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Effects of Maternal Immune Factors and Gut Microbiota on Neonatal Peyer's Patch Development: A Research Protocol

Akshita Nair, BHSc Student [1]*, Abiramee Kathirgamanathan, BHSc Student [2], Livia Su, BHSc Student [1]

[1] Faculty of Health Sciences, Queen's University, Kingston, Ontario, Canada K7L 2V5
[2] Faculty of Health Sciences, McMaster University, Hamilton, Ontario, Canada L8S 4L8

*Corresponding Author: anair3030@gmail.com

Abstract

The neonatal immune system is constantly surrounded by new antigens and must learn to balance defense against threats with tolerance for the gut microbiome. Since improper neonatal immune development is associated with conditions like asthma, allergies, and chronic bowel inflammation later in life, understanding factors that influence immune development is crucial to preventing these diseases. Intestinal immunity is managed by the Peyer's patches of the small intestine, where microfold cells (M cells) sample antigens from the intestinal lumen to stimulate B cells to produce secretory immunoglobulin A (sIgA), which protects the body from potential pathogens. Neonatal Peyer's patch development is encouraged by maternal immune factors in breast milk and the presence of bacterial genera *Bifidobacterium*, *Lactobacillus*, and *Staphylococcus* in the gut microbiome, but these interactions have not been thoroughly researched. Therefore, we propose an *in vitro* study to determine the effects of breast milk and each genus of bacteria on neonatal Peyer's patch development. Microfluidic devices will permit interactions between Peyer's patches, M cells, and cultures of *Bifidobacterium infantis*, *Lactobacillus salivarius*, or *Staphylococcus epidermidis* in the presence of breast milk or a control formula. Peyer's patch development will be measured by increased sIgA production and M cell maturation markers, which will be compared between experimental groups. It is predicted that samples involving breast milk will have the greatest immune development, likely due to the presence of maternal immune factors which encourage sIgA production. Additionally, it is predicted that *Bifidobacteria* will induce more development than *Lactobacilli* and *Staphylococci* since it is the predominant bacterial genus in the neonatal gut microbiome. This study aims to present evidence that breastfeeding and probiotics can improve neonatal immune development to help prevent inflammatory disease later in life.

Keywords: Peyer's patches; neonatal immune development; neonatal gut microbiome; breast milk; M cells; secretory IgA; microfluidic devices

Introduction

The neonatal period is a crucial time for development, especially for the immune system. Transitioning from the sterile environment of the uterus to an outside world full of new antigens, the neonatal immune system must defend against threats without causing constant inflammation by targeting the trillions of commensal bacteria within the gut. The first weeks of life are essential in developing tolerance towards harmless antigens, and improper immune development leads to conditions ranging from necrotizing enterocolitis to allergies and chronic bowel inflammation [1-3]. Neonatal immune development is key to a lifetime of overall good health, and it has long been established that breastfeeding helps lead to better health outcomes by modulating the neonatal gut microbiome and supporting the development of intestinal immunity [3].

Unlike the diverse adult gut microbiome, the neonatal microbiome is dominated by the bacterial genera *Bifidobacterium*, *Lactobacillus*, and *Staphylococcus* [4]. These commensal bacteria are beneficial because they take up space and use resources that otherwise could have been used by pathogenic bacteria, preventing them from overpopulating and causing disease [4]. They are also thought to help with neonatal immune development by interacting with the immune system in specialized lymphatic follicles in the small intestine called Peyer's patches (PPs) [5]. The structure of a PP is presented in Figure 1.

