digestion of human milk oligosaccharides (HMOs) by *Bifidobacterium* and *Lactobacillus* species in the neonatal intestine is associated with increased lactate production SCFAs, such as butyrate, acetate, and propionate [212]. These SCFAs serve as sources of energy for enterocytes, which enhance epithelial proliferation, gut barrier function and intestinal motility (**Figure 1-4**) [213].

Collectively, the normal function and maturation of intestinal immune cells and the GIT are highly dependent on colonization with healthy microbiota early in life and their metabolites, supporting the hypotheses of a role for dysbiosis in the development of several immunological diseases, including food allergy.

Figure 1-4: Breast milk and oral tolerance

(A) Breast milk is rich with dietary and environmental antigens processed by the maternal digestive system and transformed into more tolerogenic forms. Formula milk replacement is mainly derived from cow's milk with less dietary antigens than breast milk. **(B)** Breast milk is also rich in IgA, IgG, tolerogenic factors, soluble receptors, including sTLR2, and microbiota. Antigens are transferred to neonates either free or bound to sIgA or IgG. Free antigens can be transported by M cells in the PPs and by transepithelial dendrites of APCs, such as $CX3CR1^{+}$ DCs and macrophages that deliver the antigens to migratory $CD103^{+}$ DCs, which reside in PPs and LP. sIgA-antigen complexes prevent microbial translocation into the neonatal intestine and enhance dietary-antigen degradation by pancreatic enzymes. IgG-antigen complexes translocate through the intestinal barrier after binding to the neonatal Fc receptor (FcRn) receptor to be captured by DCs. DCs migrate to MLN where they prime naïve T cells to differentiate into antigen-specific Tregs. These Tregs migrate back to PPs and LP and enhance oral tolerance by reducing inflammation and allergic responses. Tregs prevent allergy development by inhibiting IL-4 production from Th2 cells, IgE class switching by B cells. Breast milk contains sTLR2, which can act as a decoy receptor and compete with TLR2 on the ligands, thus, regulate signalling and immune response.

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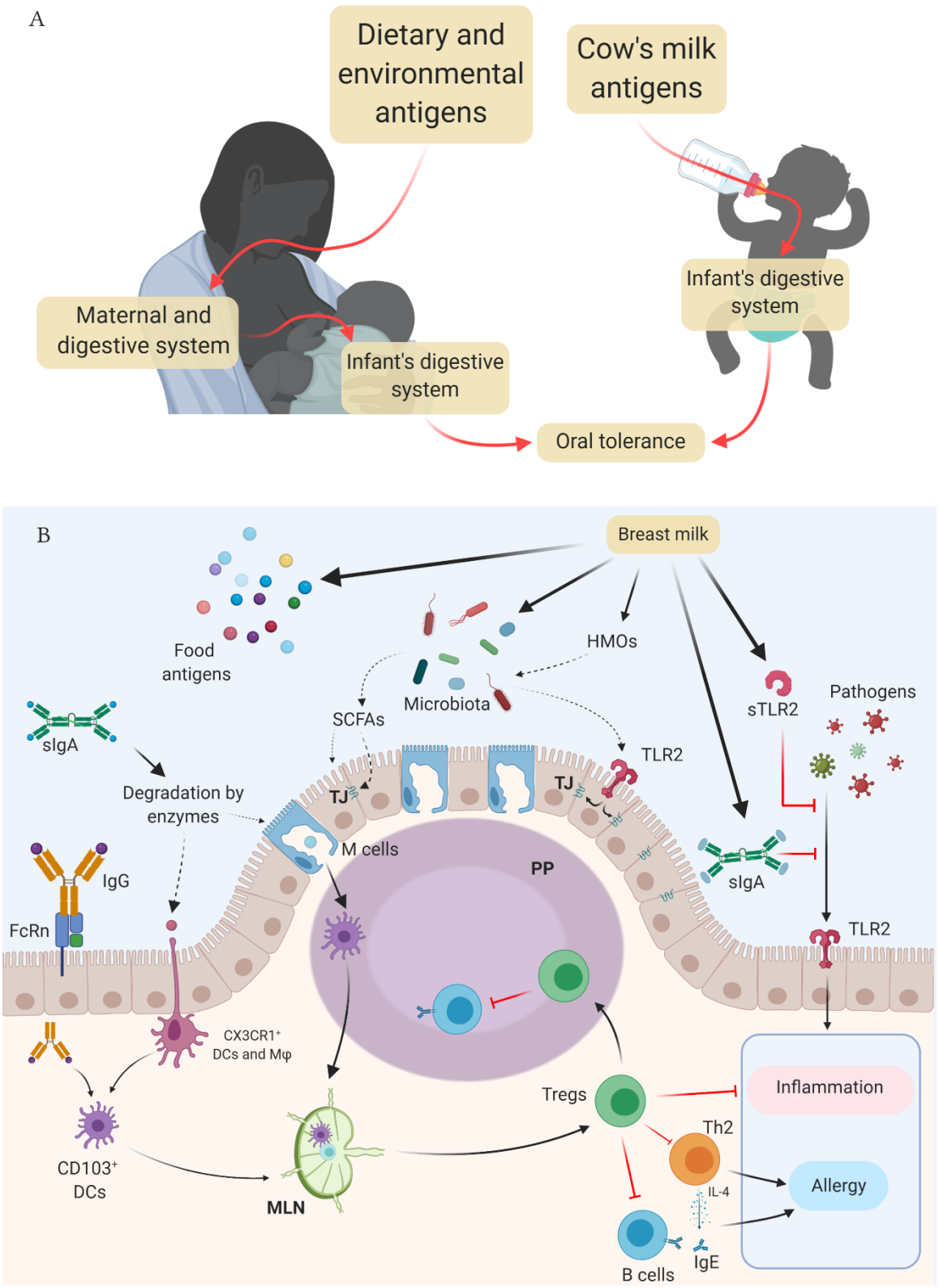
A
Dietary and environmental antigens
Cow's milk antigens
Maternal and digestive system
Infant's digestive system
Infant's digestive system
Oral tolerance
B
Breast milk
Food antigens
HMOs
Microbiota
SCFAs
sIgA
Degradation by enzymes
TJ
TJ
TLR2
sTLR2
Pathogens
M cells
PP
sIgA
IgG
FcRn
TLR2
CX3CR1⁺ DCs and Mφ
CD103⁺ DCs
MLN
Tregs
Th2
IL-4
B cells
IgE
Inflammation
Allergy

1.4. The development of oral tolerance in early life

The mechanisms involved in oral tolerance development in early life are different from those in adulthood due to the immune system's and GIT's immature states [214]. Animal studies have shown that oral tolerance in rodents is more difficult to induce in newborn infants than in adults [215]. Feeding OVA to 1-day-old mice induced an effector immune response to OVA [216]; whereas, tolerance was inducible in mice that were older than seven days with decreased susceptibility to tolerance induction around the time of weaning [216,217]. In adults, oral tolerance develops with exposure to an adequate dose of antigens that translocate through the intestinal barrier, gets sampled by APCs, and presented to naïve T cells, leading to antigen-specific development tolerance by induction of Tregs or clonal anergy of effector T cells [214]. Unlike adults, the primary source of antigens for neonates and infants is maternal milk or alternatives, before solid foods are gradually introduced until weaning (**Figure 1-4**). Understanding the impact of breast milk and its components on the development of the immune system and GIT in childhood expands our knowledge about oral tolerance processes in early life.

1.4.1. Breast milk and oral tolerance

Under healthy conditions, the neonatal immune system is biased toward Th2 responses influenced by the maternal Th2 state during pregnancy, which decreases the risk of miscarriage [218,219] by limiting cellular immune responses against the fetus. The advantage of Th2 responses in fetal and early life is reducing maternal alloantigen rejection and allowing for commensal bacteria colonization, which contributes to the gradual transition into more Th1/Th2 balanced immune response [220]. During pregnancy and breastfeeding, maternal immune cells, including DCs, translocate to the fetus or infants,

and due to the immunosuppressive and Th2 polarized microenvironment, these cells will be tolerated [221,222]. Maternal DCs and molecules transferred across the placenta and via breast milk are potent stimulators for the expansion of $CD4^+CD25^+FOXP3^+$ Tregs, which further suppresses antimaternal immunity in the uterus and during early life [222,223].

Before dietary allergens reach breast milk, they pass through the maternal digestive system where they are processed and digested, usually into more tolerogenic forms (**Figure 1-4A**) [224]. However, some allergens are found intact in breast milk [225]. The low gastric pH levels in adults (pH 1-2) compared to neonates (pH 3-5) are highly involved in developing these tolerogenic forms [226]. Breast milk is enriched with several types of immunoglobulins (Ig), primarily IgA with less IgG and IgE [225], some of which are specific for dietary antigens, such as cow's milk proteins [227] and gluten [228].

sIgA, which includes a dimer of IgA molecules, is the predominant form of IgA at the intestinal mucosa. It can inhibit microorganism adherence to the mucosa and regulate microbiota colonization (**Figure 1-4B**) [229]. Mucosal sIgA can also bind allergens and facilitate their degradation by pancreatic enzymes and enhance their endocytosis via the intestinal epithelium (**Figure 1-4B**). Furthermore, IgA's inability to fix complement by classical or alternative pathways leads to protection of trapped allergens from immunological reactions [229]. Although animal models have shown that oral tolerance is inducible in the absence of sIgA [230], there are controversial reports of increased susceptibility to allergic diseases in IgA-deficient individual [231]. Additionally, maternal milk IgA level was found to be inversely correlated with the later development of allergic diseases, such as cow's milk allergy, in breastfed children, indicating an essential role of IgA in neonatal oral tolerance [232,233].

According to a recent report by Ramanan *et al.* [234], breast milk can modulate transgenerational immune inheritance via a milk-borne IgA-mediated mechanism. In a cross-fostering experiment, the dams with high IgA levels in their milk inhibited the expansion of colonic ROR-γt^+ Tregs in nursed pups, regardless of their biological mother, for multiple generations. In contrast, dams with high levels of colonic ROR-γt^+ Tregs secreted low IgA levels in milk, which was insufficient to decrease ROR-γt^+ Tregs in nursed pups, a trait that was imprinted for multiple generations. In a double-negative feedback loop, milk IgA could coat microbiota in nursed pups and reduce the microbial induction of ROR-γt^+ Tregs' expansion in the colon. Whereas the high levels of colonic ROR-γt^+ Tregs can suppress IgA^+ plasma cells in the intestine, and subsequently in the mammary gland, therefore express low levels of IgA in milk.

Allergens in maternal milk can also be complexed with IgG [235]; such complexes have been detected in the serum of both healthy and allergic mothers [236]. These complexes are transferable from serum into maternal milk using neonatal Fc receptor (FcRn) that is expressed in the epithelium of the mammary gland [237]. FcRn is also found in the neonatal intestine, and it mediates the translocation of the IgG-allergen complex across the gut barrier (**Figure 1-4B**) [238]. Researchers have found that allergens bound to IgG induced more Tregs and more profound immune tolerance than free allergens [235]. Besides Ig-mediated transport, allergens can also be transported across the intestinal epithelial barrier by M cells in the PPs and by transepithelial dendrites of APCs that reside in PPs and LP [239].

During the fetal period, the intestinal barrier is highly permeable, absorbing nutrients from the amniotic fluid [240]. Immediately after birth, intestinal permeability

decreases rapidly due to the tight junctions' maturation between IECs [241]. This process is accelerated in infants that ingest the colostrum while non-breastfed children experience a prolonged increased-permeability period [242,243]. Intestinal permeability also decreased faster in preterm infants (≤32 weeks of gestation) fed with breast milk in place of infant formula [244]. Prolonged intestinal permeability could be linked to an increased incidence of atopic and infectious diseases in non-breastfed infants [245]. Neonatal IECs have limited microbial communities and secrete low levels of cytokines and chemokines that would promote the development of tolerogenic $CD103^+$ DCs in the lamina propria [246]. However, breast milk-derived mediators, including microbiota (i.e. *B. fragilis*), vitamin A and immune factors, such as TGF-β, compensate for this deficit and enhance the expansion of tolerogenic DCs [247–251]. Accordingly, oral tolerance in children depends on dietary factors, including those derived from maternal milk, which contributes to immune regulation and maturation of the intestinal barrier. Defining breast milk's critical characteristics might expand our knowledge of the mechanisms involved in food allergy development.

1.4.2. Breast milk and allergic diseases

Breastfeeding is recommended by scientists, health organizations and governments for all infants as a natural source of multiple factors that promote healthy immune responses, nutrition, protection against infection, and development [252]. The World Health Organization recommends exclusive breastfeeding for six months before introducing solid foods [253]. The impact of breastfeeding on the development of allergic diseases has been extensively studied, with conflicting results [254]. Some have reported beneficial effects [255–261], while others have found no impact or increased risk of allergies among breastfed children, notably in infants fed by atopic mothers [262–265]. A randomized trial by Lucas *et*

al. [261], found that feeding banked human milk to preterm infants reduced the risk of cow's milk allergy when compared with formula feeding. In contrast, a cohort study by Wetzig *et al.* [266], found that exclusive breastfeeding for more than five months was associated with increased early sensitization to eggs and atopic dermatitis. This variable effect of breastfeeding on food allergy prevention may be related to differences in length of such feeding, milk components, ethnicity, diet, and other factors.

Breast milk composition is dynamic and changes dramatically over time to match the needs of the growing infant. For example, the protein concentration is initially about 1.4-1.6 g/dl in the colostrum and decreases to 0.7–0.8 g/dL after six months [267]. The most common alternative for human milk is infant formula, commonly derived from cow's milk, which contains higher protein and fat concentrations than breast milk [268]. Breast milk is enriched with allergens ingested by the mother, such as β-lactoglobulin [269], ovalbumin [233], and peanut components [270]. In a cohort study by Pitt *et al.* [271], the peanut allergy rate was significantly reduced among children whose mothers consumed peanuts while breastfeeding. Grimshaw *et al.* [272] showed that infants diagnosed with food allergies at two years of age were more likely to have received solid foods at early ages (≤16 weeks of age) and less likely to be breastfed during the introduction of these foods.

According to the Canadian Healthy Infant Longitudinal Development (CHILD) study, a delay in food allergens, such as peanut, cow's milk, and eggs, can increase the incidence of food sensitization [273]. Furthermore, a study called Enquiring About Tolerance (EAT) led by Dr Gideon Lack has found that the early introduction of peanut, boiled eggs, milk, fish, sesame, and wheat might protect children from reacting to these foods [274]. Together, the transfer of food allergens in milk and the timing of solid food introduction

relative to breast milk consumption appears to be critical in preventing allergic diseases. The presence of immunomodulatory components in breast milk are thought to be critically important in regulating these processes.

1.4.3. Breast milk immune mediators (IM) and oral tolerance

Over the past 20 years, multiple cytokines and immunomodulatory factors have been identified in breast milk. This list of IMs is continually increasing with advances in detection methods (**Table 1-1**). Many of these factors are derived from the mammary epithelial cells or milk-borne immune cells [275], while others are transferred from the mother's circulation. Such breast milk components could impact the development of neonatal oral tolerance through both immune modulation and impacts on other factors such as barrier function or the microbiome. Particular challenges for research in this area are the variability in concentrations of immune factors in breast milk and their ability to survive the infant's stomach and exert a biological effect in the GIT. Due to ethical limitations, most studies of the impact of breast milk immune factors on the host have been conducted either *in vitro* or *in vivo* using animal models. Through the analysis of such studies, it is widely agreed that TGF-β, IL-10, IL-6, and sCD14 positively impact tolerance development [276]. Other cytokines and soluble receptors are also of potential immunomodulatory importance. Besides these factors, several chemokines, including CXCL8, CCL2, CCL5, and CXCL10, and growth factors, such as EGF and IGF-(I & II), are detected in breast milk [277,278].

1.4.3.1. Cytokines

Cytokines detected in breast milk, include TGF-β, IL-10, IL-6, IL-1β, TNF, IFN-γ, IL-4, IL-5, IL-12, IL-13, G-CSF, GM-CSF, and M-CSF (**Table 1-1**) [279–283]. Many of these

cytokines can alter oral tolerance by impacting the development of the neonatal immune system and GIT and the maternal mammary gland (**Figure 1-5**). Several factors might further influence the concentration of cytokines in breast milk. For example, subclinical mastitis, a local inflammation in the mammary gland observed in 23% of nursing mothers, induces considerable changes in milk pro-inflammatory cytokines that might affect infant immune or GIT development [284].

The most abundant cytokines in breast milk are TGF-β family members, including TGF-β1 and TGF-β2. The concentration of TGF-β differs dramatically through the lactation period and between individual mothers, with TGF-β2 being more abundant in breast milk and TGF-β1 in the serum. At the same time, both are relatively scarce in the infant formula [285–287]. The majority of TGF-β1 and TGF-β2 in breast milk exist in latent forms activated by the gastric acid in infant stomach [288]. Furthermore, $CD103^+$ DCs can activate latent TGF-β, which is essential to induce Tregs [289].

TGF-β has anti-inflammatory roles, inhibiting naïve T cells from differentiation into Th1 and Th2 subtypes, thereby suppressing Th1/Th2 responses [122,123]. TGF-β also fosters the stabilization of FOXP3 expression and maintains the differentiation of Tregs [290,291]. The role of TGF-β in the GIT is multifaceted, including enhancing oral tolerance [292], promoting intestinal integrity [293], stimulating IgA class-switching in B cells [294], promoting colonization and abundance of microbiota [295], and regulating inflammatory responses [122,123]. According to a systemic review by Oddy *et al.* [296], high levels of TGF-β1 and TGF-β2 in breast milk were inversely correlated with the incidence of allergic diseases in childhood. Furthermore, the levels of TGF-β were more elevated in maternal colostrum of infants who developed post-weaning atopy compared with those with pre-weaning atopy

[297]. Moreover, levels of TGF-β1 were significantly lower in the breast milk of allergic mothers compared to non-allergic mothers, which could be linked to increased symptoms of atopic dermatitis in infants born to allergic mothers [298]. Although TGF-β can induce pathogenic Th17 responses in the presence of IL-6, the production of RA from $CD103^+$ DCs in the intestine is thought to antagonize and override IL-6-driven induction of Th17 and promote Treg differentiation [299].

IL-10 is an anti-inflammatory cytokine detected in both the whey fraction and the fat layer of breast milk with a molecular weight >80 kD, higher than that of IL-10 in serum, suggesting that it might be bound to other molecules or post-transcriptionally modified [300]. The bioactivity of IL-10 in breast milk has been confirmed [301]. IL-10 inhibits Th1 responses, increases survival and expansion of B cells, and downregulates MHC-II on monocytes; hence, limiting their APC function [121]. It has been heavily implicated in the regulation of intestinal inflammation and regulating responses to the microbiome.

IL-6 is a pleiotropic cytokine reported to have both pro-inflammatory [302] and anti-inflammatory [303] impacts. It has a vital role in regulating the acute phase response via innate anti-bacterial host defence enhancement and limiting inflammation's adverse effects. IL-6 also regulates mucous production by goblet cells [304]. It has been detected in the whey portion of breast milk in both high molecular weight ≥100kD and 25-30kD isoforms and at relatively consistent levels in breast milk for the first three months post-partum [305,306]. This cytokine has been linked to IgA production in the neonatal intestine by inducing follicular T helper cells in the germinal centres of PP [305]. It also stimulates the mammary epithelium to transport more IgA into milk [307]. IgA levels in breast milk are positively correlated with the concentrations of TGF-β, IL-10, and IL-6 in breast milk [287]. High levels

of IgA in breast milk have been reported to be protective against allergic diseases, including cow's milk allergy [233,307].

IL-1β is probably the first cytokine to be quantified in breast milk using radioimmunoassay (RIA) by Munoz *et al.* [308] who reported that IL-1β exists in high concentrations in the colostrum and day seven milk; however, more modest levels have been reported in more recent studies [280,301,309]. Although IL-1β has been shown to attenuate skewing of T cells toward Tregs, Järvinen *et al.* [307] have demonstrated that IL-1β together with IL-6, IL-10, and TGF-βl in breast milk is associated with enhanced tolerance to cow's milk. However, the impact of breast milk-derived IL-1β on tolerance development in neonates is still not clear, as both the cytokine and its antagonists exist together in the milk.

In vitro and *in vivo* animal studies have suggested a role for milk-derived cytokines, such as TNF [310], IL-10 [311] and IL-6 [312] in intestinal epithelial maturation, proliferation and repair. Also, TNF and TGF-βl have an anti-apoptotic effect on IECs [310,313], although a very high TNF concentration will also induce apoptosis [314]. Intestinal permeability, which is a crucial factor in oral tolerance regulation, could be altered by breast milk cytokines. *In vitro* experiments suggest that IL-10 enhances intestinal integrity and compromises the barrier disrupting effect of IFN-γ, a process confirmed by severe chemical-induced colitis and increased intestinal permeability in IL-10 receptor-1 null mice [315]. A study by Kuhn *et al.* [316] has shown a decrease in the expression of epithelial barrier proteins and a thinner mucus layer in the intestine of IL-$6^{-/-}$ mice, suggesting a role of IL-6 in maintaining intestinal integrity. Also, milk cytokines could impact the mammary gland itself. For example, TNF is a regulator for the development and branching of glands in the breast [317] such factors could affect both the supply and constituents of breast milk.

The extent to which breast milk-derived cytokines can affect the neonatal GIT also depends on several neonatal factors. Their milk concentrations vary dramatically during the lactation period, and commonly, concentrations of cytokines are higher in the colostrum [277]. The ability of cytokines to retain bioactivity after passage through the infant's stomach is also critical. The neonatal stomach pH is higher than in adults (pH 3-5), which might allow more cytokines to exert biological effects and compensate for the lack of cytokine responses in neonates [318]. Other factors that might impact cytokine efficacy include soluble receptors or receptor antagonists in breast milk or the neonatal GIT, which might regulate the binding of the cytokines to their receptors or compete with them, respectively [319].

1.4.3.2. Soluble receptors

Soluble receptors are thought to have immunoregulatory roles in many biological fluids, including breast milk. They regulate the signalling of milk-borne cytokines and innate immune stimulators through membrane-bound receptors in the neonates (**Figure 1-5**). Soluble cytokine receptors reported in milk include sIL-6R and sTNF-RI and sTNF-RII, receptor antagonists, such as IL-1RA, and soluble innate immune receptors, including sCD14 and sTLR2 (**Table 1-1**). In some cases, milk soluble receptors might be bound to their ligands or carrier proteins, explaining the more massive observed molecular weight of some cytokines in milk (≥100kD and 25-30kD for IL-6, 80kD to 195kD for TNF, >80kD for IL-10) [319]. However, this issue has not been well studied.

Soluble receptors for classical inflammatory cytokines are found in breast milk throughout lactation. The level of sIL-6R is low under normal conditions in both colostrum and mature milk, and its affinity to IL-6 is also low [319,320]. The exact role of this receptor

in breast milk is not clear yet; however, *in vivo* experiments have shown an augmentation of IL-6 function by sIL-6R [321].

IL-1RA is detected in human colostrum and milk in amounts higher than serum. It binds to the IL-1 receptor due to homology with IL-1α and IL-1β [319,322]. However, it is considered an antagonist as it competes with IL-1α/IL-1β for receptor binding and thus regulates their effects [319,322,323]. The importance of IL-1RA in milk has not been well studied, but it likely limits the inflammatory response in the neonatal GIT. The two soluble forms of TNF receptor are sTNF-RI and sTNF-RII, reported in human colostrum and milk and shown to modulate TNF signalling. Only a small fraction of the TNF in breast milk is free to activate cells while the majority is speculated to be neutralized by the soluble receptors [319]. High levels of TNF have been detected in milk from mothers with mastitis; however, this was accompanied with elevated levels of sTNF-RII and IL-1RA, which might protect nursing infants from high pro-inflammatory cytokine levels in the context of such breast infections [324].

Soluble forms of innate immune receptors, CD14 and TLR2, have also been detected in breast milk [325]. A single (48kD) form of sCD14 has been observed in human milk, whereas sTLR2 is observed in 6 isoforms (from 20-85kD) [68,326]. There is substantial evidence that TLR responsiveness to ligands, such as LPS and lipopeptides in the neonate's gut, is regulated by sTLRs and sCD14 leading to the inhibition of potentially adverse responses [327] and potentially allowing for more efficient development of tolerance to commensal microbiota. CD14 is a co-receptor for both TLR2 and TLR4 and facilitates recognition of their ligands [328]. Interaction between sCD14 and sTLR2 in breast milk

increases the binding capacity of sTLR2 to bacterial products, such as PG from Gram-positive bacteria [329].

Furthermore, sCD14 can complex with LPS and limits LPS-mediated cellular stimulation [326,330]. The role of TLR2 in oral tolerance is still not exact; signalling via this receptor differs between commensal and pathogenic bacteria [194]. Our group has shown that TLR2 activators in food might skew the immune system towards an allergic response by inhibiting oral tolerance development [92]. In contrast, *B. fragilis*, which contains polysaccharide A, signals via TLR2 on Tregs, leading to suppressing Th17 responses and enhanced colonization with this bacteria in the intestine [194]. Therefore, establishing or disrupting tolerance via TLR2 might require other microenvironmental ligands and/or receptors and be highly dependent on intestinal location. sTLR2 in breast milk has also been implicated in preventing HIV infection and inflammation inhibition [331], although the mechanisms whereby this occurs are not well understood. It could relate to improved intestinal barrier function or altered receptor expression by local immune effector cells vulnerable to infection. However, little work has been done examining the role of sTLR2 in oral tolerance or its impact on the neonate's developing microbiota. Also, several soluble receptors exist in serum, saliva, or urine, including sTLR4, sIL-4-R, sIL-5-R, sIFN-γ-R, sTGF-β-R, sGM-CSF-R [332]. These might be of additional potential importance in regulating the impact of milk-borne cytokines. However, these receptors have not been well studied and defined in breast milk.

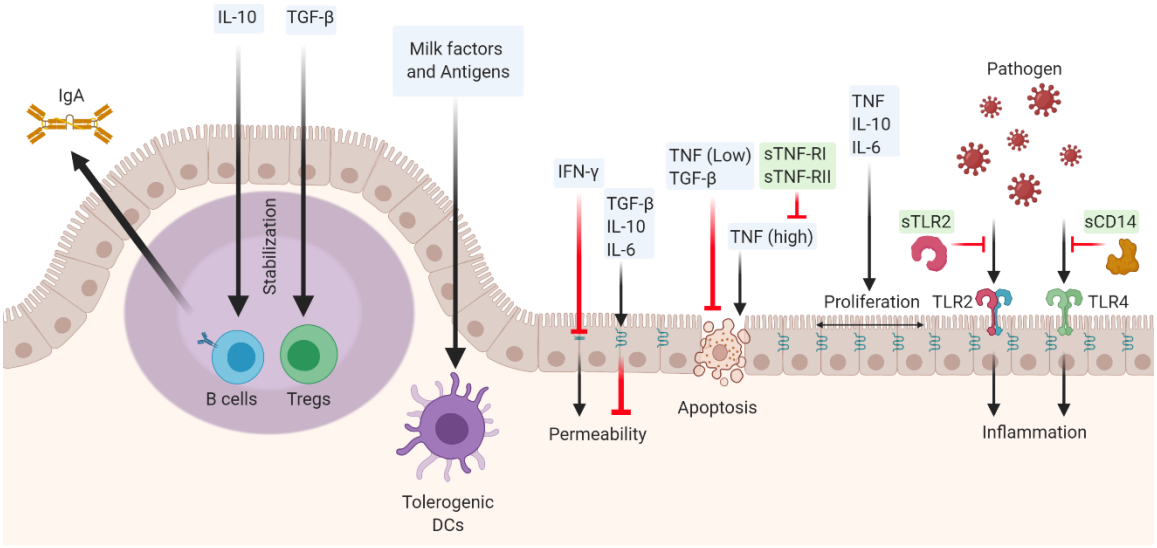

Figure 1-5: Mechanism of milk cytokines and soluble receptors in oral tolerance.
Milk factors enhance the development of tolerogenic DCs in the neonatal gastrointestinal tract (GIT), Milk derived cytokines (light blue boxes) and soluble receptors (green boxes) form a network of IM that interact together and impact oral tolerance via multiple mechanisms. Milk cytokines, including TGF-β, IL-10, IL-6, TNF, and IFN-γ, affect the integrity, proliferation, and apoptosis of intestinal epithelial cells (IECs). High levels of cytokines in breast milk could also have adverse effects, such as the high TNF concentration that can be seen in mastitis and induce apoptosis in the IECs. Effects of TNF can be attenuated via the corresponding soluble receptors, sTNF-R-I, and sTNF-R-II found in breast milk. Furthermore, sTLR2 and sCD14 in breast milk can modulate the inflammatory response towards pathogens in the neonate's GIT by regulating TLR2 and TLR4, respectively.

1.5. Rationale and Hypothesis

TLR2 is highly expressed on monocytes and mammary epithelial cells and plays an essential role in the secretion of several IMs, some of which are found in breast milk. The maturation of the immune system and the GIT in infants is highly dependent on milk IMs and other factors during the critical window of opportunity for oral tolerance development and commensal microorganism colonization. The exact role of maternal TLR2 in the modulation of milk components and, subsequently developing oral tolerance in nursed infants is not fully understood. We sought to investigate the impact of TLR2 deficiency in modifying milk components and how it impacts immune system development in nursed pups using a mouse model of cross-fostering. Also, we wanted to understand the impact of maternal factors and *TLR2* gene polymorphisms on the levels of sTLR2 and other IMs in human milk and association with allergic diseases in infants. We hypothesized that milk composition is influenced by the expression of specific innate immune receptors, such as TLR2, which mediate secretion of IMs in breast milk. The differences in these IMs between mothers might predispose some infants to immune-mediated diseases, including allergy.

