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Research Article

Dynamics of antimicrobial proteins' expression and their bactericidal activity in mouse milk

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Abstract

Mother's milk is considered as "complete edible immune system." It contains macro- and micronutrients required to maintain infant growth and provides an excellent source for innate and adaptive immune proteins that not only protects infants from enteropathogens but also aid in the initial colonization of gut microbiota. In this study, we analyzed the milk of C57BL/6J dams and found significant changes in the composition of antimicrobial and immune proteins throughout the lactation period. Innate immune proteins, serum amyloid A, soluble CD14, and notably lipocalin-2 were detected in milk at high quantities. These proteins were substantially reduced in the milk from MyD88-deficient dams. Further, adaptive immune proteins, specifically IgA and IgG, exhibit a distinct shift during postpartum lactation stages. While IgG is the dominant immunoglobulin in milk at day 5 postpartum, by day 15 its levels were surpassed by IgA whose levels increased over time. The administration of TLR4 ligand LPS to WT dams significantly increased the aforementioned milk innate and adaptive proteins. Surprisingly, the milk from WT dams suppressed *E. coli* growth more effectively than milk collected from LPS-treated mice; such suppression, however, was completely lost upon boiling. Intriguingly, IgA, but not Lcn2, serves as a predominant factor in inhibiting *E. coli* proliferation, suggesting the critical role of IgA in regulating microbial colonization in the neonatal gut. Collectively, our findings provide insight into the dynamics of various immune proteins present in breast milk and highlight their pivotal roles in determining neonatal immune responses and microbial colonization at early stage.

Keywords: *E. coli*, IgA, IgG, lipocalin-2, serum amyloid A

Introduction

The host innate immune system serves as a first line of defense against infection and also helps in regulating gut microbiota homeostasis.[1] At birth, the neonate's immune system is underdeveloped and immature, rendering infants with an increased vulnerability to enteropathogens and environmental challenges.[2] Mother's milk is considered as a "complete edible immune system" owing to its extraordinary cocktail of nutrients, proteins, antibodies, hormones, and bacteria that help develop and mature a newborn's immature GALT.[3] Such unique components of mother's milk are critical for the neonates in offering passive immunity and helping to develop tight gut epithelial barrier. In addition, milk oligosaccharides[4–6] help to establish a healthy gut microbiome that is also critical for the development of the immune system. Thus, mother's milk plays a crucial role in providing both nutrition and effector immune molecules to the newborn.[7]

The composition of mother's milk is very dynamic and how it changes over the course of lactation depends on several factors. For instance, the composition of humans' milk depends on body weight, height, protein intake, parity, menstruation cycle, and frequency of nursing.[8] Moreover, preterm breast milk contains significantly higher concentrations of some immune proteins than term breast milk.[9] Even though alterations in macro- and micronutrients during lactation had been studied extensively, how host genetics and inflammatory status influence immunoproteins and their kinetics over the course of the lactation period is less clear. The current literature documents the benefits of breastfeeding in reducing morbidity, mortality, and protection against specific infections in neonates and infants. Current scientific research greatly supports the advantages of prolonged breastfeeding, highlighting its numerous positive effects on both infants and mothers.[10] Although research on health benefits of specific factors, specifically immunometabolites in the mother's milk, has been ongoing for decades, there are still, however, unidentified factor(s) that need to be explored to redesign appropriate infant formulas.[11]

Comparative studies on human and mouse milk remain limited to date. Nevertheless, prior studies have shown that the milk of humans and mice, despite having marked differences, has substantial similarities in proteins'[12] and immune cells'[13] content. To study the lactational kinetics of milk and the genetic factors influencing milk protein composition, we employ mouse milk as an experimental model, which is more tractable than human milk for this endeavor. In this study, we analyzed the temporal dynamics of immune proteins, like Lipocalin 2 (Lcn2), serum amyloid A (SAA), and soluble CD14 (sCD14)—in C57BL/6J dams, observing significant reductions in MyD88-deficient mice. Moreover, IgA levels were significantly higher than IgG by 15 and 20 days of the lactation stage, suggesting IgA's key role in the gut homeostasis during microbiota colonization. Interestingly, we observed TLR4-ligand LPS administration elevated the immune protein levels in milk but surprisingly reduced its antibacterial activity against *E. coli*. Notably, boiling abrogated milk's antibacterial effects, and confirmed IgA, but not Lcn2, was involved for its antibacterial activity. Taken together, our

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findings demonstrate the dynamic and complex nature of milk's immunometabolites' secretion and antibacterial activity, underscoring how both innate and adaptive immune systems can orchestrate throughout the lactation stage to support neonatal gastrointestinal immunity.

Material and methods

Mice

C57BL/6J mice (stock no. 000664) were procured from Jackson Laboratory and bred in-house at the University of Toledo, Department of Laboratory Animal Resources. Immunoglobulin A–deficient (*Iga*KO) mice were a gift from Dr. Dennis Metzger (Albany Medical College). Lipocalin 2–deficient (*Lcn2*KO) mice generated by Dr. Shizuo Akira (Japan) were obtained via Dr. Kelly Smith (University of Washington). Myeloid differentiation primary response protein 88 (MyD88)-deficient (*Myd88*KO) and RAG-1–deficient mice (*Rag1*KO) were procured from Jackson Laboratory (Bar Harbor, Maine). All the mice are on C57BL/6J background and were crossed with C57BL/6J wild-type (WT) mice for more than 10 generations. This study was performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Toledo. The IACUC at the University of Toledo approved all mouse experiments. Mice were maintained under specific-pathogen-free condition, housed in cages containing corn-cob bedding (Bed-O-Cob, The Andersons Co.) and nestlets (cat. no. CABFM00088, Ancare Corp.) and fed ad libitum grain-based chow (LabDiet 5001). Mice were housed at 23 °C with 12-h light/dark cycle.