Table 1-1 Concentration of cytokines and soluble receptors in human colostrum and human milk

	Human Colostrum (0-4 days)		References	Human milk (1-6 months)		References
TGF-β1	140-3300	pg/ml	297,301	80-600	pg/ml	297,333
TGF-β2	100-3300	pg/ml	297,334	800-5300	pg/ml	287,301
IL-1β	0.29-27.7	pg/ml	301,335	0.028-23	pg/ml	301,333
IL-4	1.6-172	pg/ml	335,336	5.6-626.8	pg/ml	287,337
IL-5	6.2-79	pg/ml	287,336	6.2-142	pg/ml	287,336
IL-6	7.3-80.6	pg/ml	335,338	3.5-148.6	pg/ml	301,337
IL-10	0-3304	pg/ml	298,300	0-246	pg/ml	298,336
IL-12	3-310	pg/ml	339,340	3-40	pg/ml	339,340
IL-13	3.2-243	pg/ml	279,287	3.2-264	pg/ml	287,336
TNF	21.9-620	pg/ml	341,342	4.4-58	pg/ml	333,343
IFN-γ	2.5-708	pg/ml	301,336	0.7-175	pg/ml	301,336
G-CSF	4.38	pg/ml	282	4.2	pg/ml	282
GM-CSF	23.02	pg/ml	282	1.6	pg/ml	282
M-CSF	3740-52470	U/ml	283	1150	U/ml	283
sTNF-R-I	3703	pg/ml	319	1732	pg/ml	319
sTNF-R-II	4507	pg/ml	319	931	pg/ml	319
sIL-6R	12761	pg/ml	319	2436	pg/ml	319
sCD14	77.9-88.8	µg/ml	344	7-25	µg/ml	345,346
sTLR2	+*		68	~10	ng/ml	72

* Concentration of sTLR2 in human colostrum is not reported and assessed from the figure.

Chapter 2. Materials and Methods

2.1. Animal experiments

2.1.1. Mice

BALB/c mice, wild type (WT) or TLR2$^{-/-}$, were bred within the Carleton Animal Care Facility at Dalhousie University (Halifax, Canada). TLR2 knockout 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 microbiota variability 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.

2.1.2. 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 five days with male urine to stimulate the estrus cycles through its content of pheromones [347]. 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. Therefore, the offspring from both types of breeders were heterozygous genetically (TLR2$^{+/-}$) and phenotypically normal. Pups from each breeder were divided into two groups

within 1-3 days after birth. Half of each litter remained with its biological mother while the alternate genotype mother fostered the other half.

2.1.3. Assessment of food sensitization towards OVA

We tested the ability of newborn TLR2$^{+/-}$ mice nursed by WT or TLR2$^{-/-}$ dams, using a cross-fostering model, to develop immunological tolerance to OVA during the lactation period. We have tested our hypothesis 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 directly (20 µg per gram weight) dropwise into their mouths every second day by pipette. Alternatively, pups were nursed by 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 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.

ELISA 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. 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 synergy reader (BioTek, Winooski, VT) and analyzed

by Gen5 software (BioTek). OVA-specific IgE was quantified compared to a commercial standard (Chondrex Inc.; Redmond, WA, USA) and reported as ng/ml. OVA-specific IgA, IgG1, and IgG2a were standardized relative to a standard serum with a high titer of anti-OVA immunoglobulins and data presented as optical density at 450 nm (OD450).

2.1.4. Assessment of DC subsets, Tregs, T-helpers, and ILCs by flow cytometry

The pups from selected cross-fostering experiments were euthanized at days 15 or 21 after birth. PP, MLN, and spleen were harvested from each mouse and assessed for DC subsets, Tregs, T-helper cells, and ILCs. Collected tissues were minced, and single-cell suspensions were blocked using 10% heat-inactivated rat serum for 20 min at 4°C. Before extracellular or intracellular staining, single-cell suspensions were incubated with fixable viability dye (FVD) eFluor 450 (ThermoFisher) diluted 1:1000 in PBS for 20 min at 4°C. To determine the frequencies of DCs (adapted from [348]) and ILCs (adapted from [349]), they were stained with a cocktail of antibodies for extracellular markers indicated in (**Table 2-1**). To identify T-helper cells' subsets, they were stimulated with 50 ng/ml PMA and 1 µg/ml of Ionomycin in 10% FBS-RPMI medium for 5 hours before adding 5 µg/ml Brefeldin A for another 1 hour. In contrast, Tregs were assessed without cell stimulation. The frequencies of Tregs [350] and T-helper subsets were identified by staining with extracellular markers (**Table 2-1**), followed by fixation, permeabilization, and staining with intracellular antibodies (**Table 2-1**). Analysis of flow cytometry data was performed using Flowjo software (Ashland, OR). Viable cells (negative population of FVD-eFluor 450) were gated on $CD45^+$ cells. The frequencies of DC subsets and Tregs were calculated as indicated in the gating strategies (**Figure 3-3** and **Figure 3-5**, respectively). The levels

of DC subsets, T-helper subsets and ILCs that failed to show significant difference are summarized (**Table 2-2**).

Table 2-1: Antibody staining panels

DCs	ILCs	Treg cells	T_H cells
FITC-CD3ε*	FITC-	Extracellular	FITC-CD4†
PE-CD103†	CD5*	FITC-CD3ε*	PE-Cy7-CD3ε†
PE-Cy7-Ly6c*	CD3ε*	BV510-CD4‡	PerCP-Cy 5.5-CD45‡
PerCP-Cy 5.5-Siglec-H†	CD11c*	PE-CD25*	eF450-FVD†
APC-CD11c*	CD11b*	APC-CD45*	PE-IFN-γ*
APC-eF780-CD8α†	CD19†	eF450-FVD†	APC-IL-4§
AF700-MHC-II†	Gr-1†	Intracellular	BV510-IL-17*
eF450-FVD†	CD49b*	PE-FOXP3†	
BV786-B220‡	PerCP-Cy 5.5-CD45‡		
BV510-CD45*	APC-IL-33Rα (ST2)†		
BV650-CD317*	PE-CD117*		
PE-eF610-CD11b†	AF700-CD90.2*		
BV711-CD24‡	eF450-FVD†		
	BV786-CCR6*		
	BV510-CD4‡		
	BV605-CD127 (IL-7R)*		
	PE-CD25*		

* BioLegend (San Diego, Calif).
† Invitrogen (San Diego, Calif).
‡ BD HorizonCA (San Jose, Calif).
§ BD Pharmingen (San Jose, Calif).

Table 2-2: Frequencies of T_H cells, ILCs, and DCs subsets

Organ	Nursing dam	% of CD4$^+$			% of ILCs		% of MHC-II$^+$/CD11c$^+$		
		T_H1	T_H17	T_H2	ILC2	ILC3	cDC1	cDC2	pDC
MLN	WT	1.320 ± 0.12 (n = 4)	0.2700 ± 0.03 (n = 4)	0.1094 ± 0.02 (n = 4)	10.28 ± 3.08 (n = 5)	8.052 ± 1.69 (n = 5)	29.22 ± 0.74 (n = 5)	45.40 ± 3.84 (n = 5)	46.56 ± 6.97 (n = 5)
	TLR2$^{-/-}$	1.605 ± 0.23 (n = 4)	0.3250 ± 0.06 (n = 4)	0.1775 ± 0.030 (n = 4)	13.75 ± 2.67 (n = 8)	11.09 ± 1.37 (n = 8)	31.62 ± 0.66 (n = 8)	41.78 ± 3.03 (n = 8)	36.70 ± 3.99 (n = 8)
P-value		0.33	0.45	0.12	0.42	0.19	0.20	0.47	0.21
Spleen	WT	3.490 ± 0.31 (n = 4)	0.1500 ± 0.009 (n = 4)	0.3475 ± 0.029 (n = 4)	5.354 ± 0.42 (n = 5)	4.150 ± 1.0 (n = 5)	5.353 ± 0.76 (n = 5)	12.27 ± 0.75 (n = 5)	17.25 ± 1.33 (n = 5)
	TLR2$^{-/-}$	2.745 ± 0.30 (n = 4)	0.2050 ± 0.025 (n = 4)	0.4400 ± 0.041 (n = 4)	6.594 ± 1.24 (n = 8)	3.021 ± 0.5 (n = 8)	5.707 ± 0.61 (n = 8)	13.40 ± 0.92 (n = 8)	20.17 ± 1.2 (n = 8)
P-value		0.14	0.08	0.11	0.46	0.28	0.72	0.41	0.14

2.1.5. Digital droplet PCR (ddPCR) analysis for DCs and Tregs genes

DCs and Tregs were sorted from MLN-derived cell suspensions using FACSAria III (BD Bioscience). Lymph nodes were pushed through a 70 µm strainer then stained with fixable viability dye (FVD) eFluor-780 (eBioscience). 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 reversely 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 (Biorad) using commercially validated primer sets (Biorad and Qiagen) and normalized against *Hprt* and *Gusb* transcript levels. The results of non-significantly changed genes were summarized in (**Table 2-3**).

Table 2-3: Tolerogenic DCs and Treg cell gene' expression

Nursing dam	DC genes						Tre genes		
	Tgfb1	***Raldh2***	***Cd80***	***Cd86***	***Cd274***	***Btla***	***FOXP3***	***CCR9***	***Cd279***
WT	986.3 ± 110.0 (n = 5)	10,506 ± 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**	0.32	0.32	0.16	0.51	0.17	0.31	0.96	0.88	0.46

2.1.6. Intestinal permeability

Permeability of the small intestine in pups fed by TLR2$^{-/-}$ or WT dams was assessed *in vivo* using oral administration of FITC-Dextran 4 kD (Sigma-Aldrich, St. Louis, Missouri, USA) at the age of weaning (day 21 after birth). Briefly, the mice ingested FITC-Dextran 4 kD (44 mg/g body weight) by gavage. After two hours, the mice were anaesthetized by isoflurane, and the blood was collected via a cardiac puncture. The mice were euthanized by CO_2 and cervical dislocation following blood collection. Blood samples were centrifuged for 10 min at 4°C (12,000 x g), and plasma samples were assessed for FITC Dextran concentration using a fluorescent spectrophotometer at an excitation wavelength of 490 nm and an emission wavelength of 530 nm. Serially diluted FITC-Dextran in a non-gavaged mouse serum served as a standard curve.

2.1.7. Mouse 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. Oxytocin (Sigma Aldrich, St. Louis, MO) 2 IU in 100 µl PBS was injected intraperitoneally before milking. Mouse milk was collected at least from 6 mammary glands in each mother with yields between 150-350 µl of milk/mouse. Milk samples were depleted of lipid by centrifuging at 10,000 x g for 10 min at 4°C. Whey fractions were stored at -20°C for further analysis.

2.1.8. Mouse milk immune modulators (IMs)

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, Hercules, CA),

with calibration and standard controls. Samples were thawed only once on the ice and assayed in duplicate. Milk content of TGF-β1 and TGF-β2 was quantified by sandwich ELISA kit (R&D). 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.

Mouse soluble TLR2 and total IgA in mouse milk were assessed by using ELISA kits from Abcam (ab224880, UK) and eBioscience (88-50450, San Diego, CA), respectively, according to the manufacturers' instructions.

2.1.9. 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 four after birth and was repeated every second day until weaning on day 21.

2.1.10. Statistical analysis of mouse experiments

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 time-point by Student's *t*-test.

2.2. Human milk experiments

2.2.1. CHILD study design

In the CHILD study, mothers and infants were recruited from four different locations in Canada (Toronto, Vancouver, Edmonton, and Manitoba) in the period between 2008 and 2012 [351]. Written informed consent was acquired from the participants. Human Research Ethics Boards at the Hospital for Sick Children, McMaster University, and the Universities of Alberta, Manitoba and British Columbia have approved the study. Data were collected based on questionnaires, allergic tests, and samples taken from the mothers and the infants at different time points according to the study protocol [351]. The current sub-study used three subsets out of the full cohort.

The first subset (n=300) was chosen based on the allergic status of mothers and infants and fell within four groups; both non-allergic, both allergic, allergic mother/non-allergic infant, and non-allergic mother/allergic infant (n=75 per group). The maternal allergic status was defined by having at least one allergic disease, including food allergy, asthma, hay fever, or skin allergy, diagnosed prenatally by a medical doctor. Subsets of mother-child dyads for analysis were identified in collaboration with Dr. Meghan Azad, a lead investigator within the CHILD study. The allergic disease enriched subset was chosen to study breast milk components, with a focus on sTLR2, and how allergic status could impact the breast milk components and allergic disease outcome of nursed infants.

The second subset (n=2422) included all mothers within the CHILD cohort, for which genotyping data were available with a focus on genetic polymorphisms in the *TLR2* gene. A third group (n=262) was composed of the mothers that have both been genotyped, and their milk cytokines assessed.

Analyte concentrations that were 1.5 times higher or lower than the interquartile range (third quartile minus first quartile) were defined as outliers. One milk sample which showed more than eight outliers out of the analytes measured was excluded from the analysis. 299 samples with IMs were further studied.

2.2.2. Milk sampling

According to the CHILD Cohort Study protocol, milk samples were collected between 12.1 and 26.9 weeks after giving birth (median = 15.4 weeks). To control for variation in milk components of foremilk and hindmilk [352] and diurnal differences [353], multiple foremilk and hindmilk feeds during a day were collected and mixed. Milk samples were collected in ordinary life behaviours with no strict instructions on sanitization procedures, stored at 4°C in the home fridge for less than 24 hrs before being collected by staff. Samples were aliquoted and stored at -80°C until the first thaw for this study. Milk samples were thawed on the ice on the day of analysis, depleted from lipids by centrifuging each sample at 10,000 *g* for 30 min at 4°C. Whey fractions were removed and centrifuged again at 1,000 *g* for 10 min at 4°C to remove any carried-over lipids before analysis.

2.2.3. Milk cytokines, antibodies, growth factors and soluble receptors

The milk components to be measured were selected based on a review of the detectable immune factors in human milk [354]. The analytes that have shown levels below the lower limit of detection (LLOD) were omitted from further analysis.

A panel of 24 analytes (**Table 2-4**) was analyzed using premixed multianalyte kits according to the manufacturers' recommendations and acquired by Luminex 200 (Bio-Rad), with calibration and standard controls.

Soluble TLR2, Total IgA, TGF-β1, and TGF-β2 were quantified using sandwich ELISA (**Table 2-4**). For sTLR2 measurements, ELISA plates were coated with purified mouse monoclonal anti-human TLR2 capture antibody 4H7. This antibody exhibited greater specificity and higher range of sensitivity for sTLR2 in milk than the commercially available antibodies. The 4H7 monoclonal antibody was previously generated in Dr. Thomas Issekutz's laboratory by immunising mice with a human TLR2 transfected mouse fibroblast cell line and B cells from these mice used for routine fusion with a plasma cell line for monoclonal antibody generation. The 4H7 IgG1 antibody-producing clone was selected, following cell fusion steps based on its ability to bind selectively to TLR2 transfected cells but not TLR1, 4 or 6 expressing cells and specificity for TLR2 further confirmed by both Western blotting and flow cytometric analysis of human peripheral blood mononuclear cells. The sTLR2 standard and the detection antibody used were obtained from a commercial ELISA kit (R&D Systems). To measure the total TGF-β1, and TGF-β2, the milk samples were diluted in PBS and then treated with 1N HCl for 10 min at room temperature, and then neutralized with 1.2 N NaOH/0.5M HEPES. Samples and standards were loaded into pre-coated ELISA plates according to the manufacturer's instructions. Analytes interplate variations were adjusted by normalizing the log-transformed concentrations using the following formula: $z'_{ij} = z_{ij} - \bar{z}_{.j} + \bar{z}_{..}$, where i is the sample, j is the plate, z'_{ij} is the normalized log-transformed concentration, z_{ij} is the measured log-transformed concentration, $\bar{z}_{.j}$ is the median of samples for each plate, and $\bar{z}_{..}$ is the grand median.

Bovine sTLR2 was measured in cow's milk (homogenized and skimmed) and baby formulas (liquid, powder, and hypoallergenic) using a commercial ELISA kit

(MyBioSource, San Diego, CA). Three brands in each group were used, and powder samples were reconstituted based on the manufacturer's recommendations before analysis.

Table 2-4: List of milk IM assessed in our study

Analyte	Method	Manufacturer	Dilution factor	Catalogue #	Omitted	Comments
CCL5/RANTES	Luminex	R&D	1:2	LXSAHM		
CCL11/Eotaxin	Luminex	R&D	1:2	LXSAHM	Yes	Below LLOD
G-CSF	Luminex	R&D	1:2	LXSAHM		
GM-CSF	Luminex	R&D	1:2	LXSAHM	Yes	Below LLOD
IFN-γ	Luminex	R&D	1:2	LXSAHM	Yes	Below LLOD
IL-1B	Luminex	R&D	1:2	LXSAHM	Yes	Below LLOD
IL-4	Luminex	R&D	1:2	LXSAHM		
IL-5	Luminex	R&D	1:2	LXSAHM	Yes	Below LLOD
IL-6	Luminex	R&D	1:2	LXSAHM		
IL-10	Luminex	R&D	1:2	LXSAHM	Yes	Below LLOD
IL-12p70	Luminex	R&D	1:2	LXSAHM	Yes	Below LLOD
IL-13	Luminex	R&D	1:2	LXSAHM	Yes	Below LLOD
IL-17	Luminex	R&D	1:2	LXSAHM	Yes	Below LLOD
IL-33	Luminex	R&D	1:2	LXSAHM		
TSLP	Luminex	R&D	1:2	LXSAHM		
BDNF	Luminex	R&D	1:2	LXSAHM	Yes	Below LLOD
TNF	Luminex	R&D	1:2	LXSAHM		
IgE	Luminex	ThermoFisher	1:10	EPX070-10818-901		
IgG1	Luminex	ThermoFisher	1:10	EPX070-10818-901		
IgG2	Luminex	ThermoFisher	1:10	EPX070-10818-901		
IgG3	Luminex	ThermoFisher	1:10	EPX070-10818-901		
IgG4	Luminex	ThermoFisher	1:10	EPX070-10818-901		
IgM	Luminex	ThermoFisher	1:10	EPX070-10818-901		
IgA	Luminex	ThermoFisher	1:10	EPX070-10818-901	Yes	Above ULOD
CXCL1 (GRO)	Luminex	ThermoFisher	1:10	PPX-09-MX2W79V		
CXCL8 (IL-8)	Luminex	ThermoFisher	1:10	PPX-09-MX2W79V		
CCL22 (MDC)	Luminex	ThermoFisher	1:10	PPX-09-MX2W79V		
CX3CL1 (Fractalkine)	Luminex	ThermoFisher	1:10	PPX-09-MX2W79V		
CCL2 (MCP-1)	Luminex	ThermoFisher	1:10	PPX-09-MX2W79V		
CXCL10 (IP-10)	Luminex	ThermoFisher	1:10	PPX-09-MX2W79V		
EGF	Luminex	ThermoFisher	1:10	PPX-09-MX2W79V		
VEGF	Luminex	ThermoFisher	1:10	PPX-09-MX2W79V		

Analyte	Method	Manufacturer	Dilution factor	Catalogue #	Omitted	Comments
IL-9	Luminex	ThermoFisher	1:10	PPX-09-MX2W79V		
CD14	Luminex	ThermoFisher	1:1000	EPX010-12283-901		
Lactoferrin	Luminex	ThermoFisher	1:1000	EPX010-12250-901		
IgA	ELISA	eBioscience	1:10000	88-50600-88		
sTLR2	ELISA	R&D	1:20	DY2616		We only used the detection Ab and the standard
IGF-1	ELISA	R&D	1:2	DY291-05	Yes	Below LLOD
TGF-β1	ELISA	R&D	1:4			
TGF-β2	ELISA	R&D	1:30			

LLOD: Lower limit of detection
ULOD: Upper limit of detection

2.2.4. Toll-Like Receptor 2 (TLR2) genotype

Mother's genotyping was performed in collaboration with Dr. Qingling Duan from Queen's University. DNA for genotyping mothers in this study was acquired during pregnancy using peripheral blood monocytes, and the SNPs were defined using the CHILD study protocols and exclusion criteria [355]. Using IMPUTE2 software that imputes genotypes based on the 1000 genome project, more than 24 million SNPs of high quality (> 0.7) were identified. A total of 291 variants in the *TLR2* gene were identified, including the intronic and 5' and 3' untranslated regions (UTRs) in DNA samples obtained from mothers enrolled in the CHILD cohort study following informed consent. Out of 291 variants, 32 are common > 0.05 minor allele frequency (MAF), and 259 are rare variants. After pruning the 32 variants, 10 passed the R^2 0.6 threshold as independent variants. Sequence information was available from 262 mothers out of 299 that were studied for their milk components.

2.2.5. Statistical analysis for human milk study

Statistical analyses were performed using R (version 4.0.2 R foundation for Statistical Computing) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA). All data are presented as a median with the minimum-maximum range since they were not normally distributed. A single milk sample with more than ten outliers in different analytes was omitted from the analysis. Outliers were identified as three times the interquartile range (IQR) above or below first and third quartiles, respectively (Q1-3*IQR, Q3+3*IQR). Pearson's test was conducted to examine correlation coefficients between log-transformed cytokines. The relationship between the concentration of milk cytokines and other categorical variables in mothers and infants was assessed using unadjusted linear

regression and followed by false discovery rate (FDR) to adjust for multiple testing. All of the statistical tests were two-sided with p-values < 0.05 were regarded as significant and FDR-adjusted p-values < 0.1 were considered significant. The nonparametric Kruskal-Wallis test was used to compare the levels of sTLR2 between human milk, cow's milk brands and baby formulas. Chi-square test was used to compare the categorical variables when comparing the association between *TLR2* SNPs with maternal allergic diseases. Statistical analysis was performed in close consultation with Dr. Meghan Azad and her staff.

Chapter 3. Toll-like receptor 2 impacts the development of oral tolerance in mouse pups via a milk-dependent mechanism

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3.1. Abstract

Background: The role of breastfeeding in the development of oral tolerance and allergic diseases is controversial, which may 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 IMs 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, which 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.

3.2. Introduction

Food allergies commonly affect infants and young children, especially in Western countries [356]. According to the "window of opportunity" concept, exposure to dietary and environmental factors in early life, during early postnatal immune and GIT development, can impact susceptibility to allergic diseases [357,358]. 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 one year [253,359]. While there is evidence supporting a protective role for breast milk in preventing food allergies, there remains substantial controversy [254]. Genetic factors also play a key role in regulating susceptibility to allergic disease [360], 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 [354]. 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 PP and the MLN, or disseminated in the LP [361]. The GALT encompasses immune cells such as DCs, Tregs, Th cells, ILCs, macrophages, and B cells, subsets of which are critical for tolerance development [362–364]. Breast milk factors can induce tolerogenic properties in DC subsets, which sample food antigens from the intestinal lumen and introduce them to Tregs in the GALT [365].

Tolerogenic DCs express several immunomodulatory molecules such as CD103, indoleamine 2,3-dioxygenase (IDO), TGF-β, retinaldehyde dehydrogenase 2 (RALDH2), CD80/CD86, programmed cell death-1 ligand (PD-L1), and B- and T-lymphocyte attenuator (BTLA) [363]. $CD103^+$ DCs continuously sample antigens from the intestinal lumen, migrate to local lymph nodes, induce the gut homing receptors CCR9 on T cells [104] and promote $FOXP3^+$ Treg differentiation via RA and TGF-β1 as well as PD1-PDL1 signalling [366–368]. Antigen-specific Tregs enhance oral tolerance development and subsequently reduce class switching of B cells toward IgE secretion [369]. Milk-derived factors also enhance the maturation of the intestinal barrier [370]. Increased intestinal permeability is considered a potential risk factor for food allergy development [371]. Genetic variations, including SNPs in immunoregulatory genes, can affect the composition of human and dairy milk [372–375]. However, the impact of genetically-mediated milk composition changes on the establishment of oral tolerance has not been previously reported.

The pattern recognition receptor, Toll-like receptor 2 (TLR2), regulates IM secretion in response to microbial or endogenous ligands. TLR2-mediated responses can impact multiple immune processes [376]. It is found in both soluble and cell-bound forms in serum and breast milk [68]. TLR2 deficiency in adult mice does not impair oral tolerance development, although activation of this receptor promotes IgE and IgA responses to food antigens [92]. SNPs in *TLR2* in human subjects have been associated with increased susceptibility to diseases, including atopic dermatitis (AD) [81,82], asthma [79], inflammatory bowel diseases [84], tuberculosis infection [377] and reactive arthritis [85]. Peripheral blood monocytes from humans with common SNPs in TLR2 have a decreased capacity to

respond to some TLR2 stimuli [86]. Such monocytes are also found in human milk and act as a potent source of cytokines [275]. In dairy cattle, SNPs in *TLR2* have been associated with changes in milk components [378]. We hypothesized that milk from TLR2-deficient or control dams might 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.

3.3. Results

3.3.1. $TLR2^{+/-}$ pups cross-fostered onto WT dams have significantly less food sensitization than 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 3-1A**). OVA was introduced to the pups for one week during lactation either directly by gavage or indirectly via the dam's milk. To assess pup's tolerance, they were immunized and challenged with OVA as indicated (**Figure 3-1A**). The lactation period was restricted to 21 days. Pups not exposed to OVA pre-weaning produced higher anti-OVA IgE, IgA, IgG1, and IgG2a levels in response to subsequent immunization than those receiving OVA orally (**Figure 3-1B, 1C, Figure 3-2**). 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 3-1B, 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 from their siblings who 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. Maternal 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, 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.

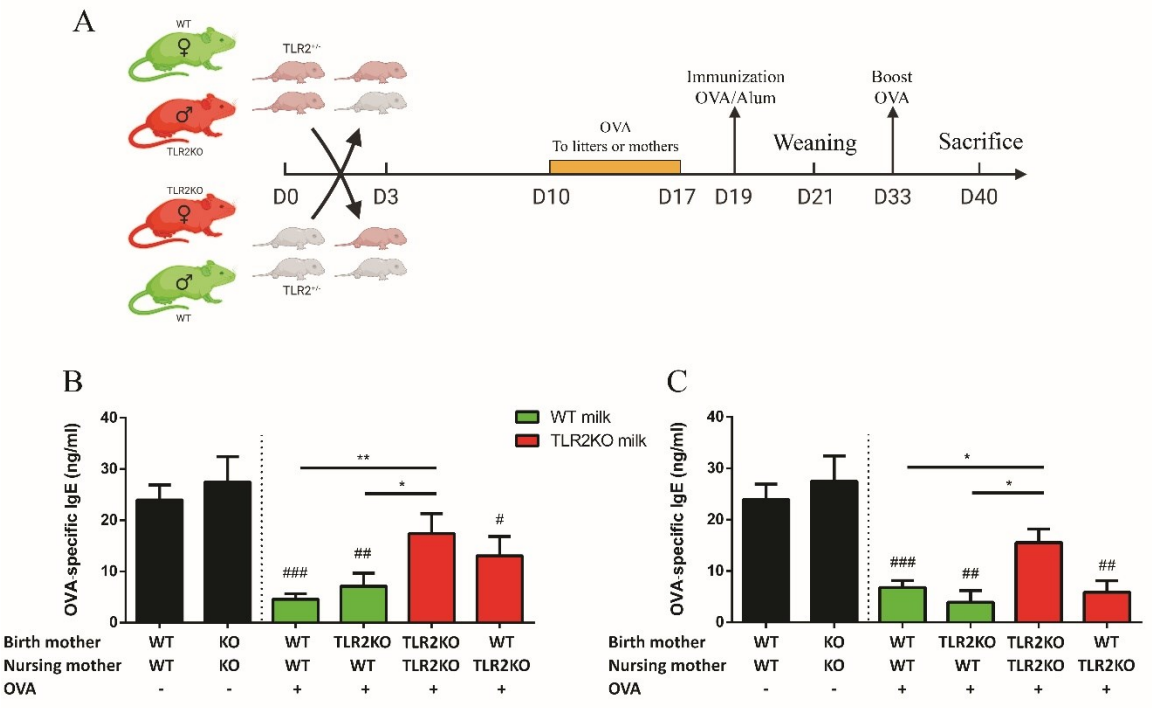


Figure 3-1: TLR2$^{+/-}$ pups cross-fostered onto WT dams have significantly less food sensitization compared to siblings that remain with their biological TLR2$^{-/-}$ dam.
(A) Schematic of the cross-fostering experiment to evaluate WT or TLR2KO milk effect on food sensitization. (B) Levels of anti-OVA 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).

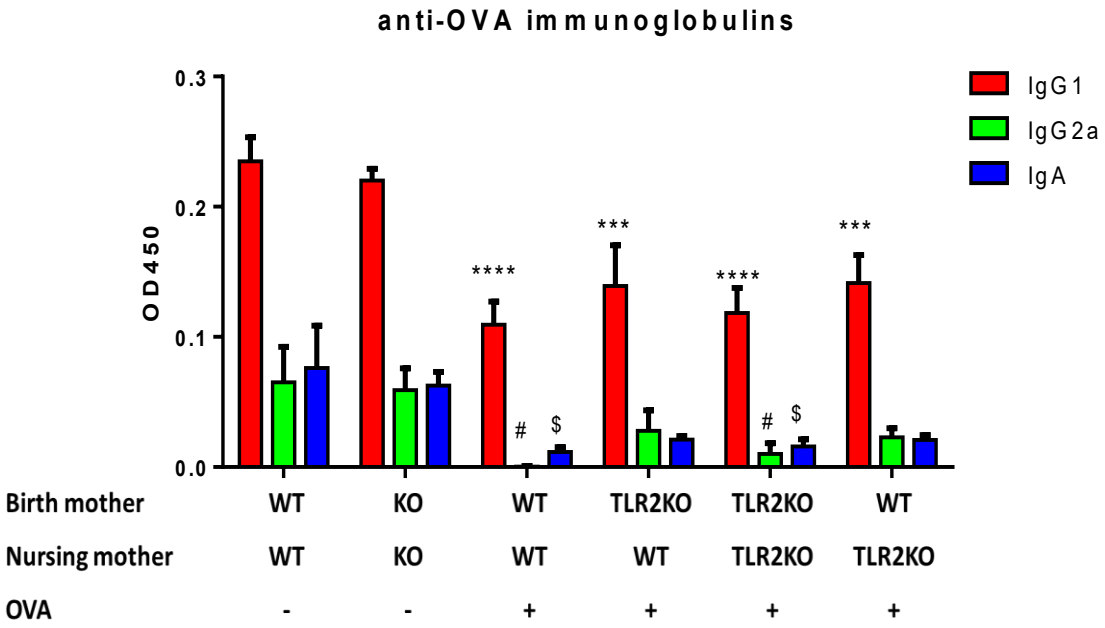


Figure 3-2: Milk from WT or TLR2$^{-/-}$ dams does not alter levels of anti-OVA IgA, IgG1, and IgG2a in the pups.