Mouse milk collection

The pups were separated from the dams 4 h before milk collection at indicated time points. Pups remained in their home cage, which was placed in an incubator during the procedure. Next, the dams were anesthetized following standard procedure using isoflurane and then injected with 2 IU oxytocin/dam intraperitoneally (Sigma-Aldrich, St. Louis, Missouri). Oxytocin, a hormone that stimulates the mammary glands to release milk, was used to facilitate milk collection. Milk secretion was stimulated by gently pressing on the dams' nipples, and the milk droplets were pipetted into the sterile microtubes and immediately frozen at –80 °C for future analysis. Dams were milked on days 5, 10, 15, and 20 of the lactation periods. Dams were subsequently returned to their home cages immediately upon recovering from anesthesia.

Lipopolysaccharide treatment

E. coli LPS (Sigma-Aldrich, St. Louis, Missouri), a Toll-like receptor 4 (TLR4) agonist, was injected (1.0 μg, i.p.) into WT dams (n = 5), and milk was collected 4 h after LPS injection.

In vitro bacteria killing assay

Escherichia coli (*E. coli*, K12 strain) was grown overnight in Luria-Bertani (LB) broth (LB broth, containing 50 μg/mL kanamycin) at 37 °C with shaking (200 rpm). Bacterial CFUs were estimated on the basis of their optical density at 600 nm, and 5×10^4 CFUs of *E. coli* were pelleted and resuspended in 200 μl LB-agar. Milk, which was collected on day 10 afterbirth from either WT, *Lcn2*KO, and *Iga*KO dams, was added to the *E. coli* culture (1:250 dilution). Culture was incubated at 37 °C for 6 to 8 h and then plated on selective LB-agar plates containing 50 μg/mL kanamycin. Plates were incubated overnight at 37 °C, followed by CFU counting on the next day.

Boiling milk

Milk was diluted in PBS (1:10) and boiled at 37 °C for 10 min and added to LB-agar media containing *E. coli* to obtain the final dilution of 1:250.

Enzyme-linked immunosorbent assay (ELISA)

Milk Lcn2 (cat. no. DY1857), SAA (cat. no. DY2948-05), sCD14 (cat. no. DY982) were measured via Duoset ELISA kits from R&D Systems according to the manufacturer instructions. Milk samples were diluted 1:50,000 for Lcn2, 1:10,000 for SAA, and 1:130 for sCD14 in reagent diluent (1% BSA in 1× PBS). Milk samples collected from LPS-given mice were further diluted in reagent diluent to obtain absorbance that falls within the median range of the standard curve.

Measurement of milk IgA and IgG

Milk IgA and IgG were measured by ELISA as described previously[14,15] with modifications. Briefly, polyclonal goat anti-mouse IgA and IgG antibodies (Southern Biotechnology, Cat. no. 1040-01 and Cat. no. 1030-01) were used as a capture antibody. Milk samples were diluted 1:10,000 for IgA and 1:5000 for IgG in blocking buffer. The bound mouse IgA and IgG in samples were detected with horseradish peroxidase–labeled goat anti-mouse IgA and IgG antibodies (Southern Biotechnology, Cat. no. 1040-05 and Cat. no. 1030-05), and developed using TMB (BD Biosciences) as the peroxidase substrate. A mouse IgA, κ-isotype monoclonal antibody (BD Biosciences, Cat. no. 553476) and mouse IgG1, κ-isotype monoclonal antibody (BD Biosciences, San Jose, California, Cat. no. 557273) were used to generate a standard curve.

Statistics

All data were represented as mean ± SEM. The statistical significance between 2 groups was calculated using an unpaired, 2-tailed *t* test. Data from more than 2 groups was compared using a one-way ANOVA followed by Tukey multiple comparison tests. The *P* values <0.05 were considered statistically significant and are denoted as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. All statistical analyses were performed with GraphPad Prism 9.0 software (GraphPad, La Jolla, California).

Results

Milk from MyD88-deficient mice display substantially reduced innate immune proteins

Mother's milk is an excellent source for macronutrients and micronutrients for newborns, but it also provides a wide array of innate and adaptive immune proteins, acting as a complete edible immune system for the neonates.[2] Mother's milk not only helps in conferring protection from opportunistic pathogens but also aids in the proper colonization of neonatal gut microbiota. Therefore, we sought to investigate the dynamics of innate immune protein secretion into the mouse milk at 5, 10, 15, and 20 days of the lactation period. We quantified three antimicrobial proteins, Lcn2 (chelator of bacterial siderophores), SAA (delivers vitamin A to immune cells), and soluble cluster of differentiation 14 (sCD14; binds LPS–

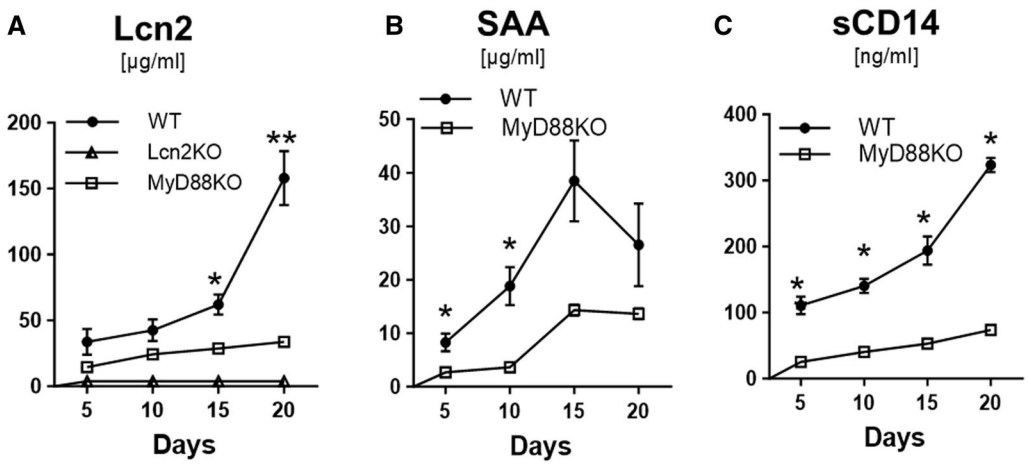

Figure 1. Milk from Myd88-deficient mice displayed significantly reduced levels of innate immune proteins. Milk was collected from WT, Lcn2KO, and MyD88KO dams (n = 4 to 10), which gave first litter at indicated time points, and we quantified (A) Lcn2, (B) SAA, and (C) sCD14 via DuoSet ELISA. *Lcn2*KO mice milk used as negative controls in ELISA (Fig A). Data represented as mean ± SEM. $*P < 0.05$, $**P < 0.01$.