ELISA was used to assess the levels of anti-OVA IgA, IgG1, and IgG2a in pups 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).

3.3.2. 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 or $TLR2^{-/-}$ dams were assessed for populations of both of these cell types in the GALT at weaning (21 days) as shown in (**Figure 3-3A**). Tolerogenic DCs have been characterized in previous studies as MHC-II^+/$CD11c^+$/$CD103^+$ cells [348]. 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 3-3C, D**).

DCs in the intestine are heterogeneous and can be divided into conventional DCs (cDC1 and cDC2) and pDCs [99]. 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 2-2**). However, the levels of tolerogenic $CD103^+$ cDC1, characterized as $CD45^+$/MHC-II^+/$CD11c^+$/$CD8a^+$/$CD24^+$/$CD103^+$/ $CD11b^-$ cells (**Figure 3-3B**), were significantly higher in the MLN and spleen of WT-nursed pups (**Figure 3-3E, F**) and such cells expressed higher levels of IDO (**Figure 3-3G**) in these mice. Furthermore, the impact of feeding WT milk on the expansion of tolerogenic cDC1, most notably in the MLN, was also observed at day 15 during the period when antigen was introduced (**Figure 3-4A, B**).

Tregs were evaluated based on the percentage of $CD3^+$/$CD4^+$/$CD25^+$/$FOXP3^+$ cells out of viable $CD45^+$ leukocytes (**Figure 3-5A**). Pups derived from $TLR2^{-/-}$ dams but nursed by WT dams had substantially more Treg cells in the PPs and MLNs than did the siblings that were not cross-fostered at weaning (**Figure 3-5B, C**) and to a lesser degree in the

spleen **(Figure 3-5D)**. Unlike $CD103^+$ cDC1, Treg levels at day 15 were not influenced by milk from WT or $TLR2^{-/-}$ dams (**Figure 3-6**), suggesting 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 food allergy susceptibility. 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 2-2**). Collectively, this indicates that milk from WT dams promotes an increase in tolerogenic $CD103^+$ cDC1 that express high levels of IDO, which promotes the expansion of Tregs in the GALT [379].

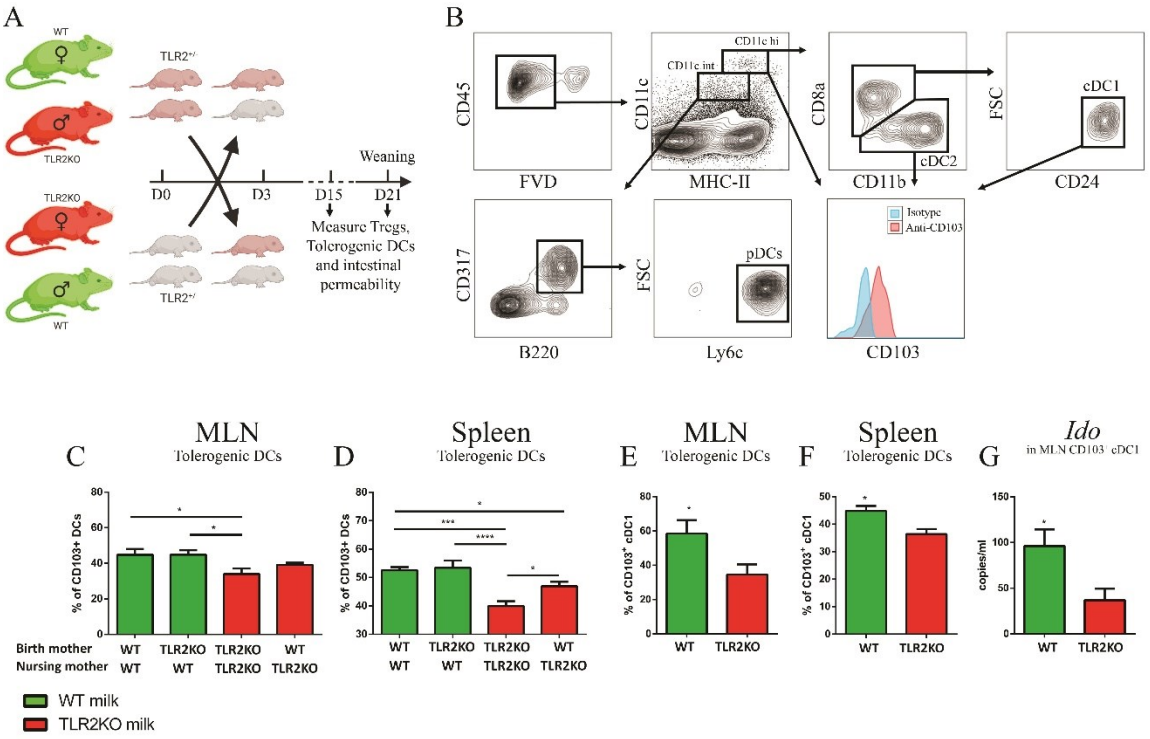


Figure 3-3: Milk from WT dams support the expansion of tolerogenic DCs in MLN and spleen

(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 $MHC\text{-}II^+/CD11c^+$ 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. $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$.

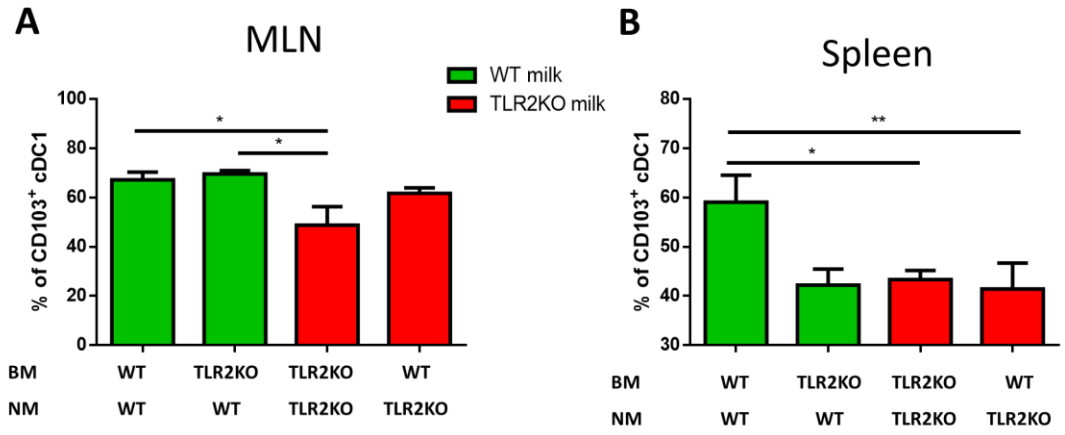


Figure 3-4: Milk from WT dams support the expansion of tolerogenic CD103$^+$ cDC1 in MLN at day 15 after birth.

(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.

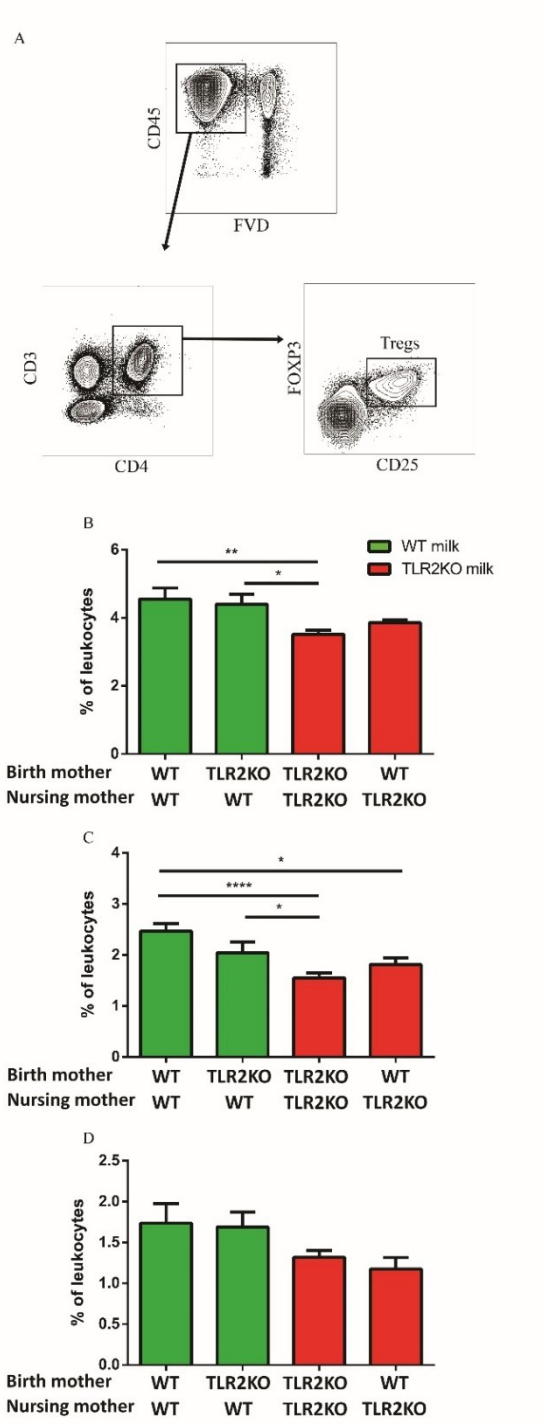


Figure 3-5: Milk from WT dams supports the expansion of Tregs in MLN and PP
(A) Representative flow cytometry plots of gating on Tregs. (B-D) Frequency of Tregs in MLN, PP, and spleen (gated on $CD3^+$, $CD4^+$, $CD25^+$ and $FOXP3^+$ cells) out of viable $CD45^+$ cells. Data are from four independent 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. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$.

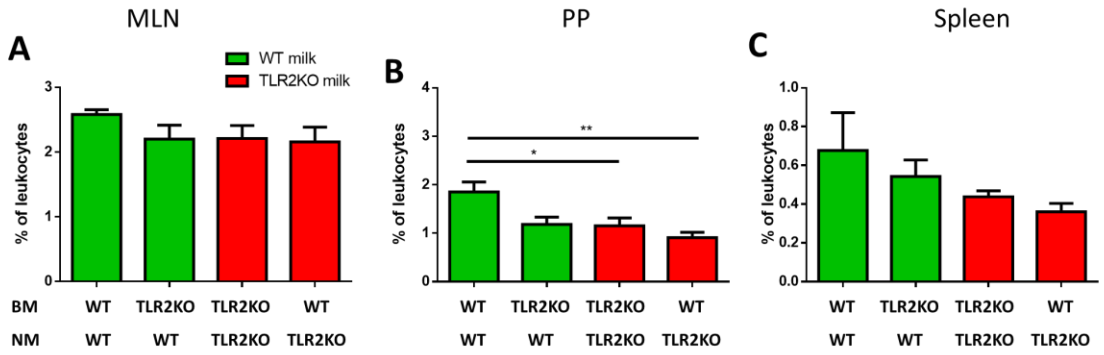


Figure 3-6: Milk from WT or TLR2 dams does not exhibit a major expansion on Tregs in the GALT at day 15 after birth.

(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.

3.3.3. 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 [371]. Intestinal maturation occurs close to weaning in rodents, while in humans, it normally occurs earlier [380]. The passage of inappropriately digested antigens via more permeable intestinal epithelial barrier might predispose to food sensitization [152,153]. Therefore, we 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 3-7**). 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.

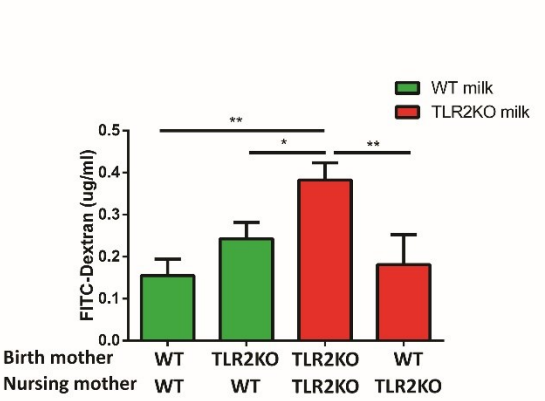


Figure 3-7: Pups born to TLR2$^{-/-}$ mothers but nursed by WT mice have decreased intestinal permeability

The levels of FITC-Dextran in the serum of pups nursed by WT or TLR2$^{-/-}$ dams were 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. *$P < 0.05$, **$P < 0.01$.

3.3.4. 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 five, IL-13, and IGF-1 at day ten compared to milk from TLR2$^{-/-}$ dams. These differences were most notable at later time points pre-weaning (**Figure 3-8**). Levels of IL-4, total IgA, TGF-β1, and TGF-β2 were not significantly different between TLR2$^{-/-}$ and WT dams (**Figure 3-8**). 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.

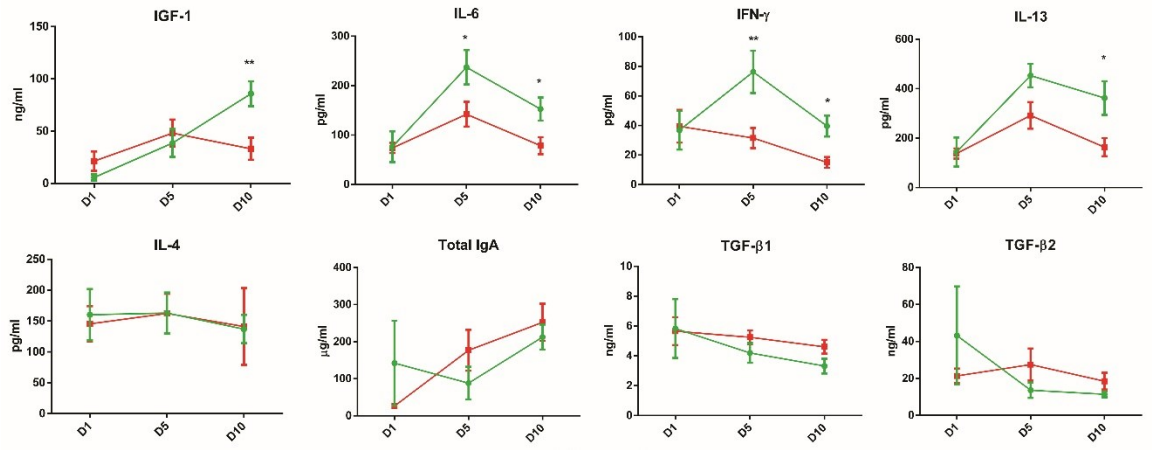


Figure 3-8: Mouse milk from TLR2$^{-/-}$ mothers has lower levels of IL-6, IL-13, IFN-γ, and IGF-1

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

3.3.5. 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 neonatal mouse diet with IGF 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 three groups that were gavaged with IGF-1, IL-6, or PBS, respectively, every second day from day four until weaning on day 21 (**Figure 3-9A**). IGF-1 supplementation was found to increase the populations of CD103^{+} cDC1 in both the MLN and the spleen and Tregs in the MLN (**Figure 3-9B**), while IL-6 supplementation had a more restricted impact in increasing CD103^{+} cDC1 in the MLN only. Notably, neither IGF-1 nor IL-6 supplementation had any significant impact on small intestine permeability. These data suggest that IGF-1 and IL-6 are important factors in milk from wild type mice and relatively deficient in TLR2$^{-/-}$ derived milk that can impact oral tolerance by expanding tolerogenic DCs and subsequently Tregs.

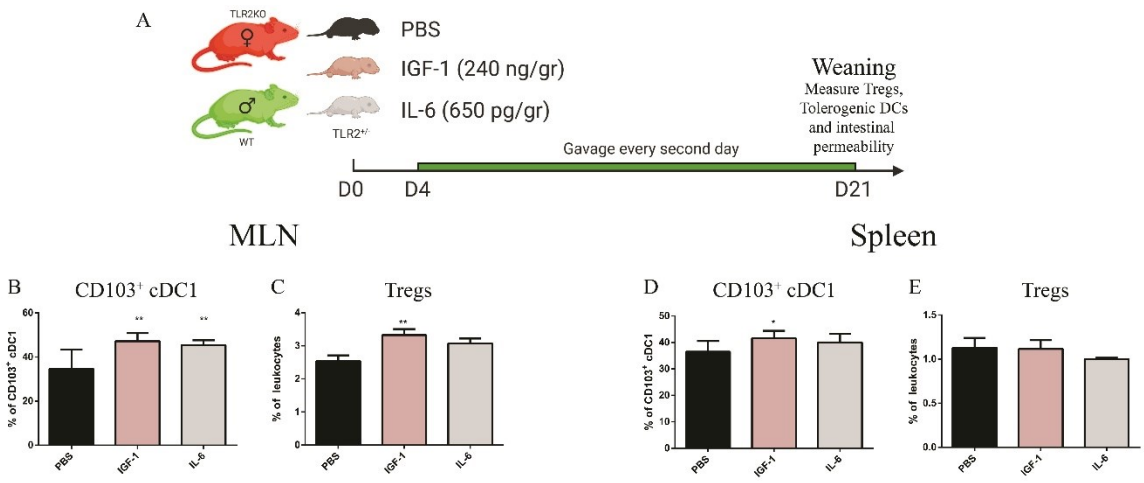


Figure 3-9: Supplementation of IGF-1 and IL-6 enhances levels of tolerogenic DCs and Tregs

(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 by one-way ANOVA followed by Fisher's LSD post-hoc test. Bars represent the mean ± SEM. $*P < 0.05$, $**P < 0.01$, IGF-1 or IL-6 vs PBS.

3.4. 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 directly to pups or maternal milk [381]. Although, the antigens in the maternal milk are delivered in more tolerogenic forms [380], 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 feeding dam's innate immune status can define the extent of later immune responses in the pups, even though they 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 [382].

This work has important implications for interpreting and designing studies of mice with deficiencies in TLR2 and related immunoregulatory molecules. It has been generally assumed that the mouse immune phenotype is genetically determined and not highly dependent on the maternal vs paternal genotype from which they are derived. Our studies demonstrate that the feeding dam's immune status 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 [383],

colitis-associated colorectal cancer [384,385] and Th1/Th2 inflammatory responses [386–388]. The feeding dam's immune status 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 [51]. TLR2 is expressed in mammary epithelial cells and milk-derived cells (**Figure 3-10**). It plays an important role in mammary gland's normal development and ductal tree formation [389]. 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 [68,390]. Levels of sTLR2 in human milk have been related to infant susceptibility to viral infection [390]. Notably, unlike the substantial levels of sTLR2 reported in human milk [72] 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; therefore, exposure to breast milk plays a significant role in maturation [391]. Critical to the development of oral tolerance are local dendritic cell populations [392]. Among several DC subsets, the frequency of tolerogenic $CD103^+$ cDC1 was substantially induced by the milk from WT dams with an increase in IDO expression. Generally, $CD103^+$ tolerogenic DCs exhibit regulatory responses, promote Treg expansion 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 [104,348,393,394]. According to Yamazaki *et al*. [395], $CD8^+$ DCs (cDC1) are stronger inducer of Tregs than other DC subsets. Increased IDO expression by the tolerogenic DCs from mice fed by wildtype mothers could

enhance Tregs expansion. Inhibition of IDO *in vivo* significantly affects the development of antigen-specific Tregs in the gut [379].

Consistently, we found a higher frequency of tolerogenic DCs in the MLN associated with increased Treg levels 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 [396,397]. 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 [398]. TLR2 $^{+/-}$ pups cross-fostered onto WT dams had significantly less permeable small intestines on day 21 than siblings fed by a TLR2$^{-/-}$ dam. Collectively, our data suggested that the milk from TLR2$^{-/-}$ mothers impair appropriate immune regulation and barrier function development.

Notably, 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 than

TLR2$^{-/-}$ dams, most notably at day ten after birth. These cytokines have pleiotropic effects, which include direct impacts on immunoglobulin responses and Tregs, DCs and intestinal epithelial cells. Their reduced level in milk from TLR2$^{-/-}$ dams is consistent with a combined impact in promoting oral tolerance in nursed pups. Variation of such milk factors might be linked to the role of TLR2 on the development of the mammary ductal tree [389].

The link between IGF-1 and TLR2 has not been well studied; however, Bohacek *et al*. [399], 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 GIT. 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. Also, the levels of Tregs were significantly increased in the MLN in IGF-1-fed pups. Although the high levels of tolerogenic DC1 might induce Tregs expansion, IGF-1 can directly stimulate human and mouse Treg proliferation *in vitro* and *in vivo* [400]. Burrin *et al*. showed that supplementation of IGF-1 in baby formula significantly enhanced small intestinal growth and maturation in neonatal pigs [401]. Furthermore, IGF-1 increased intestinal epithelial and smooth muscle cell proliferation [402,403] and decreased apoptosis rates in small intestine crypts [404,405]. In contrast, our results have shown no significant impact of IGF-1 supplementation on intestinal integrity.

Similarly, IL-6 supplementation of pups that are nursed by TLR2$^{-/-}$ dams improved the frequency 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 3-11**). IL-6 can induce the generation of tolerogenic DCs *in vitro* in synergy with IL-10 [406]. In contrast to our findings of the inability of IL-6 supplementation to improve intestinal permeability, Kuhn *et al*. [316] reported that IL-6$^{-/-}$ mice had increased intestinal permeability and a thinner mucus layer.

IL-13 promotes goblet cell proliferation in the intestine and mucus production from these cells [407–409]. This cytokine also promotes colonic wound healing [409,410] 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; however, the mechanism remains undefined [411].

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 [336]. Furthermore, activation of cDC1 (CD8^{+} DCs) by IFN-γ increases IDO expression and exhibits improved tolerogenic activity [412,413]. 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 [414].

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 oral tolerance development in early life. Likely, 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 [415], beside the long period of co-housing of WT and TLR2$^{-/-}$ mice before and throughout breeding cycles, which suggests that variation in milk composition between WT and TLR2$^{-/-}$ could be independent of bacterial signalling. However, other IMs in mouse milk might be subject to changes under the TLR2 effect, such as oligosaccharides, vitamins, and soluble innate immune receptors, requiring further investigations.

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

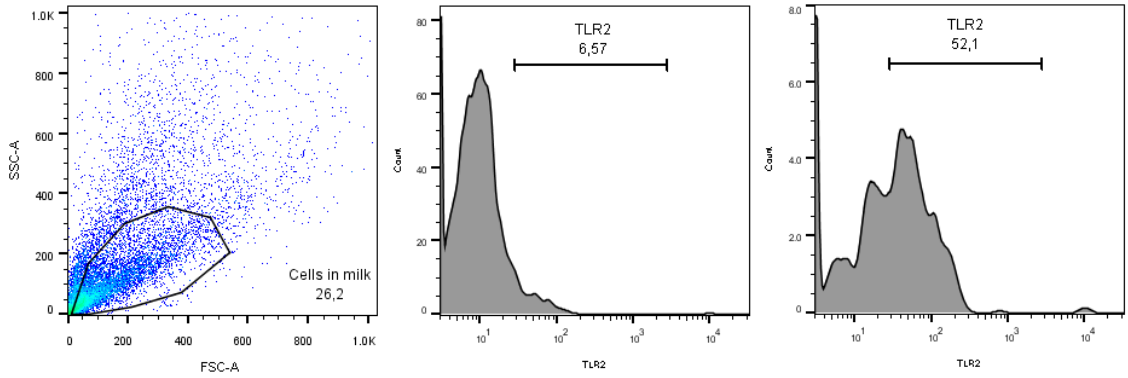


Figure 3-10: The leukocytes in the milk of TLR2$^{-/-}$ dams do not express TLR2 compared to the leukocytes in the milk of WT dams.
Milk from WT or TLR2$^{-/-}$ mice was centrifuged at 300 x g, and the cell pellets were washed and stained with FITC anti-mouse TLR2 before analysis by flow cytometry to determine TLR2 expression levels. The plots shown are representative of 1 experiment (n=3 in each group).

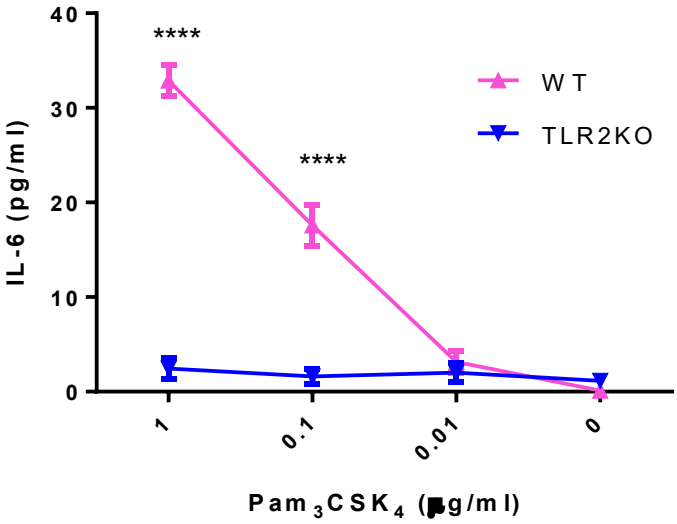


Figure 3-11: WT but not TLR2$^{-/-}$ splenocytes can respond to TLR2 agonist in a dose depending manner.

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

Chapter 4. Soluble Toll-Like receptor 2 (sTLR2) levels in breast milk are associated with selected maternal allergic status and polymorphisms in TLR2.

4.1. Abstract

Background: Soluble toll-like receptor 2 (sTLR2), is a biological regulator of membrane TLR2 activity linked to oral tolerance, which is highly abundant in human milk. The factors that impact the levels of sTLR2 in milk and the development of allergy in infants are still unknown. In this study, we investigated immune mediators (IMs) in milk, with an emphasis on sTLR2 and common *TLR2* polymorphisms to identify associations with the allergic disease using samples from the Canadian CHILD Cohort Study.

Methods: In a subset of 300 mothers, half of whom had an allergic disease, sTLR2 was assessed in milk samples along with other immune factors using quantitative ELISA and Luminex. The infant allergic status was documented at one year and three years of age based on medical diagnosis, skin prick testing, and parental questionnaires. Maternal single nucleotide polymorphisms (SNPs) in the *TLR2* gene were also assessed.

Results: The level of sTLR2 in human milk was highly variable between mothers with a median of 68.54 ng/ml (5-339 ng/ml), which is 2.4-3.4 times higher than the levels observed in cow's milk and commercial baby formulas. Two non-coding SNPs, rs56346547 and rs10222800 were significantly associated with decreased levels of sTLR2 in milk. Mothers diagnosed with food allergies, but not other allergic diseases, had substantially higher levels of sTLR2 in their milk, along with IgE and IL-9. Infants who were exclusively breastfed by non-allergic mothers until 6 months of age and high breast milk sTLR2 exhibited increased susceptibility to allergic diseases. However, this increased susceptibility was not observed in partially breastfed infants.

Conclusion: This study demonstrates that the levels of sTLR2 in milk are associated with maternal polymorphisms in *TLR2* and allergic status. Elevated sTLR2 in breast milk may associate with increased allergic incidences in infants if breastfed exclusively for a prolonged period. Further work is needed to identify the mechanisms by which sTLR2 in milk may influence infant allergic development.

4.2. Introduction

Breast milk is a dynamic fluid that fulfils the newborn child's need for nutrients and immune components essential for normal development and protection against environmental and infectious agents [416]. Numerous immune modulators (IMs) in breast milk are significant contributors to host defence for infants until their immune systems mature [354,417]. Breast milk IMs include innate and adaptive immune factors, often derived from the mother's immune cells, including cytokines, immunoglobulins, growth factors, and soluble receptors [354]. After birth, the infant immune system's maturation is influenced by nutritional, environmental, and genetic factors, which can have a long-term impact [418,419]. With breast milk or alternatives being the primary source of nutrients during that window in life, it is reasonable to believe that susceptibility to immune-mediated diseases, such as allergy, might be affected by IMs in milk.

Although, for many reasons, breast milk is recommended by World Health Organization (WHO) as the best exclusive source of nutrients for infants until the age of six months [253], its protective effect against allergic disease is still controversial [254,420]. Some have reported that breastfeeding is protective against allergy development in infants [255–261]. In contrast, others warned that there is an increased risk for allergy in breastfed infants, most notably by atopic mothers [262–265]. Such controversy could be attributed to the wide variation of breast milk components influenced by several maternal factors, including genetics, age, diet, health, environment, and lactation stage [354,355]. One of the linked factors to breast milk IM profiles is the allergic status of the mother [287]. However, the impact of allergy-associated changes in breast milk mediators on the infant predisposition to allergic diseases has not been well defined.

Toll-like receptor 2 (TLR2), is an innate immune pattern recognition receptor expressed in the mammary gland [389] and multiple immune cells [68,72]. It regulates the secretion of several pro and anti-inflammatory, immune factors [421], some of which are observed in breast milk. A feature of TLR2 is that it can be found in a soluble form produced due to extracellular domain shedding. This sTLR2 is observed in several body fluids, including breast milk [67,68] where it acts as a decoy receptor with immunomodulatory functions [73]. The level of sTLR2 is reported to be elevated in patients with inflammatory conditions, including HIV-1 infections [72], cardiovascular diseases [422], and IBD [423], when compared to healthy individuals. SNPs in the *TLR2* gene have been associated with susceptibility to non-communicable immune-mediated disorders, such as asthma [77–79], atopic dermatitis [80–82], IBD, and type 1 diabetes [78].