lipopolysaccharide binding protein [LBP] complexes), that play diverse roles in immune defense, and are also known as acute phase proteins.[16–18] Interestingly, in milk from WT dams, Lcn2, SAA, and sCD14 levels were increased gradually in a time-dependent manner. Notably, Lcn2 and sCD14 levels were approximately 3-fold higher on day 20 compared to day 5 in WT milk (Fig. 1A–C). Next, we asked to what extent the MyD88 (an adapter protein for all TLRs except TLR3) is accountable for the maintenance of innate immunity in the mucosal compartment, ie milk.[19] As expected, milk from MyD88-deficient dams showed substantially reduced levels of Lcn2, SAA, and sCD14, which remained low at all time points (5, 10, 15, and 20 days postpartum) compared to milk from WT dams (Fig. 1A–C). Thus, these findings suggest that the secretion of innate immune proteins into milk are MyD88 dependent.

Milk from adaptive immunity–deficient mice exhibits elevated levels of innate immune proteins

To further investigate how the adaptive immunity, primarily mediated by T and B cells, contributes to maintaining the optimal levels of innate immune proteins in milk, we exploited recombinase activating gene 1–deficient (*Rag1*KO) dams. Milk was collected on day 5 postpartum, and Lcn2, SAA, and sCD14 were quantified by ELISA in those milk samples. Surprisingly, milk collected from RAG-1–deficient dams displayed ∼2-fold increase in the levels of Lcn2 and SAA, but not sCD14, when compared to milk from WT dams (Fig. 2A–C). The observed increase in Lcn2 and SAA levels in *Rag1*KO mice milk may represent a compensatory mechanism whereby innate immunity is heightened in the absence of adaptive immunity.

Distinct kinetics of IgA and IgG secretion in milk at different lactation phases

Mother's milk is enriched with IgA and IgG, which are passively transferred into the neonatal gut lumen, promote gut microbiota homeostasis, and assist in the maturation of gut-associated lymphoid system.[20] To understand their kinetics, we quantified IgA and IgG levels during early, mid, and late periods of lactation in WT mice. Intriguingly, in WT dams' milk, on day 5 postpartum, IgG level was significantly higher than IgA levels (Fig. 3A). However, IgG levels progressively decreased over time, with a substantial reduction observed on days 10, 15, and 20. By day 20, IgG levels were reduced by approximately 50% in WT dams' milk compared to day 5 (Fig. 3A). In contrast, milk IgA levels exhibit a gradual and substantial increase across the lactation period. By day 20, IgA levels were increased approximately 3-fold compared to day 5 (Fig. 3A). As expected, both IgA and IgG levels were undetectable in the milk from *Rag1*KO mice compared with WT dams' milk during all lactation stages (Fig. 3A). We also analyzed milk from IgA-deficient (*Iga*KO) dams on day 5 and compared IgA and IgG levels with WT dams' milk. As expected, IgA levels were undetectable in *Iga*KO dams' milk (Fig. 3B). Intriguingly, IgG levels were ∼9.2-fold higher in *Iga*KO dams' milk compared to WT dams' milk, suggesting a potential compensatory mechanism in the absence of IgA (Fig. 3C). Collectively, these results suggest that IgA becomes more critical during mid and late lactation period, coinciding with active neonatal gut colonization (via coprophagy and solid food consumption) and reshaping neonatal gut microbiota both qualitatively and quantitatively at adult stage.[21]

Increasing and suppressing the levels of innate and adaptive immune proteins in milk influence its antibacterial activity

Systemic inflammation is well-known to induce an array of effector immune molecules, including acute phase proteins. We first titrated the doses of LPS (a gram-negative bacterial cell wall component and a potent agonist for TLR4) and found that a low dose of LPS (1.0 µg/mouse, i.p., after 4 h) was sufficient to effectively induced Lcn2 and SAA in the serum of nonlactating WT dams (Fig. 4A, B). Next, to investigate the extent to which LPS-driven inflammation could affect the levels of innate immune proteins in milk, we performed the above experiment using lactating WT dams at day 5 postpartum. Milk was collected 4 h post–LPS treatment. Intriguingly, we observed a marked increase by approximately 2- to 3-fold in the levels of Lcn2, CD14, sCD14, IgG, and IgM in milk from LPS-treated dams when compared to untreated WT control dams' milk (Fig. 4C–G).

Next, we sought to investigate the impact of milk processing on innate immune proteins. The processing of milk, which includes its exposure to high temperature, is a routine practice that may affect the composition and functions of milk immune proteins.[22] Generally large-scale suppliers

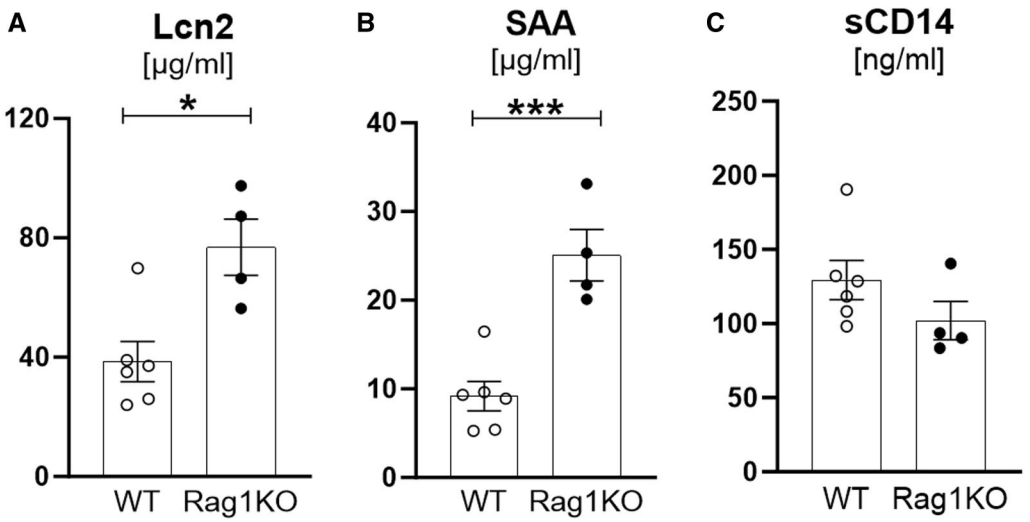

Figure 2. Mice with adaptive immune deficiency display elevated innate immune proteins. Milk was collected from WT and *Rag1*KO dams (n = 4 to 6, on day-5 lactation stage), and we quantified (A) Lcn2, (B) SAA, and (C) sCD14 via DuoSet ELISA. Data represented as mean ± SEM. $*P < 0.05$, $**P < 0.001$.

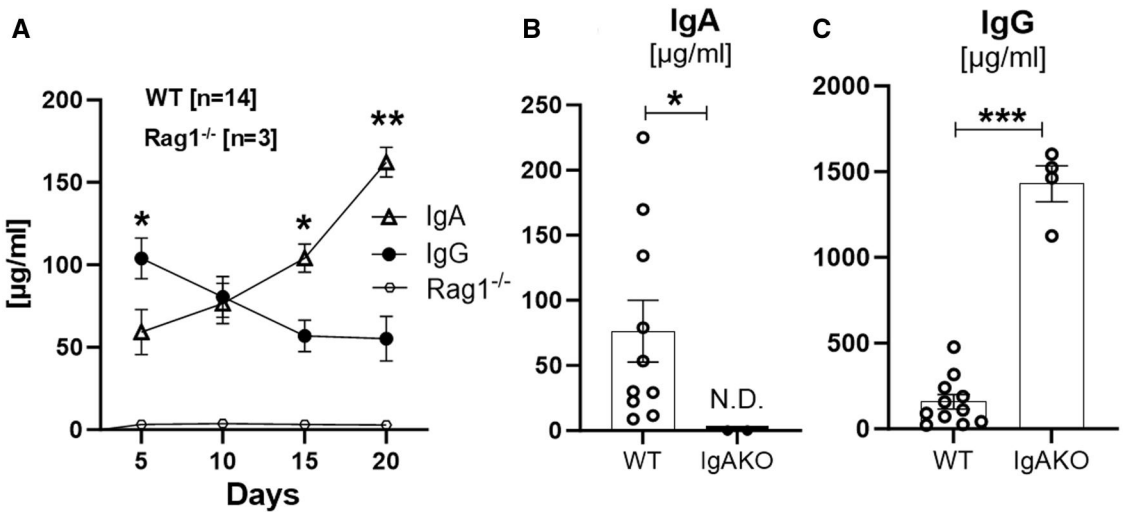

Figure 3. Inverse trend in milk IgA and IgG levels during different phases of lactation. Milk was collected from WT and *Rag1*KO dams (n = 3 to 10) at indicated time points of lactation stage, and we quantified (A) IgA and IgG via ELISA. *Rag1*KO mouse milk was used as negative control. Milk was collected from WT and *Iga*KO dams (n = 3 to 12, on day-5 lactation) and measured for IgA and IgG via ELISA. Milk (B) IgA and (C) IgG. Data represented as mean ± SEM from 3 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

typically sterilize milk through pasteurization, whereas boiling at high temperature is commonly practiced in the households, mainly in developing countries. Importantly, in the production of infant formulas, dairy milk is exposed to high temperatures during spray-drying process.[23] Therefore, to explore how heat treatment influences the innate immune proteins, we boiled the WT dams' milk samples for 10 min at 99 °C and quantified Lcn2, CD14, and sCD14. Surprisingly, milk Lcn2 was barely detectable in boiled milk, but the levels of SAA and sCD14 remained unchanged in boiled milk compared to nonboiled milk (Fig. 5A–C). These results suggest that Lcn2 is heat labile, whereas SAA and sCD14 are heat stable, to the denaturing effects of boiling. While comparing nonboiled milk samples from WT and *Iga*KO mice, we observed that Lcn2 levels were significantly reduced in *Iga*KO milk, albeit their levels of SAA and sCD14 were comparable. Similiar to observations in WT milk, boiling IgaKO milk resulted in an almost undetectable levels of Lcn2, while SAA and sCD14 levels remain unchanged (Fig. 5A–C).

Having demonstrated that milk contains an array of innate and adaptive immune proteins, we next sought to investigate their antibacterial role. To address this, we selected a nonpathogenic, laboratory adapted *E. coli* K12 strain and performed in vitro studies. *E. coli* (5×10^4 CFUs) was incubated in either PBS or milk collected on day 10 postpartum from WT dams', LPS-treated dams', or WT dams' boiled milk (1:250 dilution in PBS) for 8 h at 37 °C and plated on selective Luria Bertani (LB) agar plates containing kanamycin and incubated for overnight at 37 °C. As expected, *E. coli* grown in PBS after 8 h proliferated normally (Fig. 6A, C–E). The incubation of *E. coli* with WT dams' milks strikingly inhibited bacterial growth (Fig. 6A–E) and inhibited approximately 60% proliferation (Fig. 6B). On the basis of these results, we hypothesized that milk collected from LPS-treated dams, which contains 2 to 3 times more