Although sTLR2 in breast milk has been investigated in the context of protection against infant inflammatory responses and HIV infection [72,331], it has not previously been studied in the context of allergy. In this work, we aim to understand the maternal factors that influence the expression of milk sTLR2 and other IMs using samples from the CHILD cohort study and association with infant allergy.

4.3. Results

Three subsets of the CHILD cohort were studied in this research (**Table 4-1**). In the first subset of 2422 dyads, polymorphisms in *TLR2* gene were identified. In the second, allergic disease enriched, a subset of 299 dyads, milk samples collected from non-allergic and allergic mothers was analyzed to determine the levels of several immune factors, including sTLR2. Allergic mothers were defined as having at least one diagnosed allergic disease prenatally including, food allergy, asthma, skin allergy (e.g. atopic dermatitis or hives), or hay fever. The third group of 262 dyads, is composed of the mothers that were both genotyped and their milk samples analyzed (**Figure 4-1**).

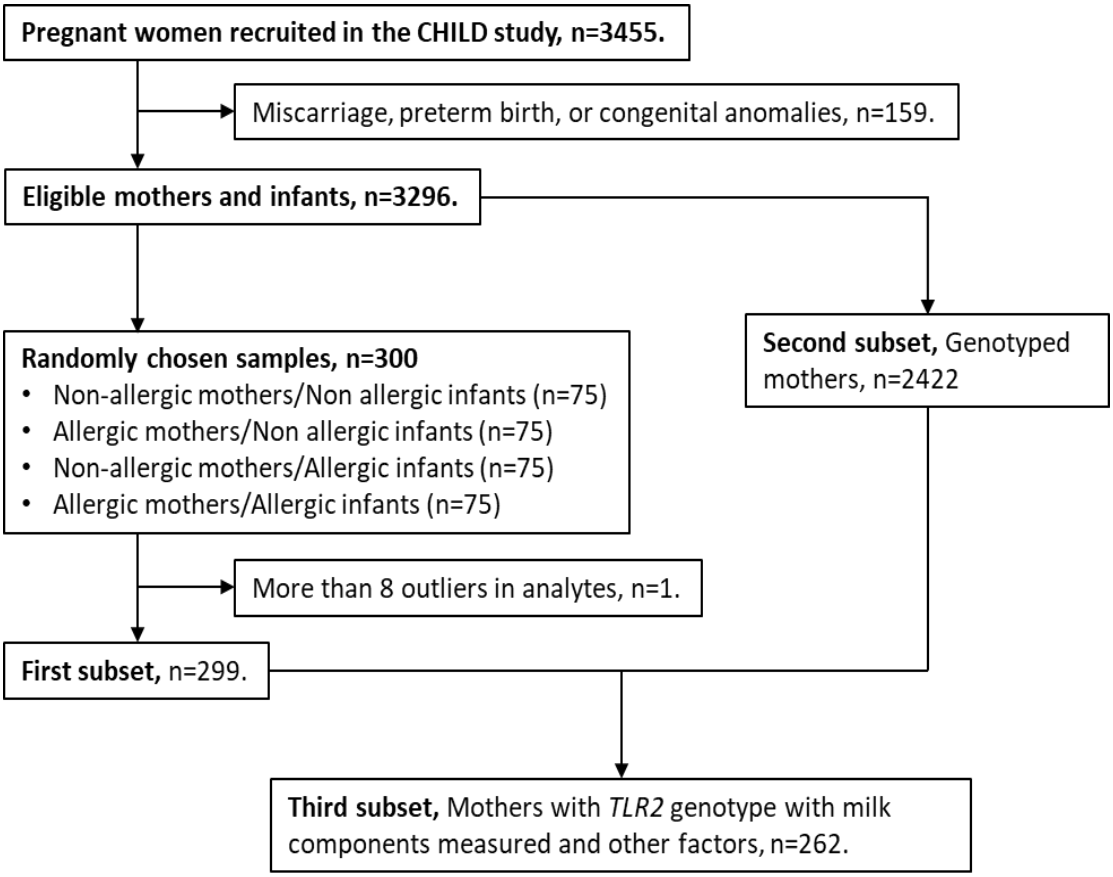


Figure 4-1: Flowchart of the study participants

Table 4-1: Characteristics of the subsets of mothers and infants taken from the CHILD cohort

Maternal characteristics	Dyads with SNPs data n = 2422	Dyads with IM n=299	Dyads with IM and SNPs n=262
Age, y	32.59 (24.2 - 40.1)	32.58 (25.7 - 40.5)	32.5 (25.6 - 40.7)
Parity, n (%)			
1	911 (38.0)	113 (38.5)	99 (38.5)
2	745 (31.1)	94 (32.0)	82 (31.9)
≥3	736 (30.7)	86 (29.3)	76 (29.5)
Completed post-secondary education, n (%)	1831 (76.8)	237 (80.8)	205 (79.7)
Marital status, married, n (%)	2276 (94.9)	284 (95.6)	248 (95.3)
Ethnicity, n (%)			
Asian	365 (15.1)	83 (27.7)	72 (27.4)
Caucasian	1798 (74.4)	176 (58.8)	153 (58.4)
First Nations	95 (3.9)	10 (3.3)	9 (3.4)
Other	157 (6.5)	30 (10.0)	28 (10.6)
Study site, n (%)			
Edmonton	536 (22.1)	106 (35.4)	93 (35.5)
Toronto	582 (24.0)	56 (18.7)	47 (17.9)
Vancouver	541 (22.3)	82 (27.4)	73 (27.8)
Manitoba	763 (31.5)	55 (18.3)	49 (18.7)
Having Cat	583 (24.5)	73 (25.3)	65 (25.6)
Having Dog	725 (30.5)	69 (23.9)	60 (23.7)
Prenatal smoking, n (%)	202 (8.4)	15 (5.02)	14 (5.3)
Pre-pregnancy BMI, kg/m2	23.19 (18.9 - 35.9)	23.54 (19.1 - 35.2)	23.44 (19.1 - 35.2)
Underweight, n (%)	71 (3.1)	7 (2.3)	7 (2.7)
Normal weight, n (%)	1377 (60.5)	174 (59.1)	153 (59.0)
Overweight, n (%)	497 (21.8)	61 (20.7)	55 (21.2)
Obese, n (%)	327 (14.3)	51 (17.3)	43 (16.6)
Weight gain during pregnancy (lb)	31 (6 - 14)	30 (12.4 - 55)	30 (13.2 - 53.3)
Maternal health conditions, n (%)			
Food allergy prenatal	340 (14.1)	38 (12.7)	35 (13.3)
Asthma	495 (20.6)	58 (19.4)	50 (19.0)
Hay Fever	858 (35.65)	89 (29.77)	76 (29.01)
Skin allergy	773 (32.13)	92 (30.77)	82 (31.3)
Atopy	1582 (65.7)	169 (56.5)	145 (55.3)
Birth and infant characteristics			
Cesarean delivery, n (%)	598 (2)	74 (25.0)	58 (22.3)
Sex, male, n (%)	1273 (52.5)	164 (54.8)	143 (54.5)
Birth weight, g	3436 (2660 - 4265.2)	3428 (2710 - 4326)	3438 (2710 - 4323.8)
Breastfeeding characteristics at sample collection			
Lactation time, wk	15.07 (11.4 - 28.1)	15.43 (12.1 - 26.9)	15.43 (12.1 - 26.2)
Breastfeeding duration, months	11 (0.5 - 24)	12 (5 - 24)	12 (5 - 24)
Exclusive breastfeeding 3m, n (%)	1455 (60.75)	204 (68.2)	185 (70.6)
Exclusive breastfeeding 6m, n (%)	431 (18.4)	60 (20.2)	54 (20.7)

Maternal characteristics	Dyads with SNPs data n = 2422	Dyads with IM n=299	Dyads with IM and SNPs n=262
Season of milk sampling, n (%)			
Winter	579 (23.9)	81 (27.1)	71 (27.1)
Spring	650 (26.9)	80 (26.8)	71 (27.1)
Summer	602 (24.9)	75 (25.1)	64 (24.4)
Fall	586 (24.2)	62 (20.8)	56 (21.3)
Feeding			
Bfs	1105 (48.92)	160 (54.79)	147 (56.76)
BFs	667 (29.53)	75 (25.68)	62 (23.94)
BfS	207 (9.16)	32 (10.96)	30 (11.58)
BFS	280 (12.39)	25 (8.56)	20 (7.72)
Breastfeeding at six months			
Exclusive	431 (18.48)	60 (20.27)	54 (20.69)
Partial	1372 (58.83)	214 (72.3)	186 (71.26)
No	529 (22.68)	22 (7.43)	21 (8.05)
TLR2 SNPs			
rs56346547	WT 927 (38.29) Het 1140 (47.09) Hom 354 (14.62)	Same as the third group	WT 114 (43.51) Het 115 (43.89) Hom 33 (12.6)
rs4696480	WT 633 (26.15) Het 1180 (48.74) Hom 608 (25.11)	=	WT 68 (25.95) Het 115 (43.89) Hom 79 (30.15)
rs10222800	WT 1060 (43.78) Het 1076 (44.44) Hom 285 (11.77)	=	WT 121 (46.18) Het 112 (42.75) Hom 29 (11.07)
rs1898831	WT 1878 (77.57) Het 491 (20.28) Hom 52 (2.15)	=	WT 208 (79.39) Het 50 (19.08) Hom 4 (1.53)
rs1898832	WT 1626 (67.16) Het 710 (29.33) Hom 85 (3.51)	=	WT 159 (60.69) Het 91 (34.73) Hom 12 (4.58)
rs13123230	WT 908 (37.51) Het 1151 (47.54) Hom 362 (14.95)	=	WT 108 (41.22) Het 107 (40.84) Hom 47 (17.94)
rs1439164	WT 1719 (71) Het 617 (25.49) Hom 85 (3.51)	=	WT 197 (75.19) Het 55 (20.99) Hom 10 (3.82)
rs62323856	WT 1672 (69.06) Het 659 (27.22) Hom 90 (3.72)	=	WT 191 (72.9) Het 67 (25.57) Hom 4 (1.53)
rs3804099	WT 797 (32.92) Het 1187 (49.03) Hom 437 (18.05)	=	WT 91 (34.73) Het 130 (49.62) Hom 41 (15.65)
rs3804100	WT 1990 (82.2) Het 397 (16.4) Hom 34 (1.4)	=	WT 205 (78.24) Het 50 (19.08) Hom 7 (2.67)

Values are n (%) for categorical variables, medians (5%-95% range). Maternal atopy includes hay fever, skin allergies, or aeroallergen, drug, insect, food, pet, or other allergies at milk collection. Maternal pre-pregnancy BMI (in kg/m2) was calculated from measured height and self-reported pre-pregnancy weight, and it was classified as underweight (<18.5), average weight (18.5 to <25.0), overweight (25.0 to <30.0), and obese (≥30.0).

IM: Immunomodulators. **Bfs**: Breastmilk only. **BFs**: Breastmilk & baby formula. **BfS**: Breastmilk & solid food. **BFS**: Breastmilk & baby formula & solid food.

4.3.1. Human milk IMs ranges and correlations

Out of the 39 milk IMs analyzed in our research, 28 of them were within the ranges of detection for our assays, with the IgA the most abundant (median ~600 µg/ml) and IL-6 the least (median of ~ 4.9 pg/ml) (**Table 4-2**).

Regarding sTLR2, the range was between 5-339 ng/ml (median of ~ 68 ng/ml), which makes it one of the most variable milk IM between mothers in our study (**Table 4-2**). Levels of TGF-β1, CX3CL1, CCL22, CCL5, TGF-β2, and CD14, most of which are derived from monocytes and macrophages, were highly positively correlated with sTLR2 (correlation coefficients = 0.57, 0.5, 0.46, 0.43, 0.39, and 0.37, respectively) (**Figure 4-2**). This correlation is consistent with reports that monocytes are the primary source of sTLR2 in milk, in addition to mammary epithelial cells [68,72]. The levels of sTLR2 in human milk were 2.4 to 3.4 times higher than the levels of bovine sTLR2 detected in commercial baby formulas and 2-2.8 times higher than the levels detected in skimmed or homogenized cow's milk (**Figure 4-3**).

IgE, which is a key functional mediator of allergic diseases, is detectable in human milk (median of ~207 pg/ml), with a range (53.84-49196.69 pg/ml) and exhibited a trend towards being elevated in allergic mothers ($P = 0.051$) (**Table 4-2**) (**Figure 4-4**). The levels of sTLR2 and IgE in milk were not correlated in healthy or allergic mothers (**Figure 4-2B, C**). The top IMs positively correlated with IgE levels in milk were IgG4 and IgG2 (correlation coefficients = 0.4 and 0.37, respectively) (**Figure 4-2A**). In contrast, IgG3 was significantly lower in the milk of allergic mothers ($P = 0.012$) with a range (200.17-6258.57 ng/ml) in our cohort (**Table 4-2**) (**Figure 4-4B**).

Table 4-2: Concentrations and range of IM in breast milk in the CHILD cohort study.

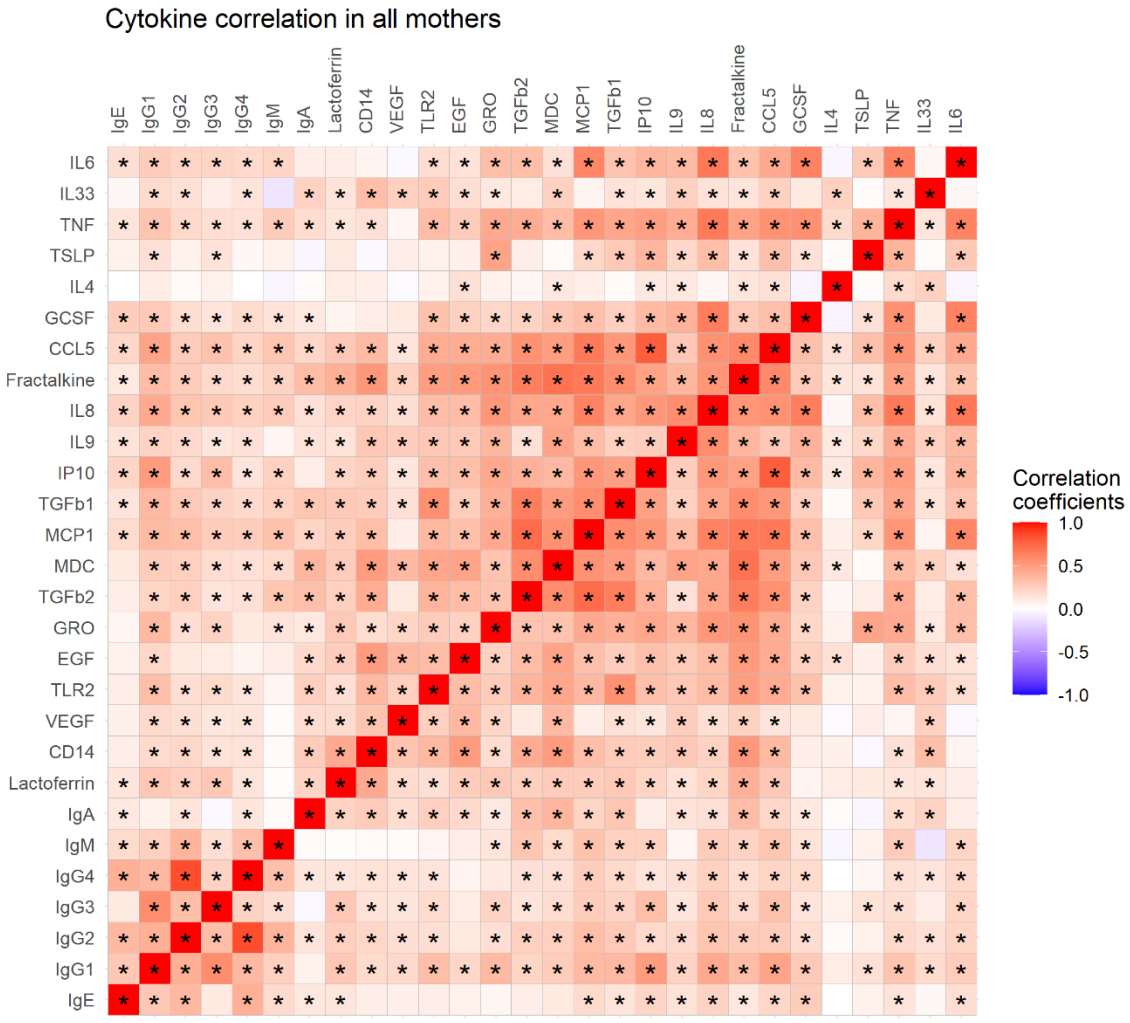
	Healthy Mothers Median (Range)	Allergic Mothers Median (Range)	Fold change	Unit	P-Value
IgA	614.56 (463.37-3100.4)	595.42 (173.54-2346.1)	17.9	µg/ml	0.641
Lactoferrin	308.8 (248.82-1645.13)	306.82 (82.38-1047.5)	20	=	0.959
IgM	17.74 (10.48-144.26)	17.91 (2.26-116.48)	63.8	=	0.997
IgG1	9.36 (6.41-90.81)	9.14 (1.82-46.15)	49.9	=	0.328
CD14	6.57 (5.52-23.1)	6.49 (2.16-17.39)	10.7	=	0.366
IgG2	3.3 (2.65-33.6)	3.22 (0.95-21.56)	35.4	=	0.466
IgG3	817.96 (584.67-6258.57)	717.77 (200.17-3753.58)	31.3	ng/ml	0.012 *
IgG4	588.53 (411.94-8887.33)	574.04 (136.06-7204.65)	65.3	=	0.865
VEGF	73.93 (61.68-271.54)	73.1 (30.19-295.83)	9.8	=	0.654
TLR2	66.44 (49.49-286.59)	68.71 (5-339.03)	1614.4	=	0.317
EGF	39.27 (33.39-115.88)	39.99 (19.1-91.13)	6.1	=	0.788
CXCL1 (GRO)	2.04 (1.43-8.7)	2.04 (0.52-9.03)	17.4	=	0.423
TGF-β2	1.93 (1.22-78.75)	2 (0.24-38.96)	328.1	=	0.548
CCL22 (MDC)	1.71 (1.37-7.6)	1.7 (0.5-20.9)	41.8	=	0.692
CCL2 (MCP-1)	1 (0.63-34.04)	1.04 (0.14-25.82)	243.1	=	0.925
TGF-β1	571.92 (421.84-3428.72)	586.01 (9.26-2263.42)	370.3	pg/ml	0.403
CXCL10 (IP-10)	331.1 (159.83-4599.84)	347.41 (71.47-25305.42)	354.1	=	0.47
IL-9	321.76 (265.39-1077.47)	323 (127.74-1396.42)	10.9	=	0.95
CXCL8 (IL-8)	315.06 (224.16-3979.47)	312.95 (80.41-7622.17)	94.8	=	0.787
IgE	196.65 (146.34-2803.3)	212.74 (53.84-49196.69)	913.8	=	0.051 ~
CX3CL1 (Fractalkine)	169.12 (110.86-536.62)	171.2 (40.65-953.97)	23.5	=	0.605
CCL5	57.21 (40.6-356.1)	59 (21.57-515.44)	23.9	=	0.619
G-CSF	43.59 (38.14-268.63)	43.98 (26.25-1842.02)	70.2	=	0.114
IL-4	35.85 (28.68-198.79)	35.85 (4.37-83.19)	45.5	=	0.9
TSLP	26.69 (17.71-161.98)	27.92 (0.51-1386.51)	2718.7	=	0.996
IL-33	6.36 (5.17-116.15)	6.36 (0.13-307.11)	2362.4	=	0.53
TNF	6.5 (5.34-28.55)	6.32 (1.47-76.68)	52.2	=	0.848
IL-6	4.91 (4.04-116.76)	4.91 (1.54-132.84)	86.3	=	0.991

The median concentration of IM in milk from non-allergic and allergic mothers sorted based on their abundance. The range is defined by (minimum to maximum) values detected.
GRO: Growth-regulated oncogene. **MDC**: Macrophage-derived chemokine. **MCP-1**: monocyte chemoattractant protein-1. **IP-10**: Interferon gamma-induced protein 10.
* $P < 0.05$. ~ $P < 0.1$

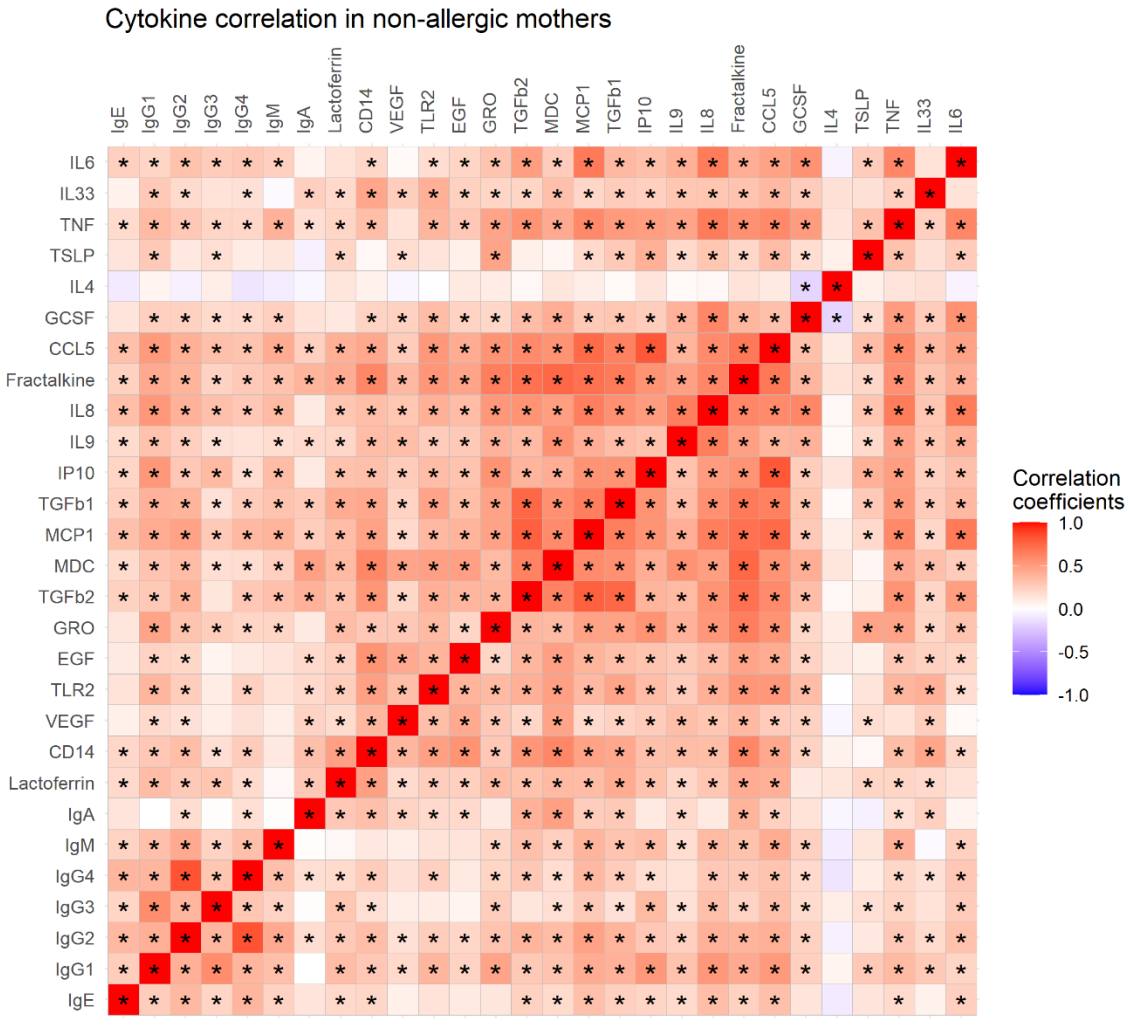
Figure 4-2: Correlation matrix of human milk IMs

Correlation coefficients of log-transformed concentrations of human milk IM in (**A**) all of the mothers (n = 299), (**B**) non-allergic mothers (n = 150), (**C**) allergic mothers (n = 149), and (**D**) food-allergic mothers (n = 38). Pearson's correlation coefficients represented as a colour gradient, with * indicating a significant correlation when *P*-value < 0.05.

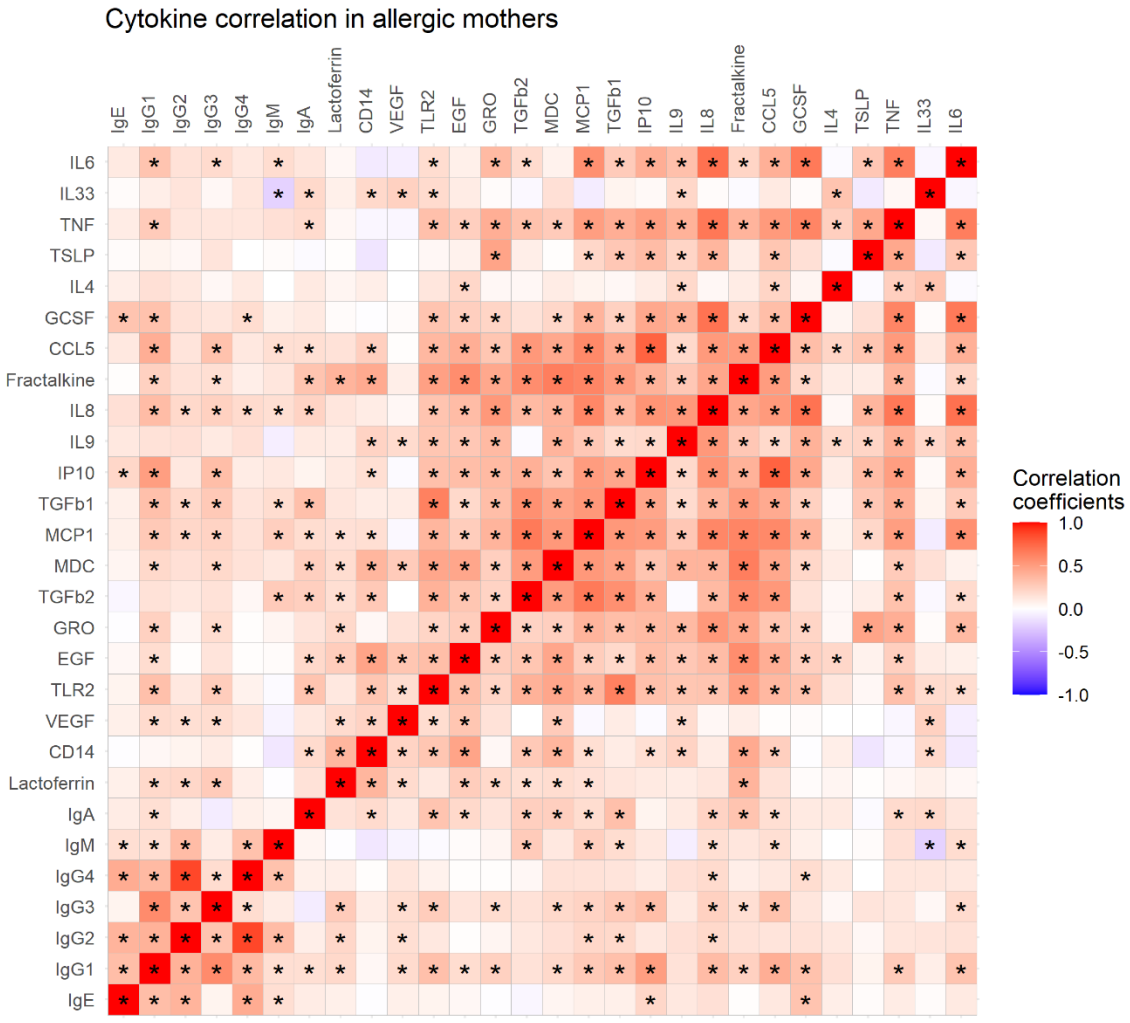
A


Cytokine correlation in all mothers
IgE
IgG1
IgG2
IgG3
IgG4
IgM
IgA
Lactoferrin
CD14
VEGF
TLR2
EGF
GRO
TGFb2
MDC
MCP1
TGFb1
IP10
IL9
IL8
Fractalkine
CCL5
GCSF
IL4
TSLP
TNF
IL33
IL6
Correlation coefficients
1.0
0.5
0.0
-0.5
-1.0

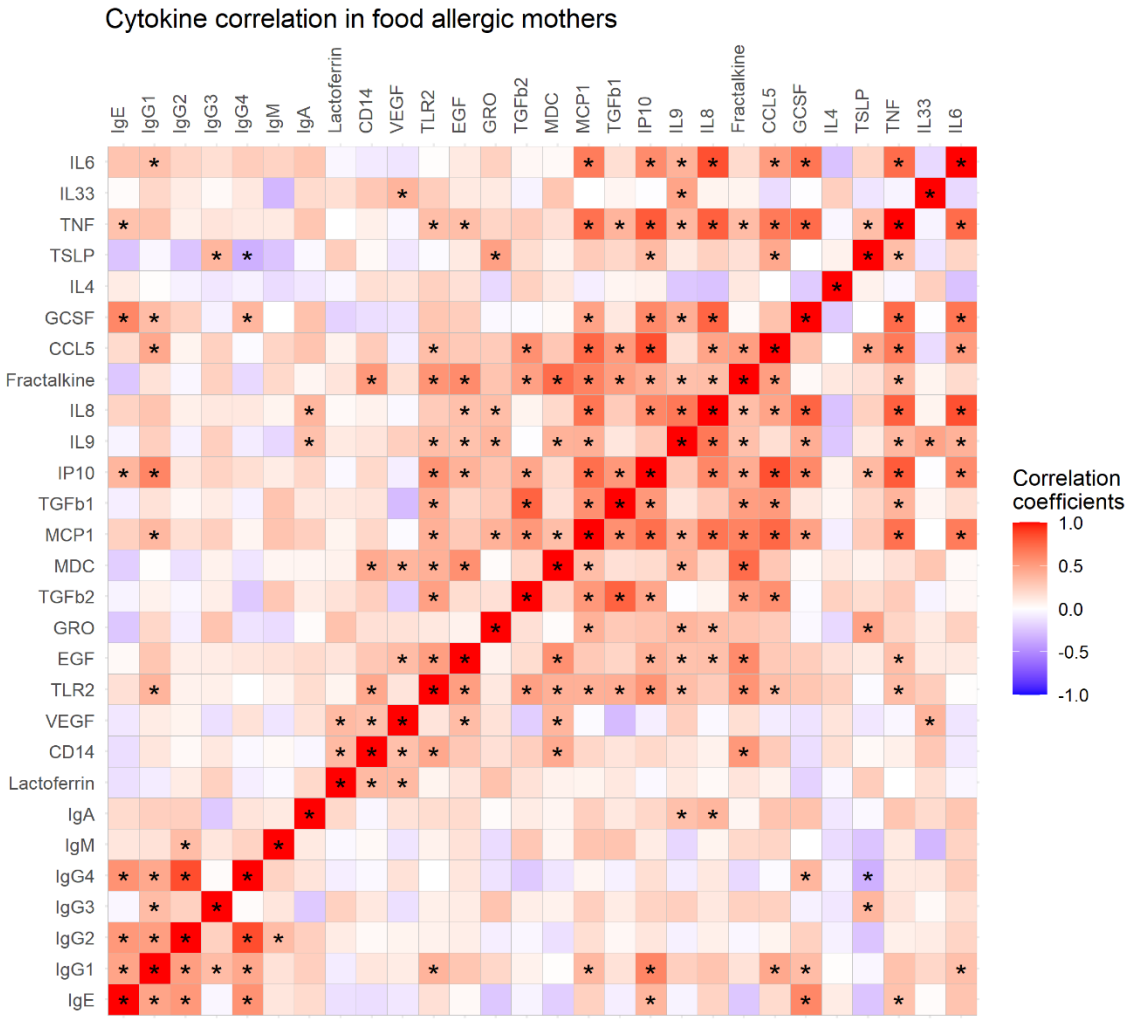
B


Cytokine correlation in non-allergic mothers
IgE
IgG1
IgG2
IgG3
IgG4
IgM
IgA
Lactoferrin
CD14
VEGF
TLR2
EGF
GRO
TGFb2
MDC
MCP1
TGFb1
IP10
IL9
IL8
Fractalkine
CCL5
GCSF
IL4
TSLP
TNF
IL33
IL6
Correlation coefficients
1.0
0.5
0.0
-0.5
-1.0

C


Cytokine correlation in allergic mothers
IgE
IgG1
IgG2
IgG3
IgG4
IgM
IgA
Lactoferrin
CD14
VEGF
TLR2
EGF
GRO
TGFb2
MDC
MCP1
TGFb1
IP10
IL9
IL8
Fractalkine
CCL5
GCSF
IL4
TSLP
TNF
IL33
IL6
Correlation coefficients
1.0
0.5
0.0
-0.5
-1.0

D


Cytokine correlation in food allergic mothers
IgE
IgG1
IgG2
IgG3
IgG4
IgM
IgA
Lactoferrin
CD14
VEGF
TLR2
EGF
GRO
TGFb2
MDC
MCP1
TGFb1
IP10
IL9
IL8
Fractalkine
CCL5
GCSF
IL4
TSLP
TNF
IL33
IL6
Correlation coefficients
1.0
0.5
0.0
-0.5
-1.0

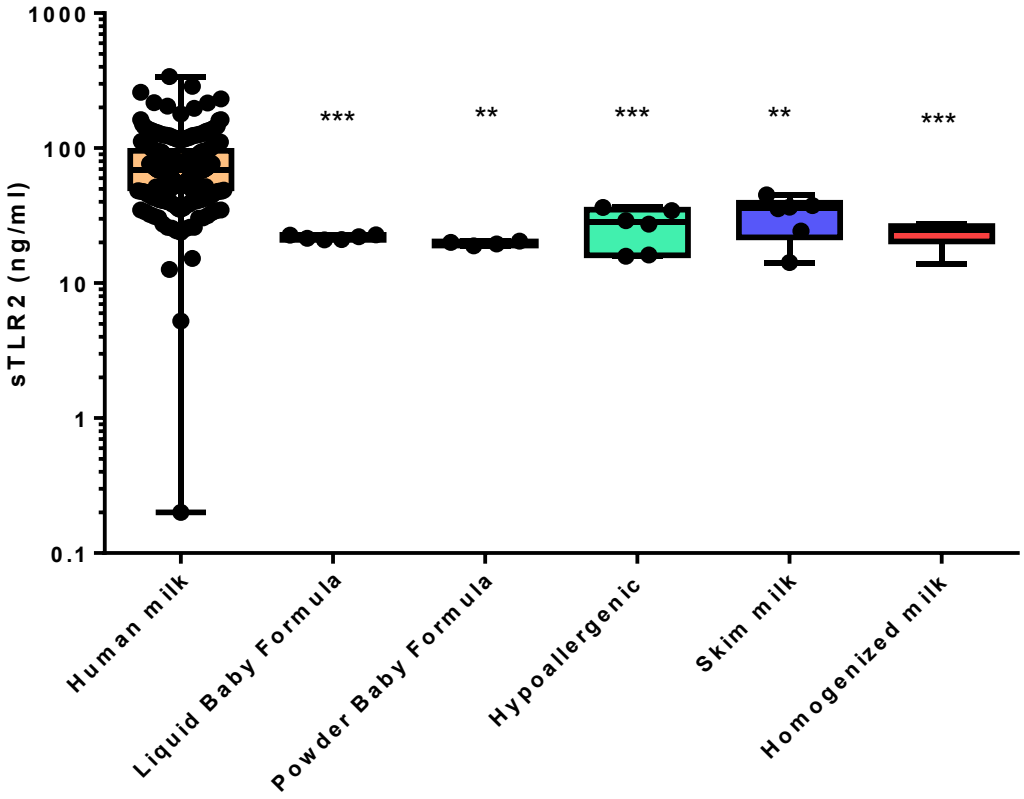
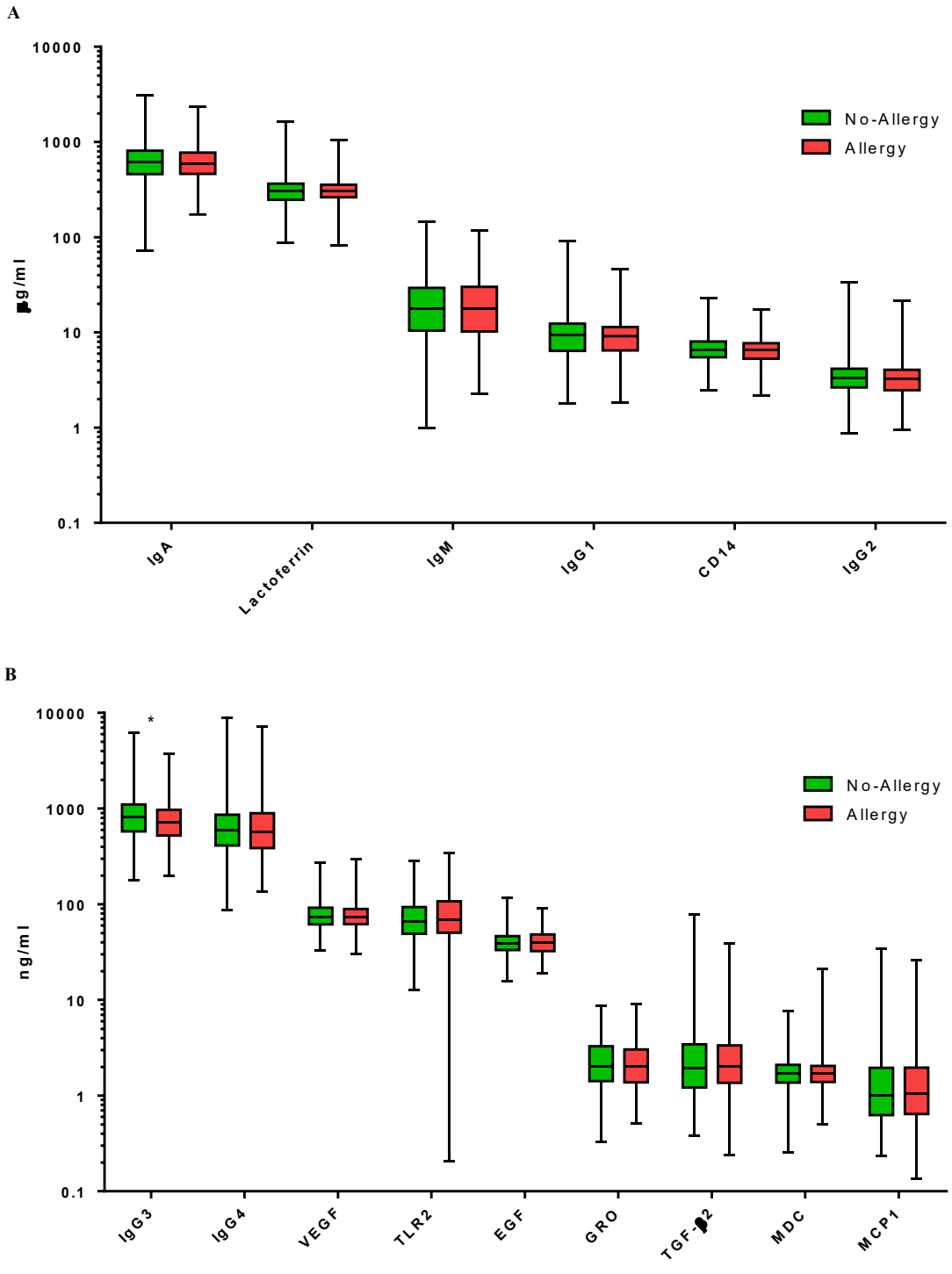


Figure 4-3: sTLR2 concentrations in human breast milk, cow's milk and baby formulas

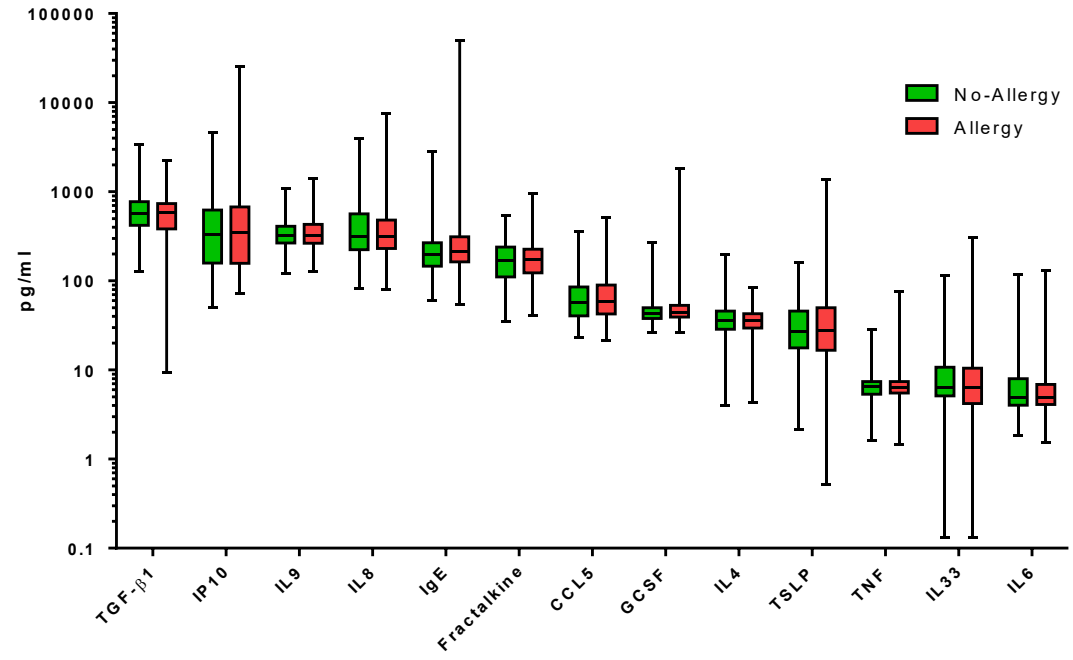
The level of sTLR2 was measured by ELISA in Human milk (n=299), liquid baby formula (n=6), reconstituted powder baby formula (n=4), hypoallergenic baby formula (n=6), skimmed cow's milk reconstituted from powder (n=6), and homogenized cow's milk (n=6). Commercial baby formulas and cow's milk were measured from 2-3 manufacturers for each type. Statistical analysis was performed by a nonparametric test (Kruskal-Wallis test) to compare all other groups to human milk. Each box is defined by 25% and 75% quartiles, with the median indicated; whiskers represent the range. $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

Figure 4-4 The concentrations of milk IM

Milk IM measured in μg/ml (A), ng/ml (B), and pg/ml (C). Each box is defined by 25% and 75% quartiles, with the median indicated; whiskers represent the range. Statistical analysis was performed by a nonparametric test (Mann-Whitney test). * P <0.05.



C


100000
10000
1000
100
10
1
0.1
pg/ml
No-Allergy
Allergy
TGF-β1
IP10
IL9
IL8
IgE
Fractalkine
CCL5
GCSF
IL4
TSLP
TNF
IL33
IL6

4.3.2. The maternal determinants of human milk sTLR2 and other IMs

The impact of maternal factors (allergic diseases, age, BMI, ethnicity, parity), environmental factors (Site, smoking, education, pets, seasons of milk collection), and infant factors (sex, birth weight, and feeding type) on milk sTLR2 and other IM was investigated. Interestingly, sTLR2 was shown to be increased in the milk of mothers diagnosed with a food allergy prenatally in the unadjusted analysis ($P = 0.031$) and but did not reach satistical significance in the FDR adjusted multiple comparisons ($P = 0.153$)along with IgE and IL-9 (**Table 4-3**). Levels of sTLR2 were not elevated in other types of allergies, such as atopic dermatitis, which was positively associated with food allergies ($P < 0.001$) (**Figure 4-5A),** indicating that a potential increase in milk sTLR2 was selective for the food allergy group.

IgE in milk was also increased in mothers diagnosed with a food allergy, asthma, and hay fever prenatally in the raw and adjusted multiple comparisons (**Figure 4-5A, B)**. In contrast, milk IgG3 was significantly decreased in mothers with skin allergies, atopy, and hay fever (**Figure 4-5A, B)**.

Although sTLR2 has shown not to be affected by the mother's ethnicity, other IMs were associated with certain races (**Figure 4-5A, B)**. Notably, the mothers of Asian ethnicity had an array of increased IMs in their milk compared with Caucasian women, including the immunoglobulins (IgE, IgG1, IgG2, IgG3, IgG4, and IgM) as well as other IMs (**Figure 4-5A, B),** supporting the impact of genetics on milk components. When mothers were considered a single cohort, sTLR2 levels in breast milk were not correlated with the mother's age, BMI, city of residence, smoking, or education. They were also not

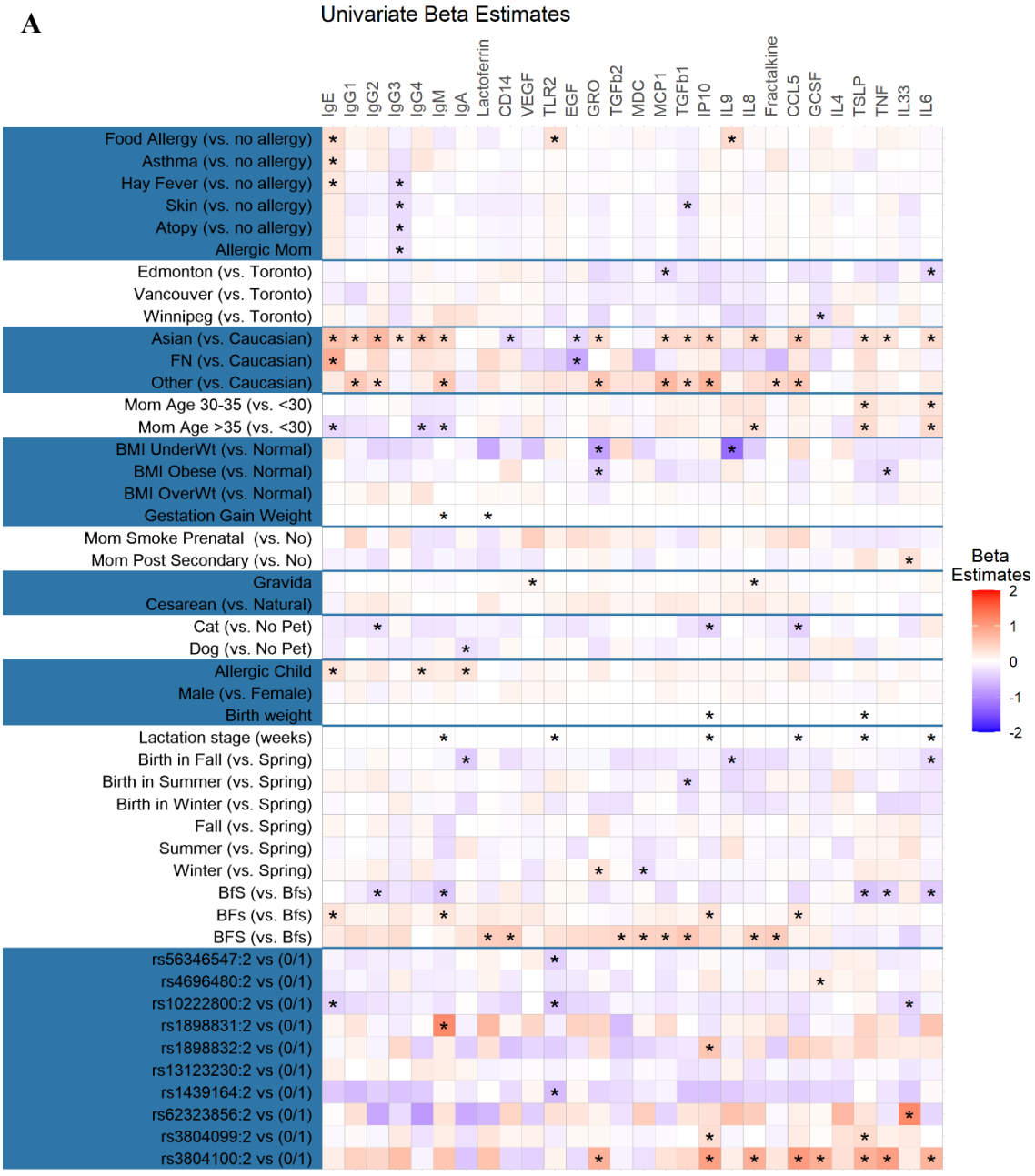
influenced by the infant sex, lactation stage, and feeding style, contrasting with some other breast milk IMs.

Table 4-3: The association between maternal allergy and sTLR2 in milk.

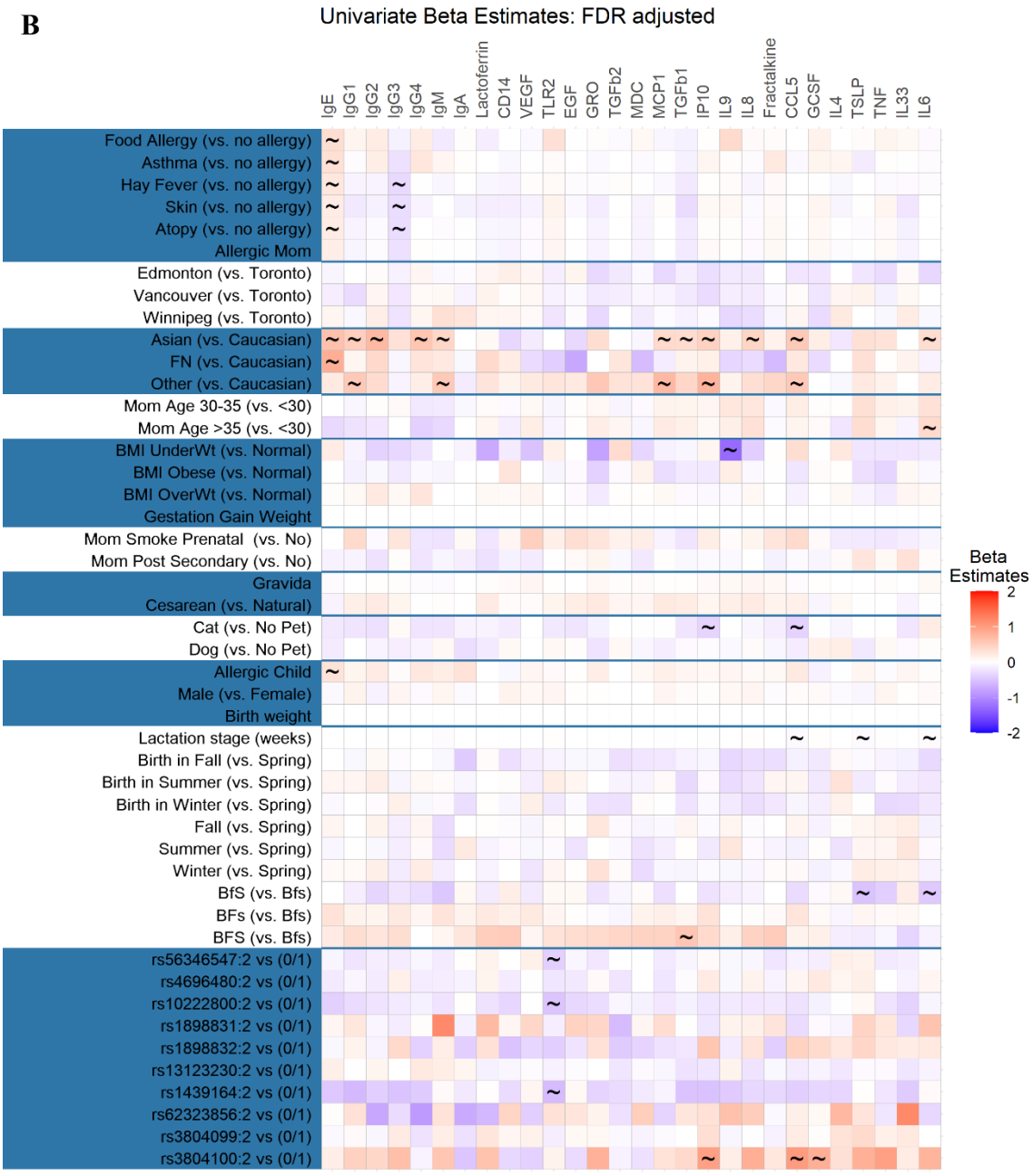
	Beta estimate	P-Value	Adjusted P-Value
Food Allergy (vs. no allergy)	0.319	0.032 (*)	0.153
Asthma (vs. no allergy)	0.045	0.737	0.737
Hay Fever (vs. no allergy)	0.06	0.58	0.726
Skin (vs. no allergy)	0.126	0.261	0.595
Atopy (vs. no allergy)	0.092	0.357	0.595

Figure 4-5: Association between milk IM and individual factors
Heat map that represents raw **(A)** and adjusted **(B)** association of human milk IM with individual maternal, infants, and environmental factors (n=299). The colors represent the B estimates from a linear regression model for each IM. $*P < 0.05$. $\sim P < 0.05$.

A


Univariate Beta Estimates
IgE
IgG1
IgG2
IgG3
IgG4
IgM
IgA
Lactoferrin
CD14
VEGF
TLR2
EGF
GRO
TGFb2
MDC
MCP1
TGFb1
IP10
IL9
IL8
Fractalkine
CCL5
GCSF
IL4
TSLP
TNF
IL33
IL6
Food Allergy (vs. no allergy)
Asthma (vs. no allergy)
Hay Fever (vs. no allergy)
Skin (vs. no allergy)
Atopy (vs. no allergy)
Allergic Mom
Edmonton (vs. Toronto)
Vancouver (vs. Toronto)
Winnipeg (vs. Toronto)
Asian (vs. Caucasian)
FN (vs. Caucasian)
Other (vs. Caucasian)
Mom Age 30-35 (vs. <30)
Mom Age >35 (vs. <30)
BMI UnderWt (vs. Normal)
BMI Obese (vs. Normal)
BMI OverWt (vs. Normal)
Gestation Gain Weight
Mom Smoke Prenatal (vs. No)
Mom Post Secondary (vs. No)
Gravida
Cesarean (vs. Natural)
Cat (vs. No Pet)
Dog (vs. No Pet)
Allergic Child
Male (vs. Female)
Birth weight
Lactation stage (weeks)
Birth in Fall (vs. Spring)
Birth in Summer (vs. Spring)
Birth in Winter (vs. Spring)
Fall (vs. Spring)
Summer (vs. Spring)
Winter (vs. Spring)
BfS (vs. Bfs)
BFs (vs. Bfs)
BFS (vs. Bfs)
rs56346547:2 vs (0/1)
rs4696480:2 vs (0/1)
rs10222800:2 vs (0/1)
rs1898831:2 vs (0/1)
rs1898832:2 vs (0/1)
rs13123230:2 vs (0/1)
rs1439164:2 vs (0/1)
rs62323856:2 vs (0/1)
rs3804099:2 vs (0/1)
rs3804100:2 vs (0/1)
Beta Estimates
2
1
0
-1
-2

B


Univariate Beta Estimates: FDR adjusted
IgE
IgG1
IgG2
IgG3
IgG4
IgM
IgA
Lactoferrin
CD14
VEGF
TLR2
EGF
GRO
TGFb2
MDC
MCP1
TGFb1
IP10
IL9
IL8
Fractalkine
CCL5
GCSF
IL4
TSLP
TNF
IL33
IL6
Food Allergy (vs. no allergy)
Asthma (vs. no allergy)
Hay Fever (vs. no allergy)
Skin (vs. no allergy)
Atopy (vs. no allergy)
Allergic Mom
Edmonton (vs. Toronto)
Vancouver (vs. Toronto)
Winnipeg (vs. Toronto)
Asian (vs. Caucasian)
FN (vs. Caucasian)
Other (vs. Caucasian)
Mom Age 30-35 (vs. <30)
Mom Age >35 (vs. <30)
BMI UnderWt (vs. Normal)
BMI Obese (vs. Normal)
BMI OverWt (vs. Normal)
Gestation Gain Weight
Mom Smoke Prenatal (vs. No)
Mom Post Secondary (vs. No)
Gravida
Cesarean (vs. Natural)
Cat (vs. No Pet)
Dog (vs. No Pet)
Allergic Child
Male (vs. Female)
Birth weight
Lactation stage (weeks)
Birth in Fall (vs. Spring)
Birth in Summer (vs. Spring)
Birth in Winter (vs. Spring)
Fall (vs. Spring)
Summer (vs. Spring)
Winter (vs. Spring)
BfS (vs. Bfs)
BFs (vs. Bfs)
BFS (vs. Bfs)
rs56346547:2 vs (0/1)
rs4696480:2 vs (0/1)
rs10222800:2 vs (0/1)
rs1898831:2 vs (0/1)
rs1898832:2 vs (0/1)
rs13123230:2 vs (0/1)
rs1439164:2 vs (0/1)
rs62323856:2 vs (0/1)
rs3804099:2 vs (0/1)
rs3804100:2 vs (0/1)
Beta Estimates
2
1
0
-1
-2

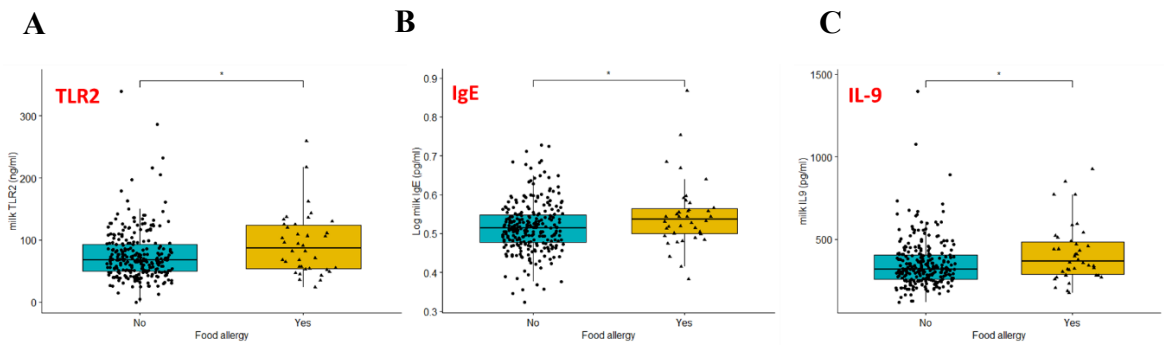


Figure 4-6: Mothers with food allergies have significantly higher levels of sTLR2 and IgE in breast milk

The level of sTLR2 in breast milk was significantly higher in mothers diagnosed with food allergies prenatally (n=38) compared to mothers with no allergies (n= 149) (A). The levels of IgE (B) and IL-9 (C) were also increased in the milk of food allergic mothers (B). Statistical analysis was performed by a nonparametric test (Kruskal-Wallis test). Each box is defined by 25% and 75% quartiles, with the median inside; whiskers represent the low and the high values. *P ≤ 0.05.