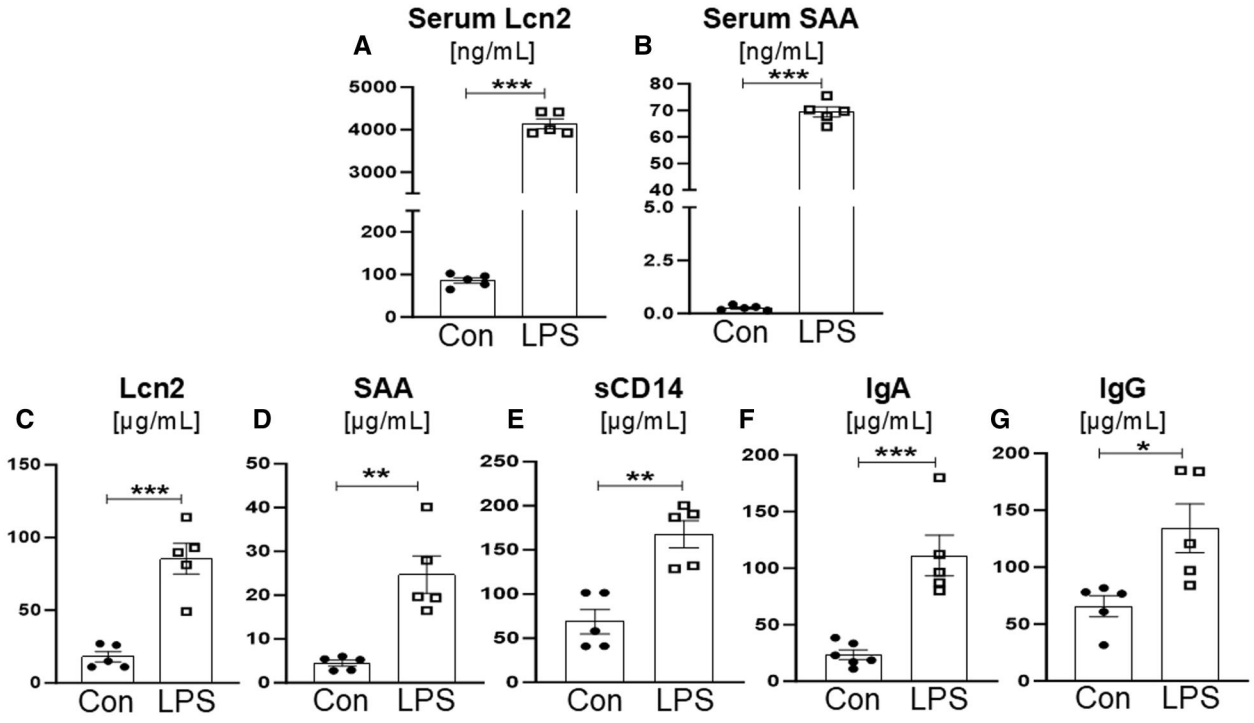

Figure 4. Administration of TLR4 ligand LPS significantly increased systemic and mucosal innate and adaptive immune proteins. PBS or LPS (*E. coli* LPS, 1.0 µg/mice, i.p.) was injected to into nonlactating WT females (8 weeks old, n = 5), and blood was collected after 2.5 h, and sera were analyzed for (A) Lcn2, (B) SAA. In a separate experiment, PBS or LPS (1.0 µg/mice, i.p.) in PBS was injected into lactating WT dams on day 5 postdelivery (n = 5), and milk was collected after 2.5 h, and we quantified (C) Lcn2, (D) SAA, and (E) sCD14, and (F) IgA and (G) IgG via ELISA. Data represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$.

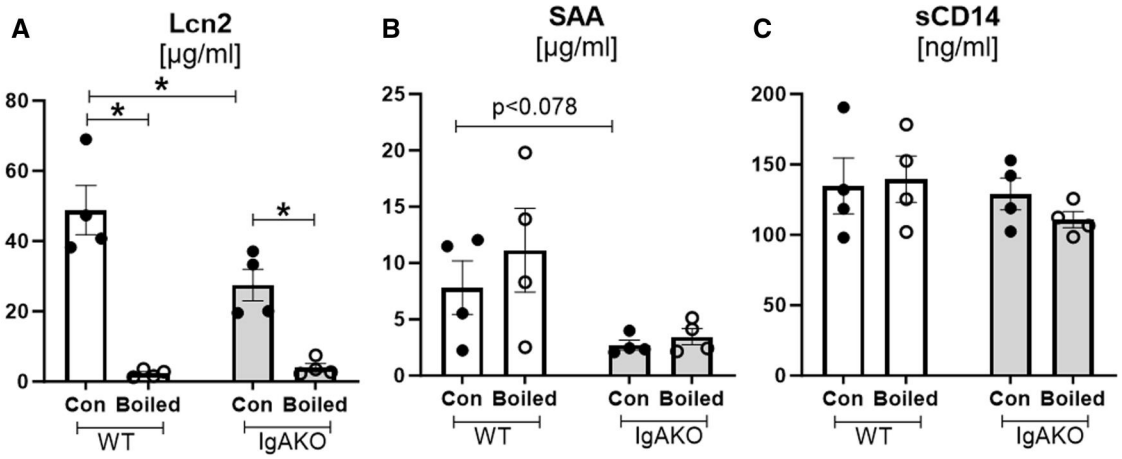

Figure 5. Milk Lcn2 is heat labile, whereas SAA and sCD14 are heat stable. Milk samples were collected from WT and *Iga*KO dams (n = 4) on day-5 lactation stage and were diluted (1:10) in sterile 1× PBS. One set of milk samples were boiled at 99 °C for 10 min. Both control and boiled milk samples were appropriately diluted for (A) Lcn2, (B) SAA, and (C) sCD14 via DuoSet ELISA. Data represented as mean ± SEM. $^{*}P < 0.05$.

immune proteins (Fig. 4C–G), may offer greater antibacterial effects. However, in contrast to our hypothesis, milk collected from LPS-treated dams displayed only 50% of antibacterial activity compared to milk from healthy WT dams (Fig. 6A–D). Surprisingly, boiled milk completely lost its antibacterial activity against *E. coli* and allowed them to proliferate normally (Fig. 6A–C, E). Collectively, these results suggest that milk from healthy WT dams significantly inhibits bacterial growth and proliferation. However, systemic inflammation introduces some unknown factor(s) that have lesser antibacterial activity. Moreover, the complete loss of antibacterial activity in boiled milk samples underscores the importance of heat-sensitive milk proteins, which are important to mediate their antibacterial properties.