4.3.3. Polymorphisms in *TLR2* are associated with altered levels of sTLR2 in breast milk

Given the wide variation of maternal sTLR2 in breast milk between mothers, their regulation at the genetic level was investigated; first, by examining the role of polymorphisms in *TLR2* gene on the levels of sTLR2 protein and other IMs in milk. Sequence information was available from 262 mothers out of 299 that were studied for their milk components. Mothers that were heterozygous for recognized *TLR2* polymorphisms exhibited no significant differences in levels of sTLR2 compared to the WT mothers, but rather higher in certain SNPs (**Figure 4-7**A), so these groups were combined for further analysis. Three non-coding SNPs, rs56346547, rs10222800, and rs1439164, were significantly associated with decreased levels in sTLR2 in homozygous mothers compared to the combined group of WT and heterozygous mothers (**Figure 4-7**B). However, the frequency of homozygous mothers with rs1439164 SNP was low in our selected cohort, with only 3.8% observed (**Table 4-1**) (**Table 4-4**).

The rs10222800 SNP was associated with decreased IgE and IL-33 in the milk in the raw analysis only (**Figure 4-5**). Analysis of milk sTLR2 by Western blot demonstrated that mothers with rs56346547 and rs10222800 SNPs had a similar distribution of isoforms of sTLR2 in their milk as the WT and the heterozygous mothers (**Figure 4-8**). Although rs10222800 was associated with decreased IgE levels in breast milk, this SNP was not associated with altered susceptibility to allergic disease in the larger CHILD cohort analyzed (n = 2422) (**Figure 4-9**). However, rs13123230 and rs1439164 were shown to associate with a lower incidence of asthma and hay fever, respectively, in the non-adjusted

analysis (**Figure 4-9**). The association between *TLR2* SNPs and mothers' milk IM changes is summarized in (**Figure 4-5**).

Table 4-4: The levels of milk sTLR2 in mothers with *TLR2* polymorphisms in the CHILD cohort

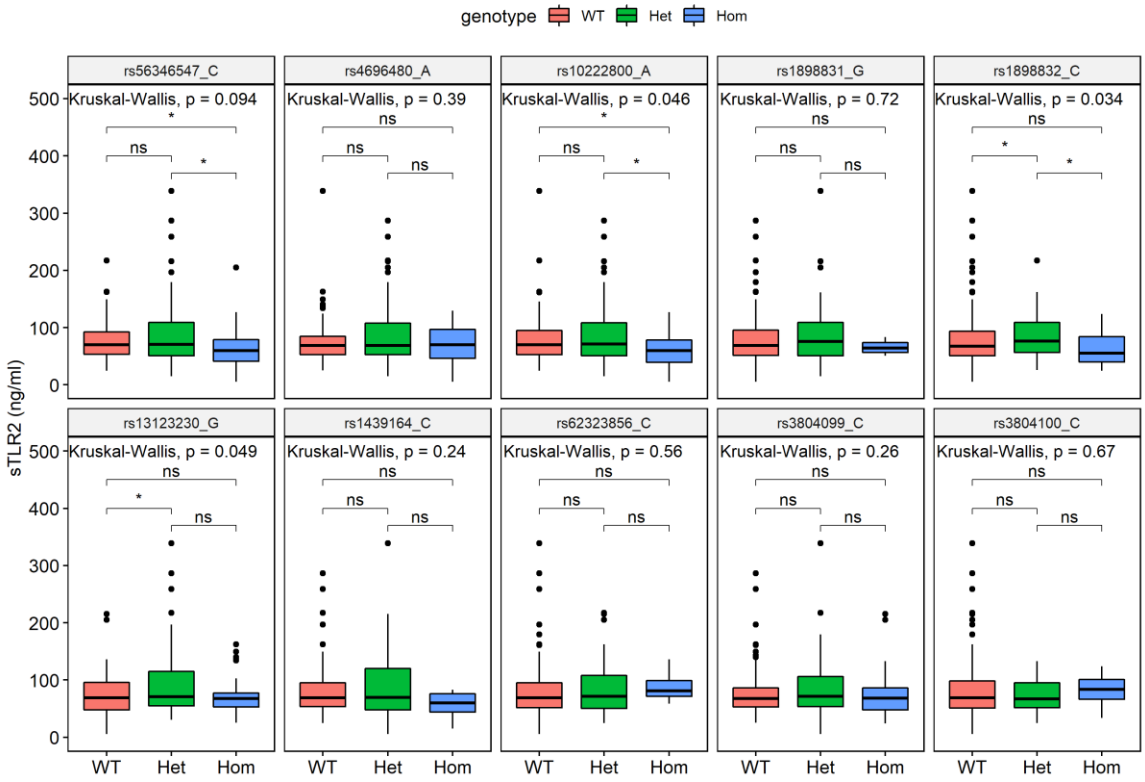
Alleles		WT		Het		Hom	
rs56346547	**A>C**	69.71 (53.27-92.38)	(114)	70.65 (50.75-109.7)	(115)	59.7 (41.31-80.34)	(33)
rs4696480	**T>A**	68.27 (51.61-84.94)	(68)	68.92 (51.27-109)	(115)	70.24 (44.99-97.08)	(79)
rs10222800	**G>A**	68.85 (52.1-85.09)	(59)	70.91 (51.2-108.4)	(174)	59.7 (39.04-79.79)	(29)
rs1898831	**G>A**	68.78 (51.52-95.81)	(208)	73.78 (50.07-109.2)	(50)	64.51 (52.83-79.99)	(4)
rs1898832	**T>C**	67.6 (50.43-93.75)	(159)	76.61 (56.16-109.7)	(91)	54.97 (35.73-85.29)	(12)
rs13123230	**A>G**	69.02 (47.68-95.86)	(108)	69.86 (53.58-115.6)	(107)	67.75 (52.1-77.14)	(47)
rs1439164	**C>T**	68.92 (52.62-95.2)	(197)	69.33 (47.63-121.1)	(55)	60.24 (36.99-78.89)	(10)
rs62323856	**T>C**	77.98 (50.98-112.2)	(35)	71.46 (48.22-108.5)	(67)	81.17 (62.75-123.7)	(4)
rs3804099	**T>C**	67.75 (52.1-86.43)	(91)	71.55 (51.35-106)	(130)	68.15 (46.31-86.19)	(41)
rs3804100	**T>C**	68.92 (50.87-98.63)	(205)	66.69 (50.74-95.44)	(50)	83.3 (61.78-116.3)	(7)

The values represent the median of sTLR2 levels (25%-75% percentiles) and (number of mothers) according to the *TLR2* polymorphisms in the CHILD cohort.

Figure 4-7: Polymorphisms in the TLR2 gene are associated with altered levels of sTLR2

(A) The levels of sTLR2 in mothers with zero (WT), one (Het), or two (Hom) alleles of the ten most common TLR2 SNPs in the CHILD cohort. Statistical analysis was performed by a nonparametric test (Kruskal-Wallis test). **(B)** The levels of sTLR2 in WT/Het mothers combined compared to Hom mothers. Two SNPs, rs56346547 and rs10222800, were associated with a significant reduction in human milk sTLR2 in homozygous mothers compared to WT and heterozygous. Statistical analysis was performed by a nonparametric approach (Mann-Whitney test). Each box is defined by 25% and 75% quartiles, with the median inside; whiskers represent the low and the high values. $*P \leq 0.05$. WT: wild-type. Het: heterozygous for SNP. Hom: Homozygous for SNP.

A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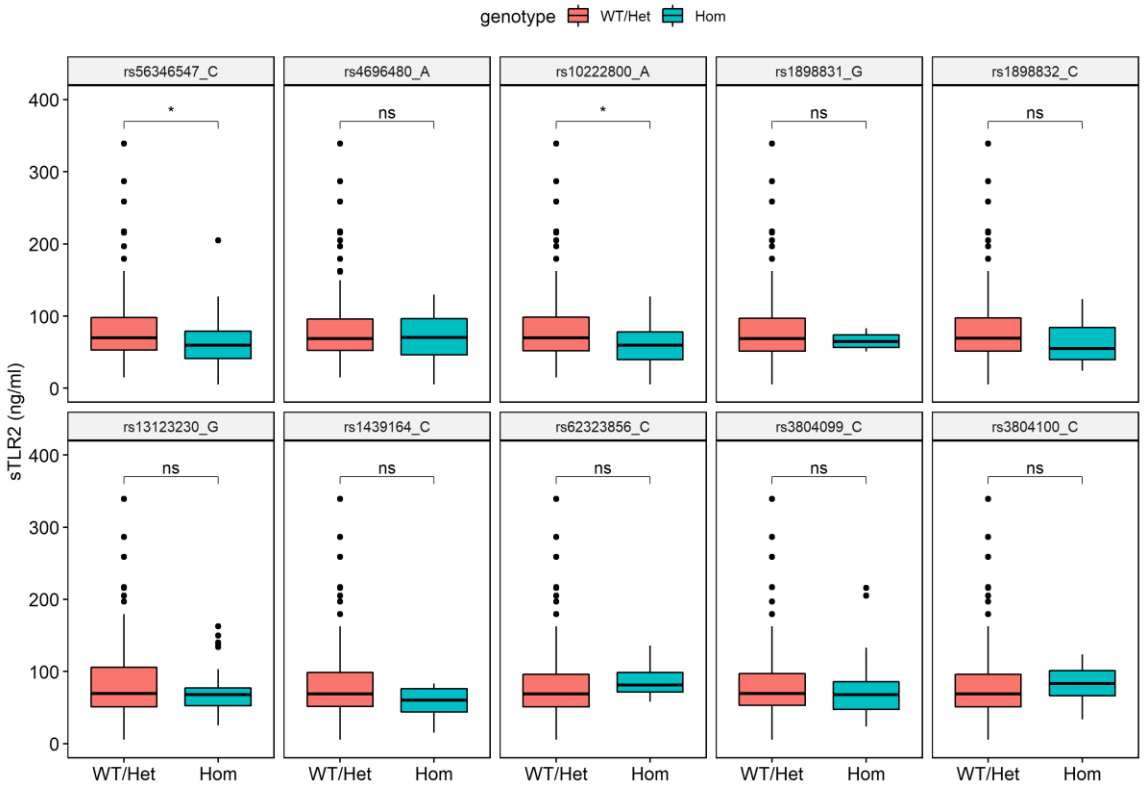
rs56346547_C
Kruskal-Wallis, p = 0.094
rs4696480_A
Kruskal-Wallis, p = 0.39
rs10222800_A
Kruskal-Wallis, p = 0.046
rs1898831_G
Kruskal-Wallis, p = 0.72
rs1898832_C
Kruskal-Wallis, p = 0.034
rs13123230_G
Kruskal-Wallis, p = 0.049
rs1439164_C
Kruskal-Wallis, p = 0.24
rs62323856_C
Kruskal-Wallis, p = 0.56
rs3804099_C
Kruskal-Wallis, p = 0.26
rs3804100_C
Kruskal-Wallis, p = 0.67
sTLR2 (ng/ml)
0
100
200
300
400
500
ns
*
WT
Het
Hom

B


genotype WT/Het Hom
rs56346547_C
rs4696480_A
rs10222800_A
rs1898831_G
rs1898832_C
rs13123230_G
rs1439164_C
rs62323856_C
rs3804099_C
rs3804100_C
sTLR2 (ng/ml)
0
100
200
300
400
ns
*
WT/Het
Hom

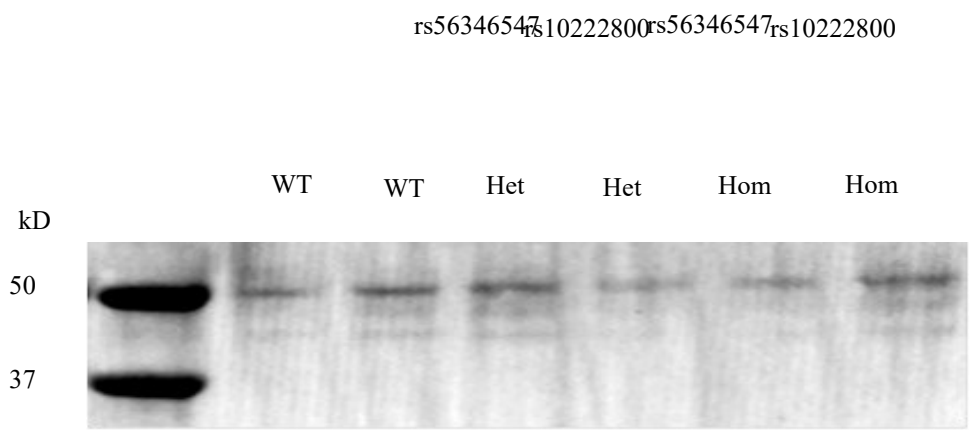


Figure 4-8: The isoforms of sTLR2, detected in the breast milk of mothers with or without *TLR2* SNPs

Milk samples were normalized based on sTLR2 levels to investigate the qualitative difference in the sTLR2 variants due to the genetic variation in mothers. Breast milk was tested from mothers harbouring no, one, or two alleles of either rs56346547 or rs10222800 that were shown to be associated with decreased levels of sTLR2.

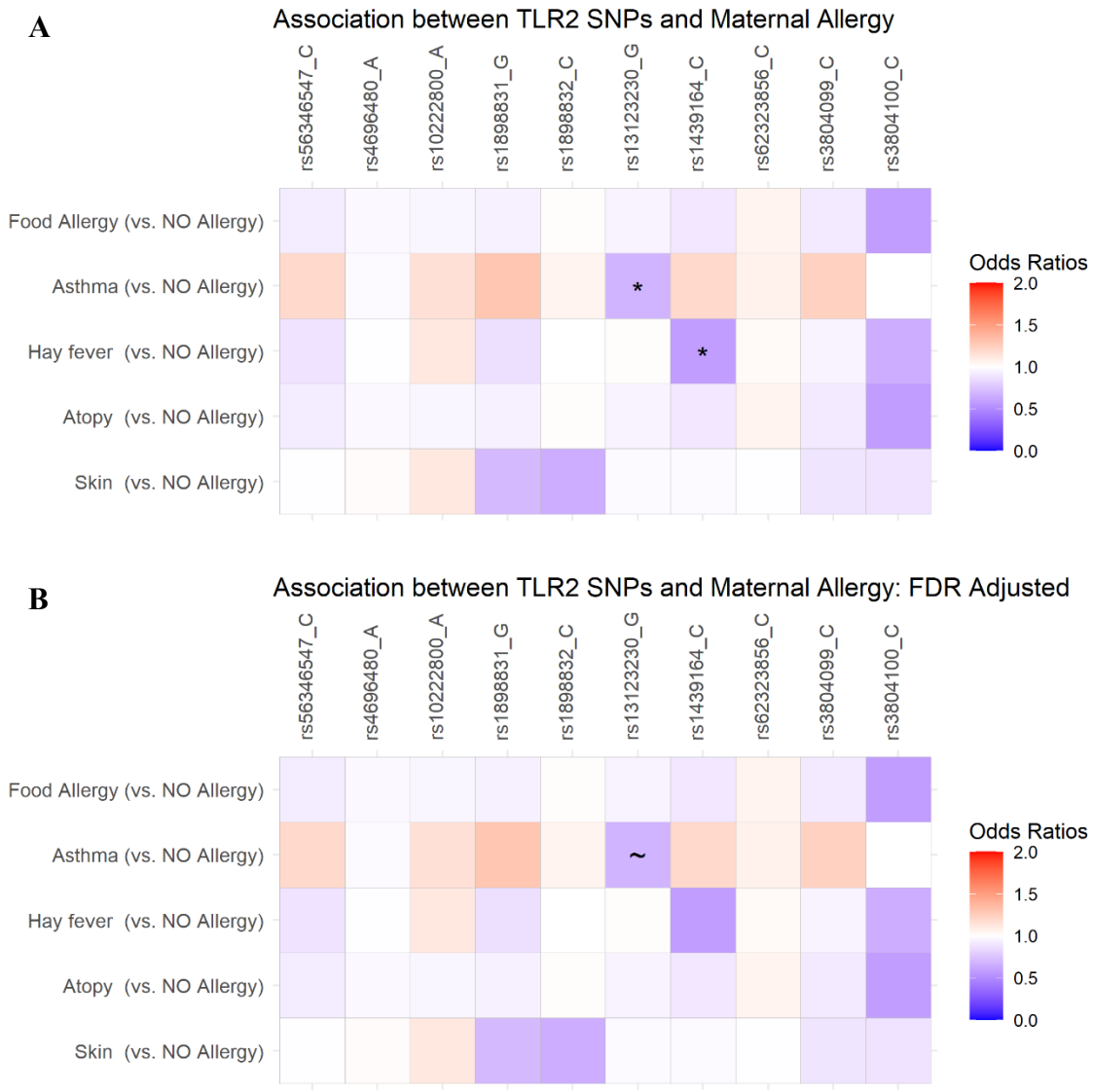


Figure 4-9: Association between TLR2 SNPs and maternal allergy
Heat map that represents the raw association of *TLR2* SNPs with allergic diseases. Colour coding represents the frequency of increases (red) or decreases (blue) in incidence compared to the control. Significance was calculated based on Chi-square analysis. *$P < 0.05$. ~ $P < 0.1$

4.3.4. Breast milk IMs and infant allergy.

Consistent with reports that maternal allergy impacts the development of infant allergy [424,425], we found, in the population-based cohort, that mothers diagnosed with a food allergy, asthma, hay fever, or skin allergy had an association with increased rates of infants' allergies at the ages of 1 and 3 years in both the raw and adjusted analyses (**Figure 4-10A, B**). Different maternal factors can be linked to this observation pre or postnatally; however, we focused on breast milk generally and sTLR2 specifically in our study.

Within the milk-analysed cohort, we found that some IM, such as IgE, were positively associated with increased rates of allergic diseases in infants in the raw and adjusted analyses (**Figure 4-11A, B**). In contrast, other IM, such as lactoferrin and CXCL8, were associated with a protective effect against food allergies at the age of three. These findings indicate that breast milk may have some risk or beneficial factors that impact allergy susceptibility in infants (**Figure 4-11A, B**). However, breast milk sTLR2 level was not shown to influence infant allergy development in this analysis category (**Table 4-5**).

Some mothers introduce alternatives to breast milk to their infants during the lactation period, such as baby formulas or solid foods, that might modify infants' milk IM effects. Therefore, to eliminate the impact of breast milk alternatives and the potential factors that allergic mothers might have transported to infants prenatally, we sought to study the effect of milk IM from non-allergic mothers in exclusively breastfed infants for at least six months of age. Interestingly, the incidences of asthma, atopic dermatitis, and atopy at the age of three were positively associated with increased levels of sTLR2 in non-allergic mothers' milk in both the raw and adjusted analyses (**Figure 4-11C, D**) (**Table 4-5**). This effect of sTLR2 was not observed in partially breastfed infants at a period of six

months (**Figure 4-11A, B**) (**Table 4-5**). But instead, sCD14, which acts as a co-receptor for sTLR2 in breast milk [68], has shown to be associated with a protective effect against infant atopy at one year of age (**Figure 4-12A, B**).

Table 4-5: The association between sTLR2 in milk and infant allergy.

	Beta estimate	P-Value	Adjusted P-Value
sTLR2 in Milk and Allergy in all Infants n=299			
Atopy 1y (vs. Healthy)	-0.014	0.895	0.903
Atopy 3y (vs. Healthy)	0.131	0.188	0.438
AD 1y (vs. Healthy)	0.088	0.44	0.617
AD 3y (vs. Healthy)	0.185	0.137	0.438
Food allergy 3y (vs. Healthy)	0.017	0.903	0.903
Rhinitis 3y (vs. Healthy)	0.172	0.357	0.617
Asthma 3y (vs. Healthy)	0.238	0.173	0.438
sTLR2 in Milk and Allergy in Exclusively Breastfed Infants for 6m by Healthy Moms			
Atopy 1y (vs. Healthy)	0.761	0.066 (~)	0.115
Atopy 3y (vs. Healthy)	0.968	0.007 (*)	0.024 (*)
AD 1y (vs. Healthy)	0.698	0.13	0.182
AD 3y (vs. Healthy)	1.136	0.006 (*)	0.024 (*)
Food allergy 3y (vs. Healthy)	0.504	0.428	0.428
Rhinitis 3y (vs. Healthy)	0.554	0.267	0.311
Asthma 3y (vs. Healthy)	1.547	0.041 (*)	0.096 (~)
sTLR2 in Milk and Allergy in Partially Breastfed Infants for 6m by Healthy Moms			
Atopy 1y (vs. Healthy)	-0.308	0.139	0.848
Atopy 3y (vs. Healthy)	0.062	0.754	0.848
AD 1y (vs. Healthy)	-0.086	0.727	0.848
AD 3y (vs. Healthy)	-0.141	0.616	0.848
Food allergy 3y (vs. Healthy)	-0.221	0.461	0.848
Rhinitis 3y (vs. Healthy)	-0.078	0.848	0.848
Asthma 3y (vs. Healthy)	0.291	0.439	0.848

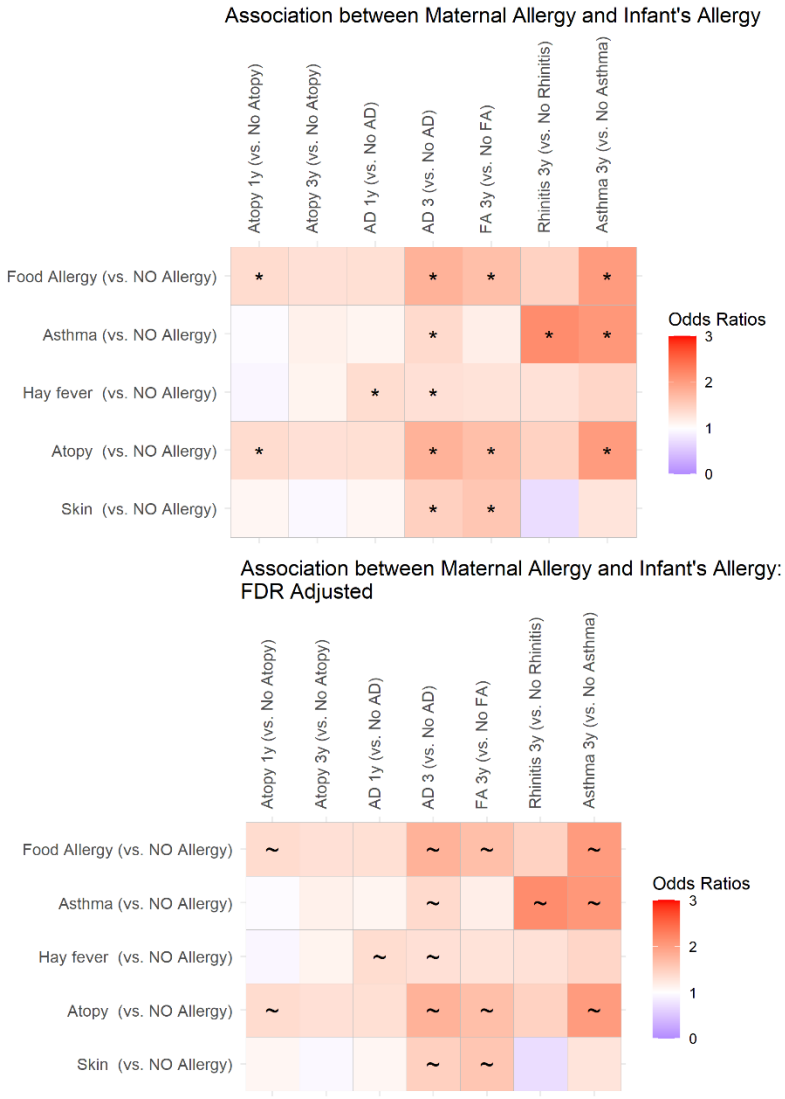
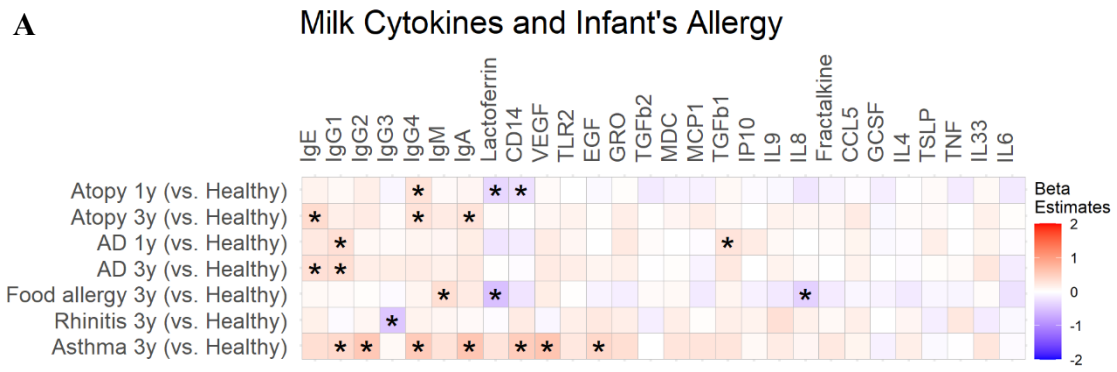


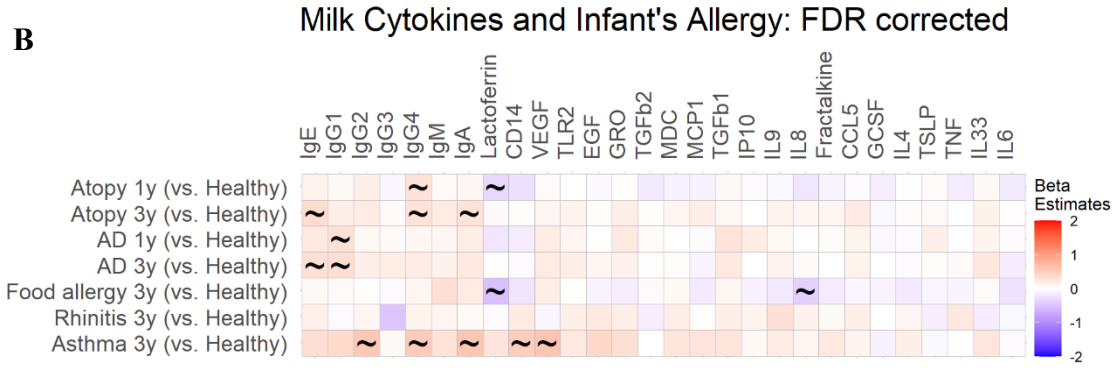
Figure 4-10: Association between maternal and infant allergy in the CHILD cohort
Heat maps represent the association between maternal allergy and infant allergy at the ages of one year and three years of age in the CHILD cohort study with raw (**A**) and FDR corrected (B) analyses. $*P < 0.05$. $\sim P < 0.1$.

Figure 4-11: Association between milk IMs and infant allergy with exclusive breastfeeding
Heat maps represent milk IM's association with allergic diseases in infants nursed by all mothers in the CHILD cohort study in raw (**A**) and FDR corrected (B) analyses. Healthy mothers who breastfed exclusively for six months in raw (**C**) and FDR corrected (**D**). The colours represent the B estimates from a linear regression model for each IM. $*P < 0.05$. $\sim P < 0.1$.