Milk IgA, but not Lcn2, is indispensable for controlling bacterial growth and proliferation

Our results demonstrate that mouse milk contains microgram levels of IgA and Lcn2, both of which increase during the late phase of lactation. IgA prevents bacterial proliferation through selective or nonselective binding,[24,25] whereas Lcn2 acts as an antibacterial protein by chelating bacterial siderophores, thereby depriving their iron uptake, a key transition metal ion vital for bacterial proliferation.[26] Next, to

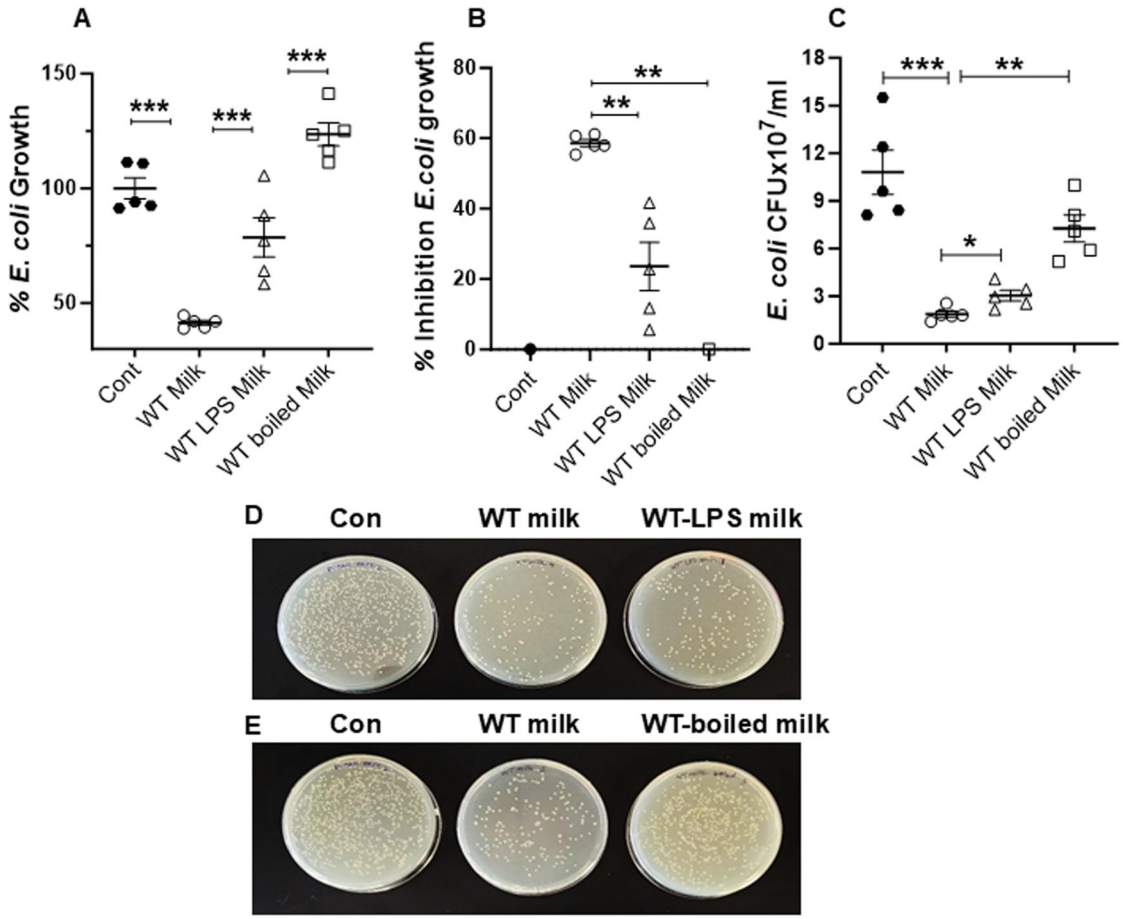

Figure 6. Milk possesses strong bactericidal activity, which was reduced in milk collected from LPS-treated mice and completely lost upon boiling. *E. coli* (strain K12; 5×10^4 CFUs) was incubated in LB media (200 µl) in the presence of milk (1:250 dilution; with and without boiling condition) that was collected at day-10 lactation period from WT dams. In a separate experiment, WT dams were treated with LPS (1.0 µg/mice, i.p.) or PBS; after 4 h, milk was collected and was incubated with *E. coli* as above. After 8 h, *E. coli* growth was determined via measuring the optical density at 600 nm. Results were calculated and shown as (A) percentage of growth and (B) percentage of growth inhibition. Bacteria were also plated on LB agar containing kanamycin (50 µg/mL), incubated at 37 °C for 18 h, and counted for CFUs. (C) *E. coli* CFUs counted from LB plates. (D, E) Representative images of LB agar plates. Data represented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

determine whether IgA and/or Lcn2 is critical for inhibiting bacterial proliferation in vitro, we performed *E. coli* growth and proliferation assays by using milk collected from either WT, *Iga*KO, or *Lcn2*KO dams. After 8 h, *E. coli* grown in PBS proliferated normally (Fig. 7A, E). Consistently with our previous results, the milk from WT mice effectively inhibited *E. coli* growth (Fig. 7B, E). In contrary to our hypothesis, milk from *Lcn2*KO mice was also equally capable of inhibiting *E. coli* proliferation as observed with WT mice milk (Fig. 7C, E). Intriguingly, milk from *Iga*KO mice displayed only 40% to 50% inhibitory activity against *E. coli* growth (Fig. 7D, E). We cannot rule out the possibility that the reduced inhibitory activity in *Iga*KO mice milk could be due to reduced levels of Lcn2 and SAA (Fig. 5A, B). These results suggest that IgA is a critical, predominant antibacterial factor in milk, playing a major role in regulating bacterial growth and offering mucosal protection in the neonates.

Discussion

The neonatal gastrointestinal tract is immature, with poorly developed gut epithelial barrier integrity, making a newborn highly dependent on an array of antimicrobials to maintain gut health. Mother's milk is an exceptional amalgam of nutrients, bifidogenic factors, commensal bacteria, and a diverse array of innate and adaptive immune factors.[27] Collectively, these components nourish the newborn, facilitate the proper maturation of GALT, promote the initial colonization of the gut microbiota and protect from opportunistic gut pathogens. Evidence suggests that the health status of breastfed infants is superior to that of formula-fed infants.[28] Despite the ongoing and active research on milk, significant gaps persist in our understanding of how maternal health, inflammatory status, and genetic factors can influence the kinetics of milk proteins and their antibacterial activity. To address these gaps, this study was undertaken and mouse milk was used as a model system to uncover these findings: (i) MyD88 deficiency substantially lowers the levels of innate immune factors in milk; (ii) the secretion kinetics of IgA and IgG are distinct across the lactation period; (iii) increasing immune factors via systemic inflammation or opposingly, heat treatment reduced the antibacterial activity of milk; and lastly, (iv) IgA, but not Lcn2, is indispensable for the antibacterial activity of milk.

MyD88 is a key adapter protein that mediates intracellular signal transduction when cells are activated by all TLR ligands (except TLR3), resulting in the activation of NF-κB pathway.[29] Accordingly, MyD88-deficient condition can be considered as "innately immunocompromised" and characterized by weak

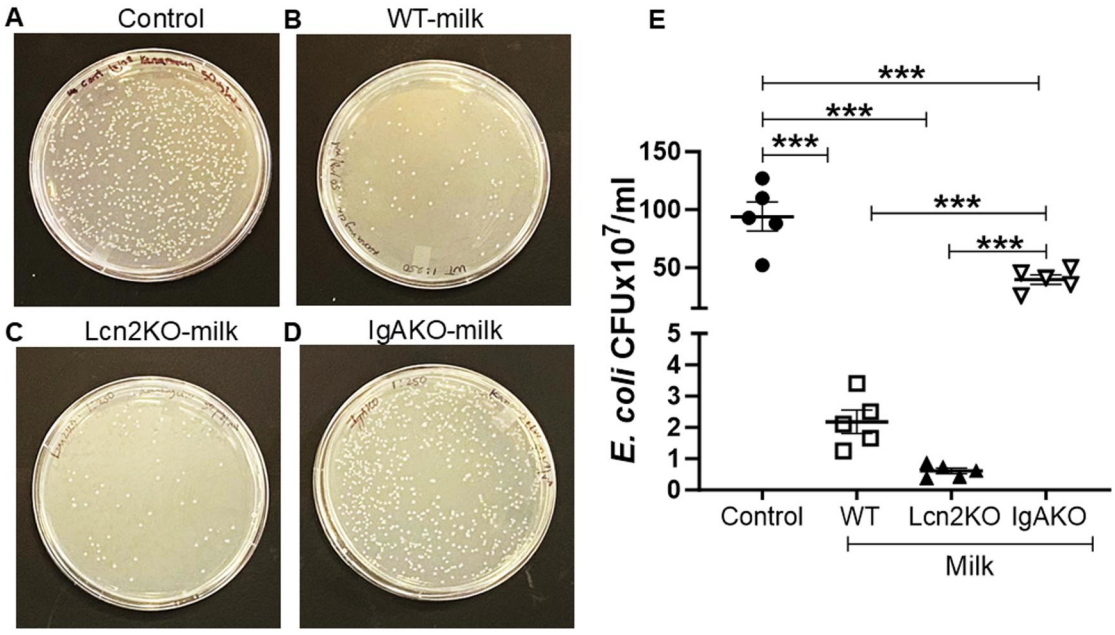

Figure 7. Milk IgA, but not Lcn2, is indispensable for inhibiting *E. coli* proliferation. *E. coli* (strain K12), 5×10^4 CFUs in 200 µl LB, was grown in presence or absence of milk (1:250 dilution 6 h) collected on day 10 from WT, *Lcn2*KO, and *Iga*KO dams. After 6-h incubation bacteria were plated in LB-agar plates containing 50 µg/mL kanamycin. After 18 h, *E. coli* colonies were counted and multiplied with the dilution factor to get absolute CFUs. (A) *E. coli* colonies without treated with milk. *E. coli* colonies treated with milk from (B) WT, (C) *Lcn2*KO, and (D) *Iga*KO. (E) *E. coli* CFUs counted from LB plates. Data represented as mean ± SEM from 3 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$.

and delayed systemic inflammatory responses.[30] *Myd88*KO mice exhibit reduced levels of both innate and adaptive immune proteins in circulation,[31–35] suggesting MyD88 signaling is required to maintain optimal induction of immune effector molecules. Previous studies, including ours, have shown that *Myd88*KO mice displayed significantly reduced levels of serum and fecal Lcn2.[31] To the best of our knowledge, our study is first to demonstrate that MyD88 deficiency not only compromises immune effector molecule levels systemically but also affects their levels in milk throughout the lactation period. Furthermore, a recent study using cell-specific *Myd88*KO mice elegantly demonstrated that mammary epithelial cell MyD88 is required for high-dose LPS-induced mastitis in lactating mice.[36] However, in this study authors did not investigate milk composition in *Myd88*KO mice, leaving a critical gap in understanding how MyD88 signaling impacts on milk's immune molecules.