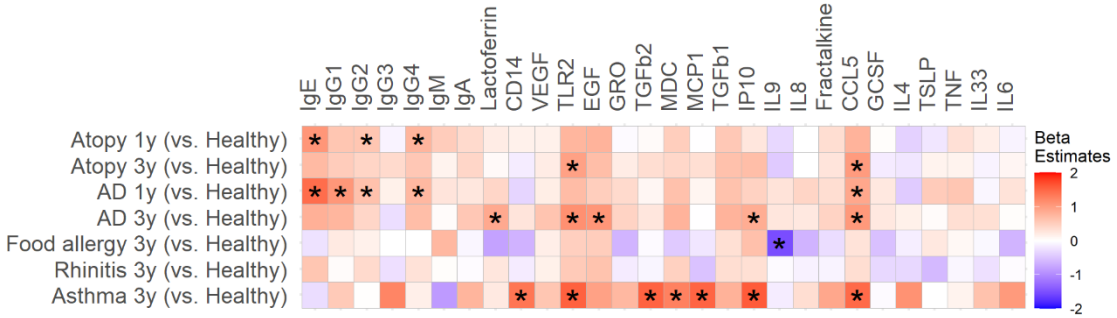
A


Milk Cytokines and Infant's Allergy
IgE IgG1 IgG2 IgG3 IgG4 IgM IgA Lactoferrin CD14 VEGF TLR2 EGF GRO TGFb2 MDC MCP1 TGFb1 IP10 IL9 IL8 Fractalkine CCL5 GCSF IL4 TSLP TNF IL33 IL6
Atopy 1y (vs. Healthy)
Atopy 3y (vs. Healthy)
AD 1y (vs. Healthy)
AD 3y (vs. Healthy)
Food allergy 3y (vs. Healthy)
Rhinitis 3y (vs. Healthy)
Asthma 3y (vs. Healthy)
Beta Estimates
2
1
0
-1
-2

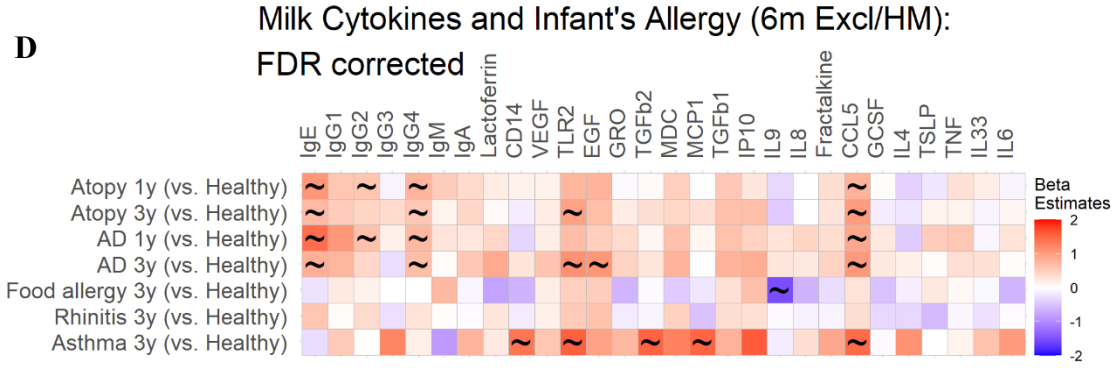
B


Milk Cytokines and Infant's Allergy: FDR corrected
IgE IgG1 IgG2 IgG3 IgG4 IgM IgA Lactoferrin CD14 VEGF TLR2 EGF GRO TGFb2 MDC MCP1 TGFb1 IP10 IL9 IL8 Fractalkine CCL5 GCSF IL4 TSLP TNF IL33 IL6
Atopy 1y (vs. Healthy)
Atopy 3y (vs. Healthy)
AD 1y (vs. Healthy)
AD 3y (vs. Healthy)
Food allergy 3y (vs. Healthy)
Rhinitis 3y (vs. Healthy)
Asthma 3y (vs. Healthy)
Beta Estimates
2
1
0
-1
-2

C


Milk IMs and Infant's Allergy (6m Excl by Healthy Mom)
IgE IgG1 IgG2 IgG3 IgG4 IgM IgA Lactoferrin CD14 VEGF TLR2 EGF GRO TGFb2 MDC MCP1 TGFb1 IP10 IL9 IL8 Fractalkine CCL5 GCSF IL4 TSLP TNF IL33 IL6
Atopy 1y (vs. Healthy)
Atopy 3y (vs. Healthy)
AD 1y (vs. Healthy)
AD 3y (vs. Healthy)
Food allergy 3y (vs. Healthy)
Rhinitis 3y (vs. Healthy)
Asthma 3y (vs. Healthy)
Beta Estimates
2
1
0
-1
-2

D


Milk Cytokines and Infant's Allergy (6m Excl/HM):
FDR corrected
IgE IgG1 IgG2 IgG3 IgG4 IgM IgA Lactoferrin CD14 VEGF TLR2 EGF GRO TGFb2 MDC MCP1 TGFb1 IP10 IL9 IL8 Fractalkine CCL5 GCSF IL4 TSLP TNF IL33 IL6
Atopy 1y (vs. Healthy)
Atopy 3y (vs. Healthy)
AD 1y (vs. Healthy)
AD 3y (vs. Healthy)
Food allergy 3y (vs. Healthy)
Rhinitis 3y (vs. Healthy)
Asthma 3y (vs. Healthy)
Beta Estimates
2
1
0
-1
-2

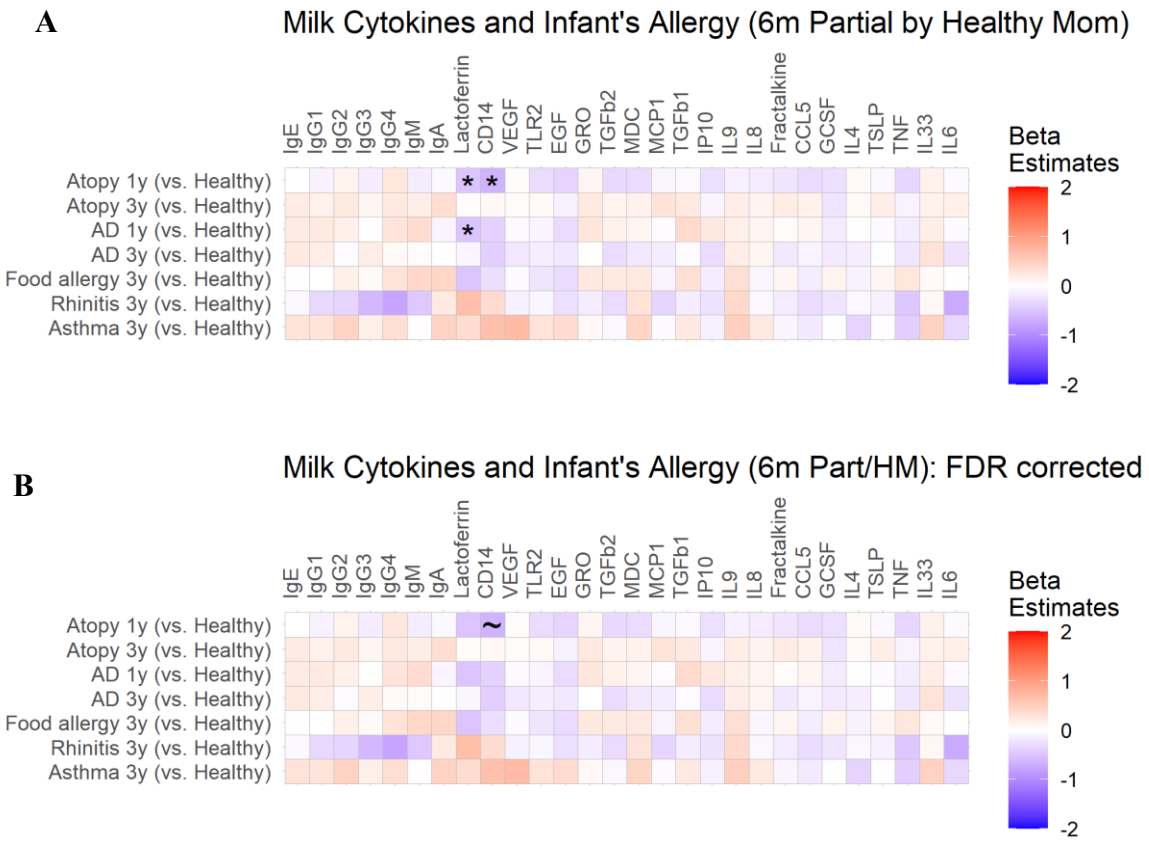


Figure 4-12: Association between milk IMs and infant allergy with partial breastfeeding

Heat maps represent the association of milk IM with allergic diseases in infants partially breastfed at six months of age in healthy and allergic mothers (**A**) or healthy mothers only (**B**). The colours represent the B estimates from a linear regression model for each IM. $*P < 0.05$. $\sim P < 0.1$.

4.4. Discussion

It has been previously observed that maternal TLR2 impacts oral tolerance development in mice via a milk-dependent mechanism [426]; therefore, we sought to investigate its role in humans. In our animal model studies (See 3.3.4), we found that dams deficient in TLR2 had different milk IMs than WT dams marked by a decrease in IL-6, IGF-1, IL-13, and IFN-γ, and associated with attenuated oral tolerance in nursed pups. We successfully detected and quantified sTLR2 and other IMs impacted by maternal and environmental factors in human milk.

Several reports have suggested that maternal atopic status can influence breast milk composition [307,427,428], and a significant role for TLR2 in predisposition and susceptibility to different allergic diseases has been described [77–82,429]. Our study cohort was enriched with allergic mothers and allergic infants to assess better the impact of maternal allergic status on milk components and susceptibility of infants to allergy. Using milk samples collected from participants in the CHILD study, we selected four groups of dyads out of the population-based cohort (**Figure 4-1**). Milk samples were randomly distributed on ELISA and Luminex plates during the analysis, which was completed with investigators blinded to the dyad groups.

In addition to sTLR2, we analyzed a wide array of IMs in breast milk and are believed to have a potential impact on the development of oral tolerance [354]. Most previous reports regarding the levels of sTLR2 in milk have been mainly qualitative and assessed by Western blot. However, Henrick *et al.* [72] reported that the average concentration of sTLR2 in healthy mothers' breast milk is slightly less than 10 ng/ml, which is seven times less than the median concentration we detected in our study. They and others have also

reported multiple isoforms of sTLR2 and its degradation products in human milk [68,72,331]. Using a novel anti-human TLR2 monoclonal capture antibody (4H7), which was developed in our laboratory, along with a commercially available detection antibody and standard, we observed a concentration of human milk sTLR2 with a range between 5 – 339 ng/ml, with a median of 67 ng/ml in all mothers. Consistent with reports concerning the source of milk-borne sTLR2 [68,72], the major breast milk IMs correlated with sTLR2 levels in our analysis were TGF-β1, CX3CL1, CCL22, CCL5, TGF-β2, and CD14, most of which are derived from monocytes and macrophages. Levels of sTLR2 in breast milk were independent of several factors, including age, obesity, having pets in the home, lactation stage, infant sex, smoking, and residence site.

Maternal allergic status was associated with sTLR2 concentrations in milk in the unadjusted analysis. Breast milk from food allergic mothers appears to have selectively higher levels of sTLR2 along with IgE. Several mechanisms might be involved in an increase of sTLR2 in milk in the context of food allergic responses, including increased activation of TLR2 [92], hyperreactivity of monocytes [430–432], or augmented shedding activity. Excess TLR2 activation can increase the shedding of the ectodomain to contain the inflammatory response [74]. Tunis *et al*. [92], showed that activation of TLR2 during OVA ingestion in mice could break oral tolerance and induce food sensitization. Therefore, TLR2-mediated food sensitization might be one of the mechanisms for increased sTLR2 levels in food-allergic mothers' milk. According to Neeland *et al*. [432], infants with persistent egg allergy have increased reactive monocytes in the circulation, capable of producing inflammatory cytokines. However, in our study, other monocyte-derived milk IMs were not altered in breast milk in a pattern related to the mother's allergic status, suggesting that

the increase of sTLR2 in food allergic mothers could be independent of monocyte activation. A report by Cooley *et al*. [433] has noted an increase in the activity of ADAM10, a primary sheddase for sTLR2, in allergic patients. In parallel, ADAM10 acts as a sheddase for sCD23, which mediates IgE augmented secretion from B cells. Increased shedding of sTLR2 in response to augmented activation of shedding enzymes might be another mechanism for the increased levels of sTLR2 in breast milk.

One of the essential factors that might impact the levels of sTLR2 in breast milk is the maternal genome; therefore, we investigated the polymorphisms in the *TLR2* gene in participating mothers. Heterozygous mothers for *TLR2* SNPs have shown comparable or higher levels of sTLR2 than WT mothers; thus, they were grouped in the analysis. Interestingly, three non-coding *TLR2* SNPs, rs56346547, rs10222800, and rs1439164 were significantly associated with lower levels of milk sTLR2 in homozygous mothers (**Figure 4-5**). In addition to sTLR2, other mediators including IgE and IL-33 were decreased in subjects with the rs10222800 SNP. These specific SNPs have not previously been reported to impact TLR2 expression or been associated with allergic traits. According to Langjahr *et al.* [74], breast milk contains multiple isoforms of sTLR2 released through post-translational modifications of TLR2 ectodomain instead of mRNA alternative splicing. We found that WT, heterozygous, and homozygous mothers with rs56346547 and rs10222800 SNPs, have a similar distribution of sTLR2 isoforms in their milk (**Figure 4-8**). Being in the non-coding regions, rs56346547, rs10222800, and rs1439164 SNPs might alter the levels of sTLR2 via impacting the regulation of expression or stability of TLR2 mRNA. Several IMs in milk have shown to be related to other *TLR2* SNPs that did not associate

with sTLR2, including rs4696480. rs1898831, rs1898832, rs62323856, rs3804099, and rs3804100 (**Figure 4-5**).

Besides altering the expression of TLR2, polymorphisms in the *TLR2* gene have been linked to susceptibility to different immune-mediated diseases, including allergy [77–82]. Using the maternal-genotyped cohort (**Figure 4-1**), we found rs13123230 and rs1439164 SNPs in the unadjusted analysis and only rs13123230 in the adjusted analysis, associated with reduced incidence of asthma and hay fever, respectively. To our knowledge, we are the first to report a link between these two SNPs and allergic diseases.

With the immature state of the infant immune system, breast milk represents the best nutritional source in early life with a vast array of IMs, nutrients, and microbiota. The role of sTLR2 in breast milk has been extensively studied as an inflammatory regulator, but little is known about its effect on oral tolerance. We wanted to use the allergic infant-enriched cohort to investigate the link between sTLR2 and other IMs and allergic diseases development in early life. Overall, there was no significant association between sTLR2 and allergic disease incidence in infants in the milk-analyzed cohort. In contrast, several immunoglobulins, including IgE and excluding IgG3, associated with increased risk of allergic diseases in infants (**Figure 4-11A, B**). These findings might help to explain the controversial role of breastfeeding in the protection against allergic diseases. In addition, we found that maternal allergy substantially associates with different types of infant allergies in the population-based cohort (**Figure 4-10A, B**). According to Wright *et al*. [434], infants breastfed by mothers with high milk IgE have increased IgE levels in their serum, which might increase their susceptibility to allergic diseases. Furthermore, as described by Msallam *et al*. [424], IgE can cross the placenta and sensitize fetal mast cells in mice and

humans, leading to transferred sensitization from allergic mothers to infants. Also, the increased incidence of allergy in infants might be genetically linked to their atopic mothers.

In addition to the allergic status of the mother, which has been shown to impact milk IM, other factors to consider when studying the effect of milk IMs on infants are the timing of liquid and solid foods introduction due to the possible manipulation of IM impact on the development of infant GIT and immune system. Therefore, we decided to check breast milk IM from non-allergic mothers who exclusively breastfed their infants for at least six months of age. Interestingly, we have seen a significant positive association between sTLR2 in milk and the incidence of infant allergies at the age of three years. This observation was absent in partially breastfed infants. However, sCD14, a co-receptor of sTLR2, was associated with a decreased atopy incidence (**Figure 4-12**).

The impact of prolonged exposure to high levels of sTLR2 and the potential link to infant allergy is worth further investigation. It is well known that proper TLR2 signalling can have a beneficial effect on oral tolerance, such as establishing intestinal integrity [87], microbial colonization [194], and expansion of Tregs [90]. Furthermore, Amoudruz *et al*. [435] have shown that maternal allergy has been associated with decreased responses in neonatal PRRs, including TLR2, to microbial stimuli. Accordingly, the excess inhibition of TLR2 signaling through prolonged exposure to high levels of sTLR2 might interfere with such pro-oral tolerance effects. Excess sTLR2 may also decrease infants' inflammatory response towards pathogens and might dampen the beneficial interaction between normal flora and the neonatal immune system, essential for tolerance development. These findings could be one of the mechanisms through which atopic mothers promote increases in their infants' allergic disease development.

4.4.1. Strength and limitations

This cohort study's strength comes from using milk samples collected from mothers of multiple ethnicities that live in different geographical locations across Canada. Analysis of milk IMs was randomized, blinded, and comprehensively assessed with several maternal covariates, including genetic, health, education, location, age, and environmental variables. Also, all infants were followed up from birth and clinically monitored for allergic disease development until the age of three in this study.

Our study's limitations include using one milk sample from each mother, representing a single time point. Also, the total number of milk samples is not big enough to provide power for more specific sub-group analysis. The limited number of subjects and the wide range of parameters examined contributed to non-significant results in some of the adjusted multiple comparison tests. Furthermore, our genetic analysis was exclusive to the most common SNPs in the maternal TLR2 gene and not extended to the infant genotype or other genes that might have impacted maternal milk factors and susceptibility to allergic diseases.

Collectively, we have shown that genetics, including *TLR2* polymorphisms, allergic status, and other maternal and environmental variables might be responsible for the wide variation in the concentration of sTLR2 in milk. Mothers with high levels of sTLR2 in their milk might not promote adequate oral tolerance development in their infants with a prolonged period of exclusive breastfeeding. Further research is required to understand the mechanisms involved in sTLR2 association with the allergic status of nursed infants and should include many milk samples collected at different time points in the analysis.

Chapter 5. General Discussion

The role of breastfeeding in supporting oral tolerance and protection against allergic diseases has long been questioned. Part of this controversy is attributed to the variation in breast milk components, which are critical for the normal development of immature neonatal immune and digestive systems to achieve optimal oral tolerance. TLR2 is an innate immune receptor that is expressed on different types of cells and mediates the secretion of several IMs [61–63], some of which are found in breast milk. However, the effect of TLR2 in changing milk components has not been previously studied.

5.1. Summary of findings

In this project, we aimed to use animal models and analysis of human clinical studies to understand how maternal TLR2 can affect breast milk composition in a way that can influence the adequate development of oral tolerance in infants.

The advantage of the animal model in our project is that we can use mouse dams that are entirely TLR2 deficient, collect their milk at different time points, analyze it, and manipulate it; something that is not possible in humans. Cross-fostering experiments in mice enabled us to swap the pups between mothers from different genotypes and compare their immune system changes to their siblings that remain with their birth mother.

We found that the pups that were nursed by a $TLR2^{-/-}$ dam, regardless of their biological mother, had an impaired tolerance towards OVA compared to those fostered by WT dams (**Figure 5-1**). Pups that ingested OVA during the lactation period, directly or via their nursing mother's milk, were more prone to food sensitization if the fostering dam was TLR2 deficient (**Figure 3-1**). Increased OVA-specific IgE in serum was used to measure food sensitization following post-weaning vaccination with OVA and an alum adjuvant.

We sought to investigate the immune and gastrointestinal factors that might expose these pups to such an increase in food sensitization. We found that pups fostered with $TLR2^{-/-}$ dams had lower levels of tolerogenic DCs in the MLN and the spleen and exhibited lower expression of *Ido* that may impact the quality of DC tolerogenic activity (**Figure 3-3**). Knowing that $CD103^{+}$ tolerogenic DCs are a potent inducer of Tregs, we observed that lower levels of $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs accompanied the decrease of GIT-tolerogenic DCs in the pups (**Figure 3-5**). The reduction in tolerogenic DCs and Tregs, which can inhibit IgE class switching, might be one mechanism of increased food sensitization. The maturation of the GIT in pups at weaning age was also assessed. We observed that pups fostered by WT dams had better intestinal epithelial integrity marked by a decrease in permeability towards ingested dextran. The importance of intestinal integrity in oral tolerance relies on forming a barrier that both protects epithelial-underlying structures from harmful microorganisms and undigested allergens and limits antigen contact to specific populations of antigen-presenting cells.

This evidence of milk's fundamental importance in regulating tolerance prompted us to investigate the differences between $TLR2^{-/-}$ and WT dams' milk. Using ELISA and Luminex, we found that the milk from $TLR2^{-/-}$ dams had decreased levels of IGF-1, IL-6, IL-13, and IFN-γ. An attempt to add-back IGF-1 and IL-6 to pups fostered with $TLR2^{-/-}$ dams demonstrated improvement in the levels of tolerogenic DCs and Tregs, most notably in the MLN and spleen. Our findings suggest a vital role for maternal genetics in changing milk IMs, some of which are important in oral tolerance (**Figure 5-1**).

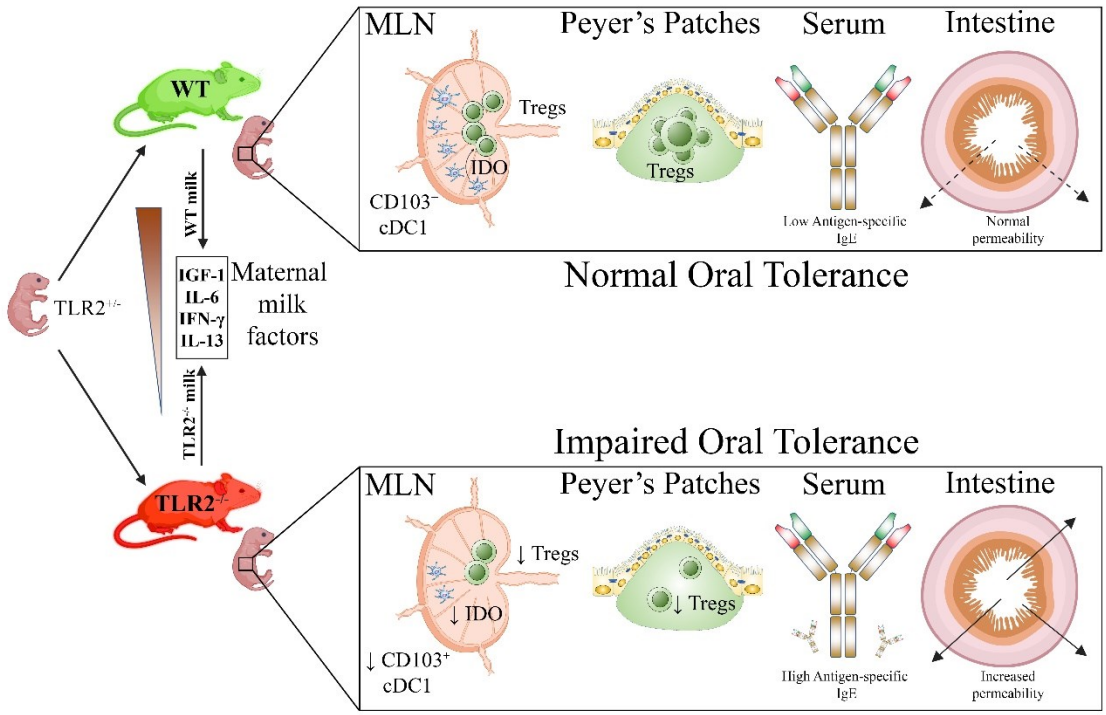


Figure 5-1: Schematic summary of the role of maternal TLR2 in alteration of milk IMs and establishment of tolerance in early life in a mouse model.
Pups that are nursed by TLR2 deficient dams have an impaired oral tolerance, marked with increased food sensitization, increased intestinal permeability, and decrease in tolerogenic DCs and Tregs in GIT, compared to their sibling that are fostered with WT dams. Milk IM such as IGF-1, IL-6, IFN-γ, and IL-13 that were deficient in the milk collected from TLR2$^{-/-}$ dams, might be essential for establishing tolerance in mice during the lactation period.

In light of our animal model studies, we decided to follow up on our findings in humans, emphasising the role of sTLR2 in milk. It has been reported that TLR2 play an essential role in infant response to inflammation but has never been studied in the context of oral tolerance. Similarly, we sought to understand the impact of human TLR2 genotype on different IMs and the subsequent association with infant allergic diseases. To answer this question, we collaborated with Dr. Meghan Azad, from the University of Manitoba to investigate the IMs in breast milk samples collected from participant mothers in the CHILD study that involved more than 3400 families from multiethnic communities and multiple geographic locations across Canada. This longitudinal study involved collecting several types of samples, including breast milk, as well as data collected from all family members at different time points before and after birth. Our study was designed to recruit equal numbers of allergic and non-allergic mothers and infants (as described in 2.2.1). This allergy-enriched cohort gave us more power to investigate maternal atopy impacts on breast milk components and assess their influence on infant susceptibility to different types of allergic diseases.

From our study, we were able to determine that human milk has higher levels of sTLR2 than previously reported in the literature as measured by ELISA using a unique capture monoclonal antibody that was raised by our lab against human TLR2. Western blotting has demonstrated that this antibody detects several isoforms of sTLR2, some of which may have remained undetected by the commercially available antibodies. The range of concentrations of TLR2 in human milk was between 5 - 339.03 ng/ml, which made it one of the most highly variable milk IMs in our study after TSLP and IL-33. The median level of human sTLR2 in milk is around 2.5 times higher than the levels observed in cow's

milk and cow's milk-based baby formulas. sTLR2 levels in breast milk were independent of several maternal and environmental factors including age, weight, location, education, smoking, stage of lactation, pets, feeding practices, and infant sex. However, sTLR2 was shown to be higher in food allergic mothers' milk, along with IgE. In contrast, polymorphisms in *TLR2*, including rs56346547, rs10222800, and rs1439164, were associated with decreased sTLR2 in breast milk. Also, the SNPs rs13123230 and rs1439164 were associated with a decreased incidence of asthma and hay fever.

Collectively, we found that exclusive breastfeeding by mothers with high levels of sTLR2 in milk was associated with increased allergic disease susceptibility in nursed infants regardless of the allergic status of the mother. Therefore, although sTLR2 can inhibit inflammatory responses to particular infections, excessive levels of sTLR2 might dampen the beneficial roles of TLR2 signaling that are critical for intestinal maturation immune regulation and subsequent oral tolerance development.

5.2. Implications and relevance of our findings

The role of breast milk in the protection against allergic diseases in infants is still a puzzle that has not yet been resolved. In this work, we have focussed on a less studied innate immune receptor in the context of oral tolerance, TLR2, which has been shown to play an essential role in changing the IM in breast milk and subsequently tolerance establishment in nursed infants.

Using the mouse model, we have shown that breast milk components are affected by maternal genotype. Although nursed pups were genetically identical, their immune response and susceptibility to food sensitization significantly shifted from their siblings

upon fostering with a mother from different genotype. Besides genetics, other environmental factors, such as diet, bacteria, and housing, can alter milk composition. Therefore, our findings indicate the importance of considering the impact of lactating mothers and their milk on studied subjects to minimize the variation in the results that highly depends on the immune response, knowing that milk effects can last for an extended period in life. Although our study focused on TLR2, it definitely can be translated to other immune receptors and signalling molecules. For example, another approach to emphasize the importance of IL-6 in milk, instead of the add-back experiment, can be achieved via fostering pups born to a WT dam with an IL-$6^{-/-}$ deficient dam and compare their immune response to their littermates that are not cross-fostered.

Regarding the design of cross-fostering experiment, we have managed to control pups' delivery at matched time points via co-housing of the females together before mating with the addition of male urine to stimulate and synchronize their estrus cycles [347]. This synchronisation method could be translated into other types of research, such as ovarian cancer field, for example, in which females' estrus cycle might influence the immune response and the growth of tumours that might be expressing estrogen receptors [436].

In humans, our study and those of other have shown the impact of multiple factors on the composition of breast milk, including maternal health, genetics, and environment, which subsequently, influence the development of allergic diseases in infants. Using a population-based cohort, we have observed a wide range of common *TLR2* gene polymorphisms. Three *TLR2* SNPs, rs56346547, rs10222800, and rs1439164 that have not been reported previously to impact the levels of sTLR2 milk were shown to modify such levels. These polymorphisms might also be of interest in other research fields such as

virology, given the previously reported role of sTLR2 in viral infections [72,390]. To our knowledge, we are the first to note that rs13123230 SNP can be associated with decreased incidence of asthma in the adjusted analysis (**Figure 4-9**), which could expand our knowledge about the genetic basis that predispose human to such allergic diseases.

Our findings were consistent with other studies [424,425] that the mother's allergic status positively associates with an increase in allergic diseases in infants, with a suggested role of milk IM, including sTLR2. These findings might partially explain why the protective role of breast milk against allergic diseases is still controversial.

Our results may inform the development of specific protocols for measuring breast milk IMs and define the normal ranges for each one to serve as a reference for milk analysis. Therefore, knowing that deficiency or abundance of particular IM in milk predispose infants to certain allergic diseases might promote the development of prophylactic arrangements, either by compensating the missing IMs or using alternatives to breast milk. In addition, defining the standard levels of essential IM in milk can also help the baby formula industry, aiming to produce substitutes that are as close as possible to human milk.

5.3. Limitations and future directions

Using both mouse models and human studies, we were able to elaborate several mysteries around the role of TLR2 in impacting the composition of milk IMs, some of which are essential in oral tolerance. However, several limitations of our project are linked to the nature of each model.

Among the limitations of the studies is the challenge in comparing results from mouse and human analyses. The milk IM profile was substantially different between mouse and human. Certain IMs that were detectable in mouse milk, such as IGF-1, IL-13, and IFN-γ were below the detection limit in our human milk analysis. In contrast, we could not detect sTLR2 in mouse milk, unlike human milk, with commercially available detection methods. To date, no publication has reported the detection of sTLR2 in mouse milk, while it has been reported in mouse plasma and peritoneal lavage [67]. So, either it is not expressed in mouse milk, masked by binding ligands, or requires alternative detection method and antibodies to those we have used.

We studied the expansion of total Tregs in the GIT with the general marker $CD4^+CD25^+FOXP3^+$, without distinguishing iTregs from nTregs, or looking for antigen-specific Tregs. It would be interesting to examine both subsets of Tregs and OVA-specific Tregs in the pups getting milk from WT vs $TLR2^{-/-}$ dams in future experiments. Furthermore, we plan to investigate the expansion of tolerance supporting immune cells in the LP, a vital site for tolerance induction. The LP contain resident $CX3CR1^+$ DCs and macrophages capable of capturing food antigens from the intestinal lumen and providing it to the migratory $CD103^+$ DCs [437]; therefore, it would be essential to follow up on those cells to better understand regulatory mechanisms involved in tolerance induction.

We have seen that WT dams that ingested OVA during the lactation period could induce better tolerance in nursed pups than $TLR2^{-/-}$ dams. However, we must consider the detection of OVA peptides in the milk of WT or $TLR2^{-/-}$ dams to assess their capacity to secrete food antigens in milk. Beside food antigens and IMs in milk, the microbiota has been detected in mouse milk [438]. However, with the current yield of mouse milk collection

(~200µl per mouse), it was too technically challenging to assess mouse milk microbiota along with other IMs in milk. Therefore, we should consider designing a separate future experiment for microbiota assessment via pooling multiple samples from the same mouse strain to increase DNA extraction ability to give sufficient yield for analysis.

Knowing that milk from TLR2$^{-/-}$ dam has lower IGF-1, IL-6, IL-13 and IFN-γ than WT dams, we demonstrated that the adding-back of only IGF-1 and IL-6 to the diet of nursed pups could induce tolerogenic DCs and Tregs. However, whether IL-13 and IFN-γ provided orally might also potentially influence nursed pups' tolerance development remains to be seen. As proposed previously, an alternative method to the "add-back" experiment is to nurse the pups with a cytokine deficient dam, such as IFN-γ$^{-/-}$ or IL-13$^{-/-}$ dams and compare oral tolerance of nursed pups to their siblings that are nursed by a WT dam. In this animal model, we have used mice with BALB/c background that are skewed towards Th2 phenotype; thus, it might be of interest to test our hypothesis using an alternative strain background, such as C57BL/6 and assess the impact of TLR2$^{-/-}$ in C57BL/6 mice on the dam's milk IMs.