In this present study we analyzed various innate and adaptive immune factors in milk. Among the innate immune proteins, Lcn2 is known to elicit antibacterial activity by chelating bacterial siderophore (eg enterobactin) and intervene with bacterial ferric iron uptake, which consequently inhibits bacterial proliferation.[37] Our findings demonstrate that systemic inflammation can upregulate the secretion of both innate and adaptive immune proteins in milk. Given this, we hypothesized that milk from LPS-treated mice, which displayed approximately 2- to 3-fold increase in Lcn2, SAA, and sCD14, would exhibit augmented inhibitory activity against *E. coli* proliferation in vitro compared to normal milk. In contrast, milk from LPS-treated mice failed to offer increased inhibitory activity against *E. coli* proliferation. These results suggested that some unknown factor (s) may either have increased or decreased, creating a condition that favors bacterial proliferation. It is also plausible that the elevated immune factors in milk from LPS-treated mice significantly change the milk metabolome[38] and fat composition,[39] potentially affecting its ability to inhibit *E.coli* proliferation. Interestingly, a previous study has shown that cross-fostering IL-10–deficient mice, which are prone to spontaneous colitis in a gut microbiota–dependent manner, with WT milk prevents colitis,[40] suggesting that milk from IL-10–deficient mice may contain elevated immune proteins associated with inflammation, further influencing gut microbiota composition and function.

In addition, our findings showed that boiled milk lacks detectable levels of Lcn2, underscoring the potential loss of critical immune factor(s) during the processing of infant formulas including heating (eg spray drying). These processing steps may negatively affect the milk bioactivity and could contribute to gut microbiota dysbiosis in formula-fed infants.[41] It is interesting to note that recent studies recommend continuing breastfeeding during illness such as the flu or even a breast infection in humans, which underscores the importance of preserving the bioactive components of milk.[42] These findings warrant further in-depth investigations to elucidate the interactions between milk immune components, microbiota composition, and metabolome, and how they collectively influence bacterial proliferation and gut health.

IgG and IgA are the predominant antibodies in systemic circulation and at mucosal surfaces, respectively, with IgA serving as a key immune factor, often reffered to as a coproantibody, in maintaining gut microbiota homeostasis.[43] Milk, secreted at the mucosal surfaces of the mammary gland, is rich in IgA and passively transferred to neonatal guts. In fact, maternal milk is the first source of IgA for a newborn, which provides the first line of defense at mucosal surfaces and regulates initial microbial colonization.[44] In this study we demonstrate that the kinetics of IgA and IgG in milk are displayed in an inverse relationship. IgG predominates during the early phases of lactation and gradually decreases over time, whereas IgA progressively increases and peaks around late phase, ie at the time of weaning (day 20).

This temporal increase in IgA levels at weaning stage may play a crucial role in shaping the colonization of mature adult microbiota, when the offspring begin consuming solid foods and exhibit coprophagic behavior,[21] which contributes to recycling nutrients and reshaping the gut microbiota. Indeed, IgA can selectively or nonselectively bind to microbiota and help in fecal shedding of certain bacteria and maintain microbiota homeostasis.

Further, we demonstrated that milk IgA is more critical for controlling *E. coli* proliferation than the siderophore-binding antibacterial protein, Lcn2. We previously demonstrated that spontaneously hypertensive rats (SHR) exhibit microbiota dysbiosis and markedly reduced IgA levels, which are almost undetectable in systemic, organ and mucosal compartments, including milk, highlighting the role of IgA deficiency in driving diverse host phenotypes.[45] An elegant study by Gopalakrishnan et al. further demonstrates the importance of breast milk IgA, showing its critical roles in protecting from necrotizing enterocolitis (NEC) in the mouse model.[46] Their human studies demonstrate that breast milk is the primary predominant source of IgA in the first month of life. Moreover, a relative decrease in IgA-bound bacteria was associated with the development of NEC, which was linked to a bloom in *Enterobacteriacea* in the IgA-unbound fraction.[46] This evidence suggests the protective role of maternal milk-derived IgA in gut microbiota regulation in neonates.

Although our study has significant clinical implications at multiple levels, there are a few limitations we need to mention. Firstly, we did not analyze milk bacteria composition present in milk samples or in fecal samples of dams or neonates with or without LPS administration. Further, we did not explore dietary interventions aimed at manipulating innate and adaptive immune factors in the milk, which is a potentially translatable way for improving milk composition. Despite these limitations, we believe our study serves as a foundation for future investigations designated to improve both quality and quantity of immune factor(s) in milk. Such advancement has the potential to benefit neonatal intestinal health not only under physiological healthy conditions but also in fatal inflammatory bowel diseases in preterm infants (eg NEC).

In conclusion, our study provides valuable insights into the kinetics of innate and adaptive immune factors secreted into the mouse milk and their implications for antibacterial activity. Of note, we emphasize that IgA is the predominant adaptive immune factor offering antibacterial protection, highlighting its critical role in neonatal health and immune defense.

Author contributions

M.V.-K. was responsible for conceptualization, formal analysis (with P.S and A.A.), funding acquisition, project administration, resources, and supervision (with P.S and A.A.). Investigation was carried out by P.S., A.A., and M.V.-K. Methodology was developed by P.S. and A.A. The original draft was written by P.S. and M.V.-K. Review and editing were done by P.S., A.A., and M.V.-K.

Piu Saha (Data curation [Equal], Formal analysis [Equal], Investigation [Equal], Methodology [Equal], Supervision [Equal], Validation [Equal], Visualization [Equal], Writing—original draft [Equal], Writing—review & editing [Equal]), Ahmed Abokor (Data curation [Equal], Formal analysis [Equal], Methodology [Equal], Validation [Equal], Visualization [Equal], Writing—review & editing [Equal]), and Matam Vijay-Kumar (Conceptualization [Equal], Funding acquisition [Equal], Investigation [Equal], Project administration [Equal], Resources [Equal], Supervision [Equal], Visualization [Equal], Writing—original draft [Equal], Writing—review & editing [Equal])

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The data underlying this article is available in the article.

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