Regarding the human study using CHILD samples, one of the most significant limitations is basing our conclusions on a single time point milk sample from each mother. It would be more valid to have at least three-time points of milk, including colostrum, three months and six months of age. Some IMs are higher in the lipid layer than in the whey section. Therefore, comparing the analytes' levels in both layers might also give a more accurate estimation of the actual concentration. Mediators with more lipid-soluble are hard to assess accurately using a multiplex system such as Luminex and may require more

specialised analysis. Knowing that sTLR2 mainly binds ligands with a lipidic backbone, we can expect to find an unspecified amount in milk's lipid layer.

As mentioned above, IGF-1, IL-13, and IFN-γ were below our assay limit of detection in human milk. However, some studies have reported IL-13 [279,287], IFN-γ [301,336], and IGF-1 [439] in human milk. Differences in results from studies examining the presence and concentrations of cytokines and soluble receptors in breast milk may relate to the studied populations, collection time, sample storage, and detection methods, as reviewed by Agarwal *et al*. [277]. Therefore, it is always better to consider validation of one method by an alternative one, such as using the ELISA of single analytes to validate the multi-array Luminex, as sensitivity differs between methods and manufacturer. Also, for analytes that were undetectable in our assays, it would be optimal to spike back the standard of this analyte into milk samples and assess the recovery concentration by the unspiked standard. This method can provide information regarding the low apparent abundance of specific analytes in milk as they might be bound to other molecules that can weaken their binding capacity to detection antibodies.

One of the crucial components of milk is the nutritional antigens that are derived from the maternal diet. Milk antigens might be complexed with immunoglobulins that might play a vital role in infants' oral tolerance induction. Therefore, we consider assessing maternal milk composition of the most common food and environmental antigens to compare it with infant responses towards these antigens.

Our study only focused on the correlation between IMs with other maternal, infant, and environmental factors. It is critically important to compare the correlation of these IMs

with other milk components, including the microbial taxa, fatty acids, HMOs, and vitamins because the overall impact of milk components on the outcome of allergy in infants might involve different types of molecules. We should also compare the levels of sTLR2 and other IMs in milk with their maternal serum concentration to increase our knowledge about the source of these milk components.

We are considering the analysis of SNPs in multiple genes because the expression of a single IM might involve several genes, as we have seen that SNPs in *TLR2* associate with variation in IMs other than sTLR2. Other genes that control the production of proteins involved in the TLR2 signalling pathway, such as MyD88 [440], or the enzymes that release sTLR2 from cells might be good candidates to confirm the importance of the whole system in oral tolerance vs allergy development. In addition to the maternal genotype, we should consider assessing the infant SNPs in TLR2 and other related genes to better understand the role of genetics in susceptibility to allergy in early life.

Having seen that intestinal permeability might be a risk factor for food sensitization in mice, it would be a breakthrough to assess it in human infants and whether milk IMs and allergic disease incidence are associated with it. Kosek *et al*. [441] have shown that neopterin, alpha-1-anti-trypsin, and myeloperoxidase in the stool can be used as useful non-invasive markers to assess intestinal permeability in infants. Stool samples have been collected in infants from the CHILD cohort and might help assess intestinal permeability and allergic diseases.

Finally, we have seen that our findings' adjusted values were not as significant as our raw analysis, which could be due to the limited number of analysed samples. In the future, we will be expanding our study to a broader number of milk samples.

5.4. Conclusions

It is undeniable that breast milk is the best choice for healthier development of infants. The establishment of oral tolerance in early life might be highly dependent on the composition of breast milk in addition to other genetic and environmental factors (**Figure 5-2**). Still, research in this field has been inconsistent mostly due to the wide variation of milk components between mothers. We utilized both mouse and human models in this work due to their unique advantages and limitations. Despite the differences between the two model systems, we successfully delineated several IMs in the milk of mice and humans that might be essential for oral tolerance or represent a risk factor for allergic disease development in infants (**Figure 5-2**). We have shown the benefits of using a mouse model to assess the importance of specific molecules or IMs in milk, relevant to tolerance establishment. Also, the wide availability of genetically modified mice can be a powerful tool to confirm or discover the link between genetics and certain diseases, including allergy, that can be translated into humans as we have seen in the case of TLR2 in our study.

Here we have seen in both mouse and human that maternal TLR2 genotype is critical in controlling several IMs, some of which were essential in oral tolerance or could be a risk factor for allergic diseases in infants. Alteration in milk components might expose infants to higher or lower than normal IM levels, which might interfere with oral tolerance development during the critical window of opportunity. In mice, a deficiency in TLR2

correlated with low levels of IGF-1, IL-6, IL-13, and IFN-γ in milk, which increased susceptibility to food sensitization. In humans, excess expression of sTLR2 in milk was associated with an increased incidence of several immune diseases in prolonged exclusive breastfeeding cases.

We believe that this work can improve our understanding of breast milk's controversial role in immune-mediated disease prevention in childhood, such as allergy. Knowing the normal ranges of IMs in breast milk might help in allergy prevention strategies' development and improve the baby formula industry. It might also improve the guidelines and the recommendation for the optimal period of exclusive breastfeeding and the introduction of alternatives to achieve the best outcome for infants.

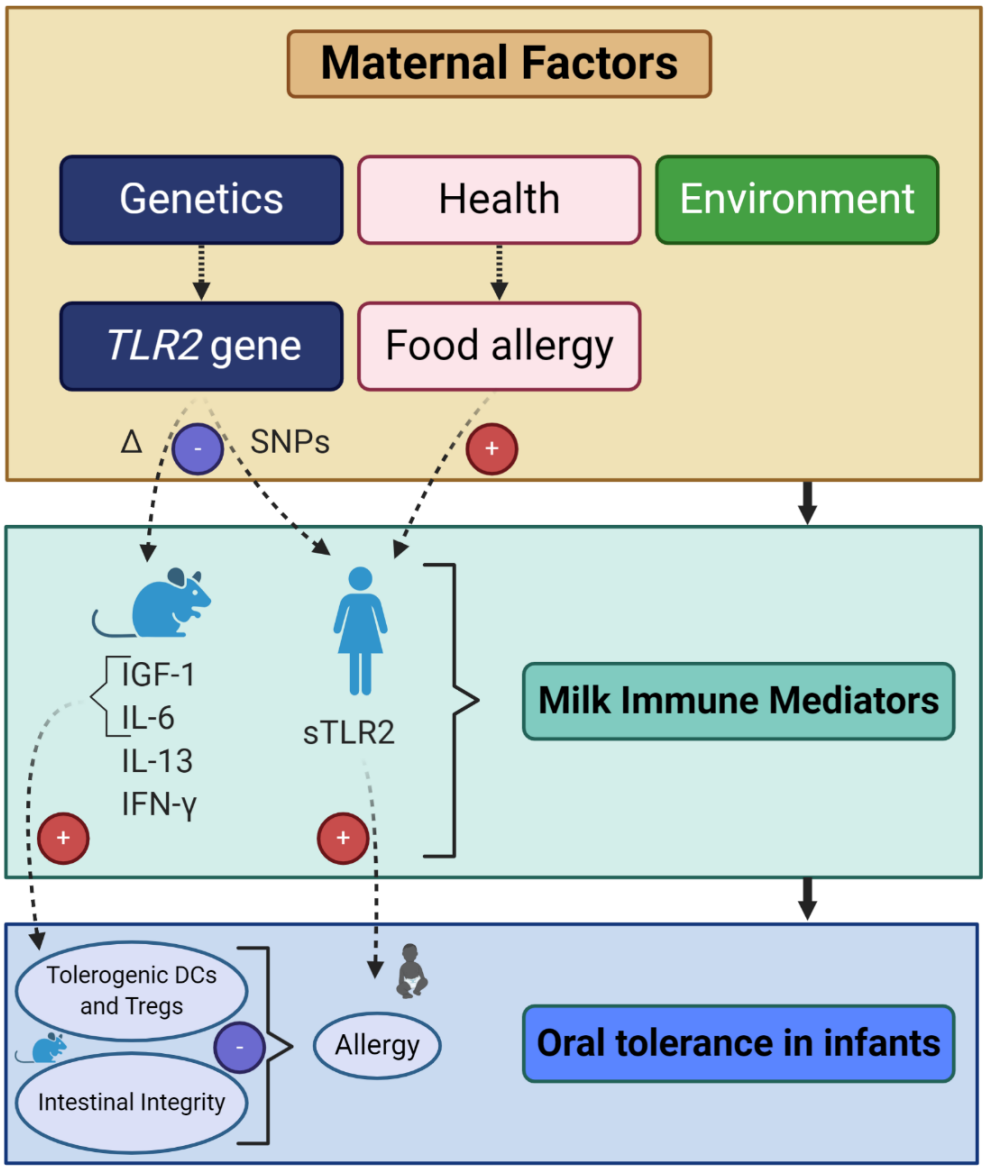


Figure 5-2: Summary figure.
Several maternal factors, including genetics, health, and environment, impact the composition of milk IMs. The relationship between TLR2 and milk composition was assessed in both humans and mice. Deficiency of TLR2 in mice was associated with decreased IGF-1, IL-6, IL-13, IFN-γ, and presumably sTLR2 (undetected). *TLR2* SNPs in humans were associated with alterations in milk components, most notably decreased sTLR2 levels. In contrast, human mothers diagnosed with food allergy had significantly higher levels of sTLR2 in their milk. The milk from dams that are TLR2 deficient showed decreased protection capacity against the development of food sensitization in nursed pups, which could be due to a decrease in tolerogenic DCs and Tregs (reversed with the add-back of IGF-1 and IL-6), and an increase in intestinal permeability. Whereas in humans, the increase in sTLR2 in milk was associated with increased incidences of allergy in infants exclusively breastfed for at least six months of age.

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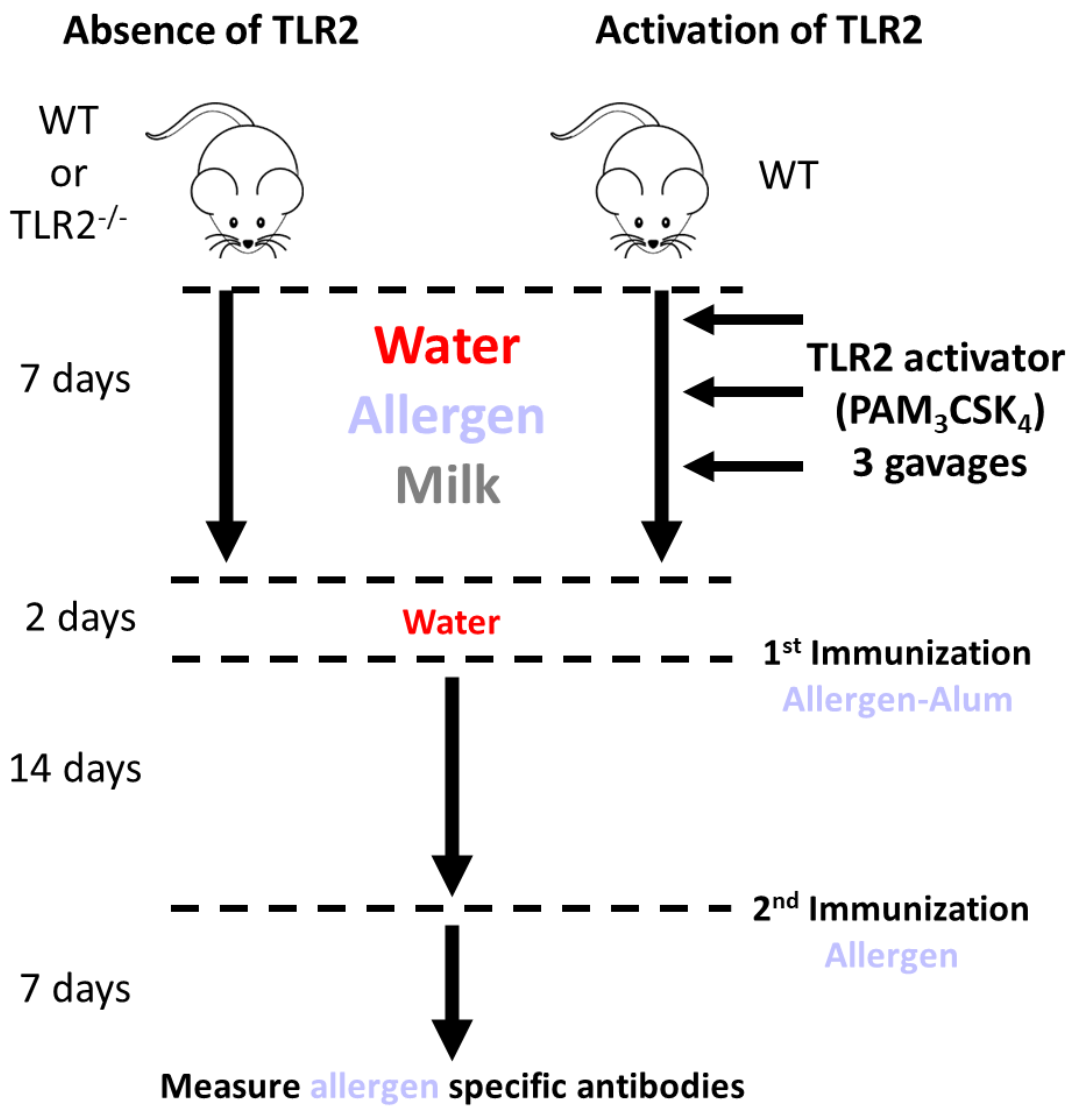
APPENDICES

Appendix A: The role of TLR2 activation on the development of oral tolerance towards milk antigens.

According to Tunis *et al*. [92], TLR2 deficiency does not impact the establishment of oral tolerance towards OVA, whereas activation of TLR2 during feeding with OVA can disrupt tolerance establishment. We investigated if this observation applies to milk antigen, β-lactoglobulin (BLG). We have seen that feeding WT and $TLR2^{-/-}$ mice with milk or BLG alone (WT only) has shown to induce tolerance towards the BLG marked by reduced anti-BLG IgE levels IgA, IgG1, and IgG2a after sensitization compared to water-fed mice (**Appendix 1A**). This finding was consistent with a previous study in our lab that TLR2 is not essential for developing oral tolerance toward OVA [92]. However, activation of TLR2 via PAM_3CSK_4 during feeding with BLG has disrupted humoral tolerance towards IgG1 and IgG2a only and did not alter anti-BLG IgE (**Appendix 1B**) similar to OVA.

Next, we wanted to test the impact of milk on tolerance disruption during TLR2 activation. As seen previously, activation of TLR2 during OVA ingestion induces food sensitization marked by increased anti-OVA IgE levels; however, adding milk during the disruption process alters the impact of the TLR2 agonist (**Appendix 1C**). This observation indicates that have wither a protective effect against TLR2 activation or induction impact towards oral tolerance.

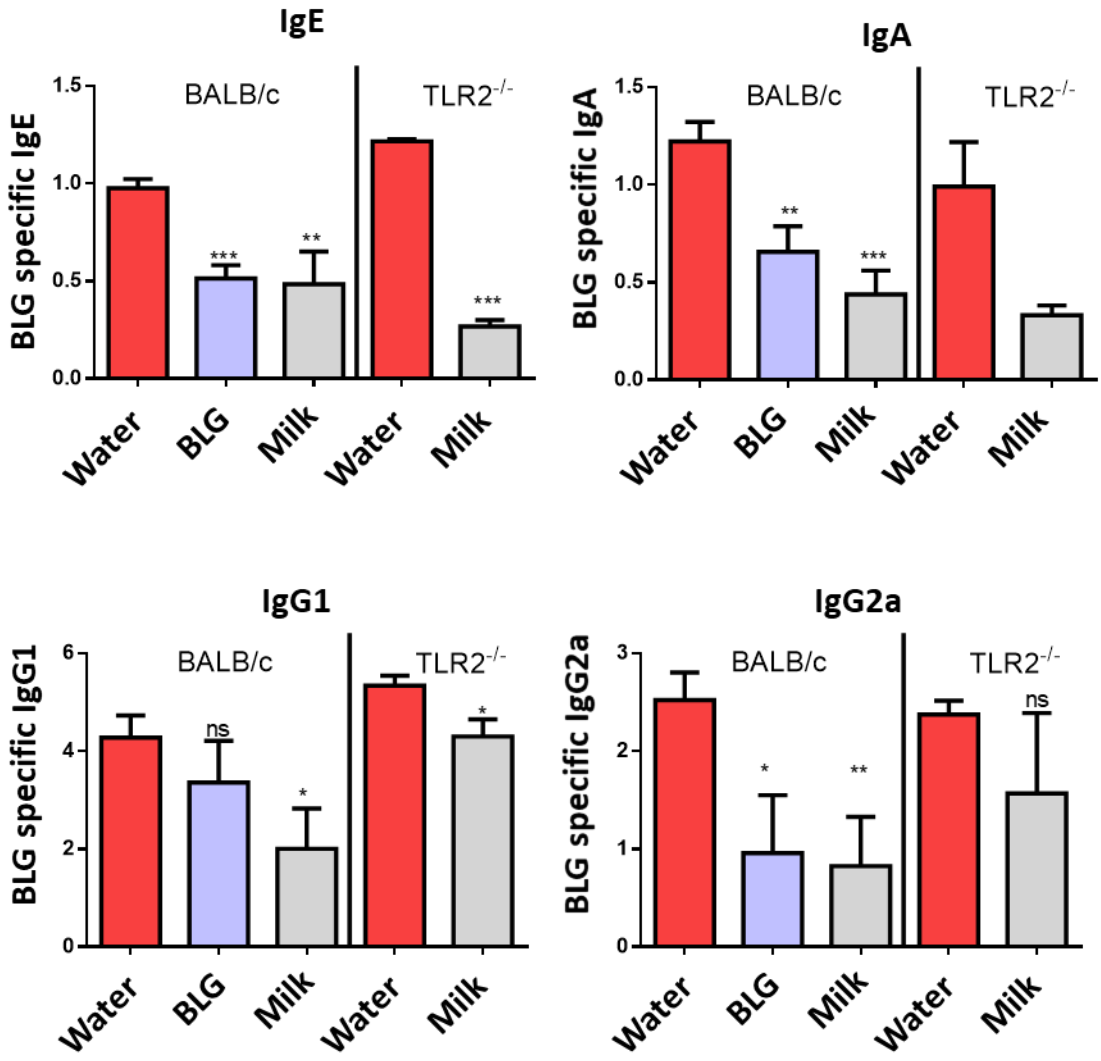


Schematic of the experimental method.
Male BALB/c mice (6 weeks old WT or TLR2$^{-/-}$) were fed with either water, BLG, or milk *ad libitum* for a week. In the experiment of TLR2 activation, WT mice were fed with BLG ± PAM_3CSK_4 (10 µg in 100µl PBS). After that, these mice were supplemented with water for two days, immunized intraperitoneal with 10 µg of BLG/Alum-adjuvant and boosted with 1 µg of BLG after two weeks. Serum was collected after a week of the second immunization and assessed for the levels of anti-BLG specific immunoglobulins (IgE, IgA, IgG1, and IgG2a).

Appendix 1, Humoral tolerance is intact in TLR2$^{-/-}$ mice and disrupted upon TLR2 activation but not when milk is introduced.

ELISA was used to assess antigen-specific immunoglobulins. (**A**) Plasma anti-βLG-specific immunoglobulins measured in WT and TLR2$^{-/-}$ mice after feeding them with milk (WT and TLR2$^{-/-}$) or BLG (WT only). (**B**) Plasma anti-βLG-specific immunoglobulins measured in WT mice after activation with PAM_3CSK_4 during BLG ingestion. (**C**) Plasma anti-OVA-specific IgE was assessed in mice that were fed with milk during the activation of TLR2. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n.s., not significant.

A


IgE
BALB/c
TLR2-/-
BLG specific IgE
1.5
1.0
0.5
0.0
***
**
***
Water
BLG
Milk
Water
Milk
IgA
BALB/c
TLR2-/-
BLG specific IgA
1.5
1.0
0.5
0.0
**
***
Water
BLG
Milk
Water
Milk
IgG1
BALB/c
TLR2-/-
BLG specific IgG1
6
4
2
0
ns
*
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Water
BLG
Milk
Water
Milk
IgG2a
BALB/c
TLR2-/-
BLG specific IgG2a
3
2
1
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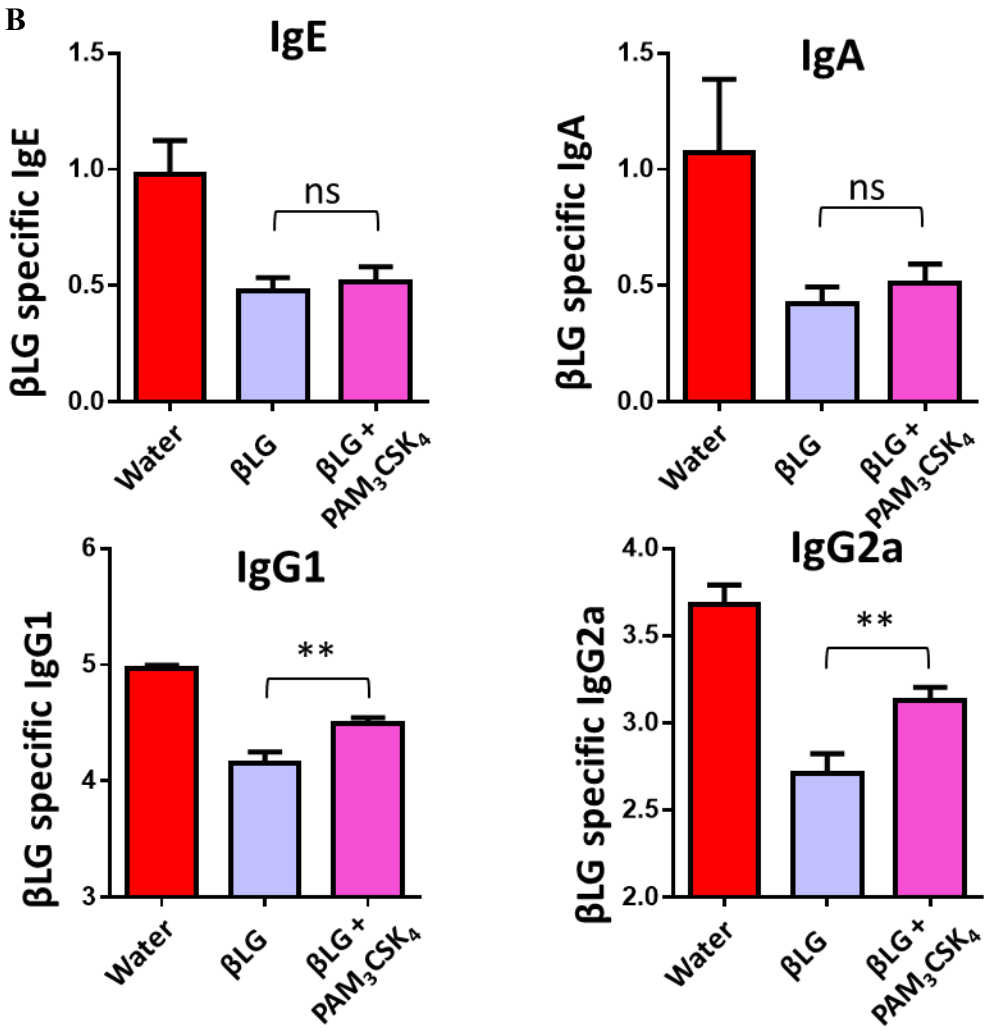
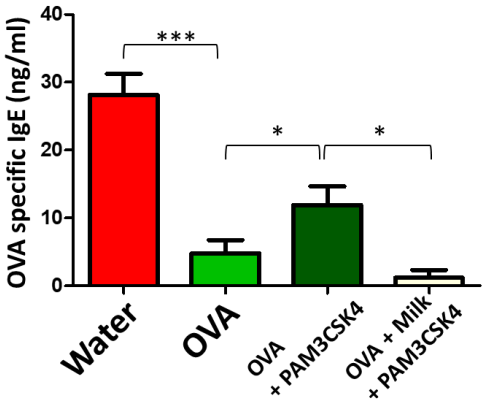
ns
Water
BLG
Milk
Water
Milk

B


IgE
βLG specific IgE
1.5
1.0
0.5
0.0
ns
Water
βLG
βLG + PAM3CSK4
IgA
βLG specific IgA
1.5
1.0
0.5
0.0
ns
Water
βLG
βLG + PAM3CSK4
IgG1
βLG specific IgG1
6
5
4
3
**
Water
βLG
βLG + PAM3CSK4
IgG2a
βLG specific IgG2a
4.0
3.5
3.0
2.5
2.0
**
Water
βLG
βLG + PAM3CSK4

C


OVA specific IgE (ng/ml)
40
30
20
10
0
***
*
*
Water
OVA
OVA + PAM3CSK4
OVA + Milk + PAM3CSK4

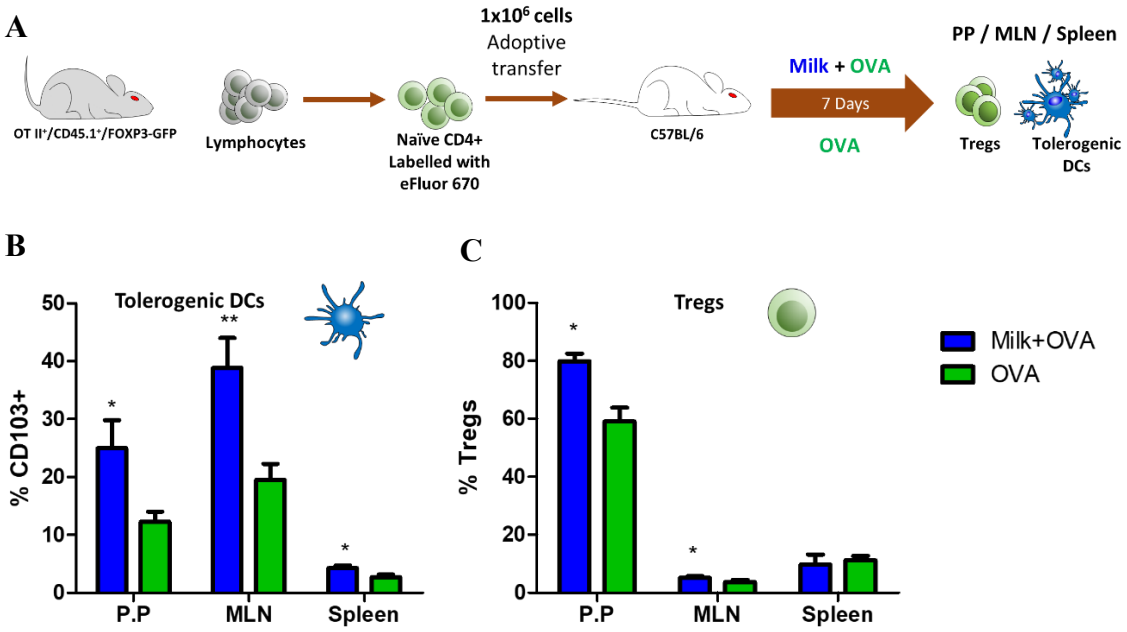
Appendix B: Milk induces oral tolerance via expansion of tolerogenic DCs and Tregs.

Milk has shown to protect against food sensitization induced via TLR2 activation. We sought to investigate the mechanism involved in this protection. Tolerogenic DCs and Tregs are important immune cells in the induction of oral tolerance (see 1.2.2, and 1.2.3). We sought to investigate the direct impact of milk on both types of immune cells. To assess milk's effect on the expansion of tolerogenic DCs, we used C57BL/6 mice fed with OVA ± milk. Interestingly, milk and OVA fed mice have significantly higher tolerogenic DCs in the MLN, PP, and spleen than OVA fed mice (**Appendix 2B**), indicating that milk promoted these cells' expansion.

To assess milk's impact on Tregs' expansion, we used naïve T cells derived from CD45.1^{+}/OT-II^{+}/FOXP3-GFP mice and adoptively transferred them into C57BL/6 WT mice and tracked their skewing towards Tregs after feeding mice with OVA ± milk (**Appendix 2A**). These cells are taken from the progeny of crossing CD45.1^{+}/OT-II^{+} males with Foxp3-GFP females. OT-II^{+} CD4^{+} T cells respond exclusively to the $OVA_{323–339}$ peptide and produce GFP once they become Tregs, which can be detected by flow cytometry. We found that the mice that ingested milk and OVA have a significantly higher frequency of CD45.1^{+}/ FOXP3-GFP^{+} cells in the MLN, PP, and spleen than OVA fed mice (**Appendix 2C**). This observation might be coming directly from milk or indirectly under the influence of tolerogenic DCs.

Collectively, milk has shown to promote oral tolerance in mice via induction of tolerogenic DCs and Tregs, which might be one of the mechanisms to protect against the disruption of oral tolerance by TLR2 agonist. Also, bovine milk has shown to have sTLR2

(**Figure 4-3**) with levels around 35 ng/ml in skimmed milk, which might be competing with membrane TLR2 and block the effect of PAM_3CSK_4.



Appendix 2, Milk enhances the expansion of tolerogenic dendritic cells and regulatory T cells in PP, MLN, and spleen.

(**A**) Schematic of the experimental design. (**B**) The level of tolerogenic dendritic cells was identified by the expression of $CD103^+$ marker on $CD11b^+/MHCII^+/CD11c^+/CD3e^-/CD8^-$ cells using flow cytometry. (**C**) The frequency of $CD4^+/CD45.1^+/FOXP3\text{-}GFP^+$ cells as representative of OVA-induced Tregs was identified using flow cytometry. Mice fed with milk + OVA were compared with mice fed with OVA alone using t-tests. Bars represent mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

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