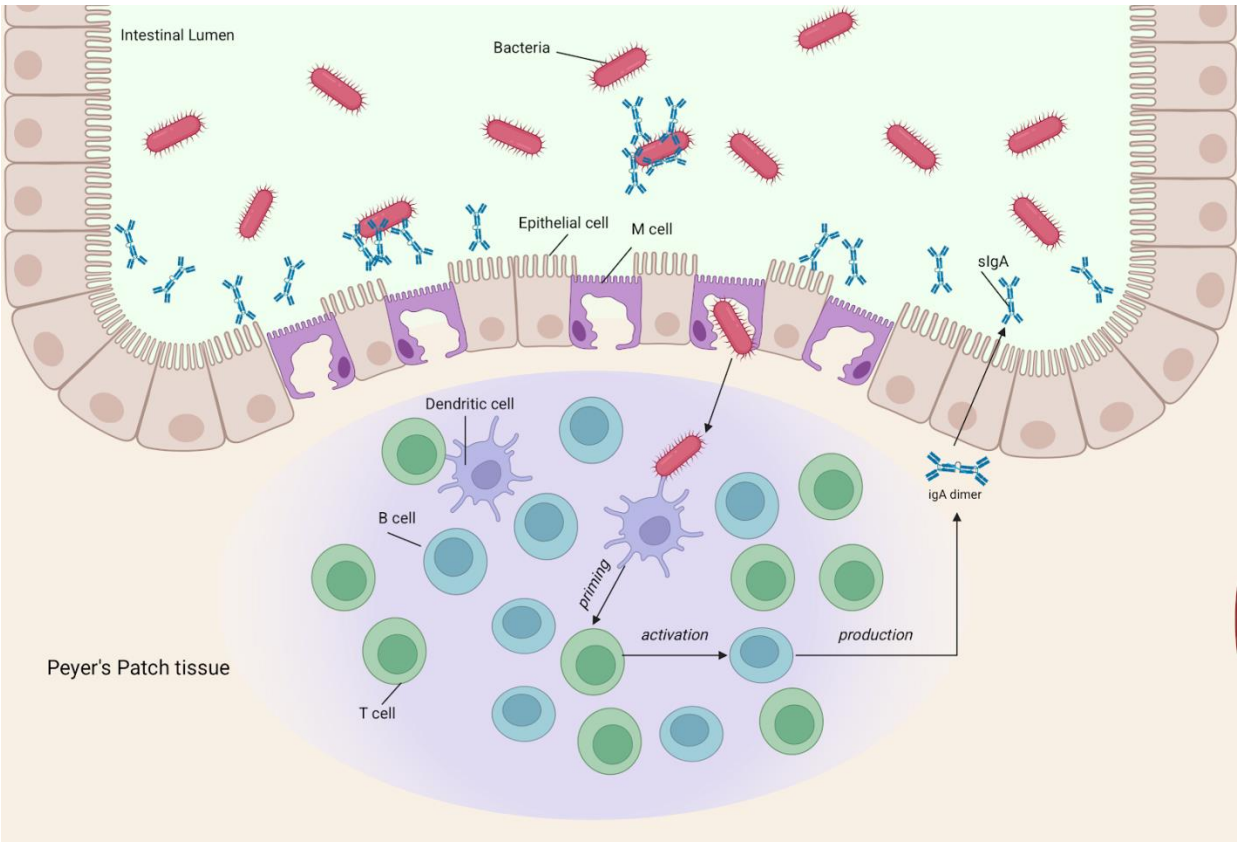

Figure 1. Peyer's patch structure and interactions with the gut microbiome. Bacteria in the intestinal lumen are sampled by M cells along the epithelium. The production of secretory IgA occurs through interactions between M cells, dendritic cells, T cells, and B cells. Peyer's patches protect the intestines from infection by preventing bacterial adhesion to the epithelium without causing inflammation. Figure created using BioRender (https://www.biorender.com).

To ensure the immune system can differentiate harmless microbiota from potential pathogens, the follicle-associated epithelium (FAE) along PPs contains microfold cells (M cells), which present luminal microorganisms to dendritic cells within PPs [6]. Dendritic cells prime T cells which activate B cells to produce secretory immunoglobulin A (sIgA) antibodies [6]. sIgA secreted into the intestinal lumen prevents bacteria from adhering to cell surfaces without triggering inflammation, preventing infection and bacterial overgrowth [6, 7]. This allows for homeostasis to be maintained between the microbiome and the body without causing intestinal damage [6, 7].

PPs are formed before birth but mature postnatally after the appearance of M cells, since homeostasis between the immune system and intestinal microbiota is only possible when B cells are able to produce sIgA against the microorganisms sampled by M cells [8]. PP development is supported by maternal immune factors in breast milk, including anti-inflammatory and antimicrobial compounds, sIgA, lymphocytes, and probiotics [1, 7, 9, 10]. Immunomodulatory factors like prolactin and cytokines increase the production of systemic and mucosal antibodies, increasing sIgA production by the infant's immune system [10]. Additionally, maternal lymphocytes brought to the neonatal intestine through breast milk are able to enter PPs to defend against pathogens and activate B cells to produce sIgA [7]. Breast milk also contains human milk oligosaccharides and peptides such as caseins which promote the rapid growth of *Bifidobacteria*, *Lactobacili*, and *Staphylococci* in the neonatal intestine, promoting better health outcomes throughout life [4, 10, 11].

Neonatal PP development is rarely studied despite its implications in inflammatory diseases [1]. Although human trials have correlated neonatal feeding methods to different gut microbiota and health outcomes, *in vitro* research is yet to be conducted on the effects of breast milk and specific microbiota on neonatal PP development [4]. Investigating these particular PP development factors could identify methods to enhance neonatal intestinal immunity, such as promoting the growth of certain bacteria through probiotic use or encouraging breastfeeding, which could help prevent later-life inflammatory disease. Therefore, we pose the research question, how does breast milk influence neonatal PP development in the presence of different intestinal microbiota?

Methods

Following previous literature, this study will measure sIgA production and the presence of M cell maturation markers as indicators of PP development to assess the influence of breast milk and specific bacteria on neonatal immune development [8]. Due to complexities in *ex vivo* tissue sampling for immunological studies, neonatal PPs can be modelled *in vitro* instead, eliminating ethical issues while allowing specific control over conditions [12]. Microfluidic devices are ideal because cells and bacteria can be co-cultured, replicating intestinal conditions, and creating a fluid flow of 30 µL/h in the device can emulate peristalsis, preventing bacterial overgrowth commonly seen in traditional culturing and organoids [13-15]. A microfluidic device model of a PP is shown in Figure 2.

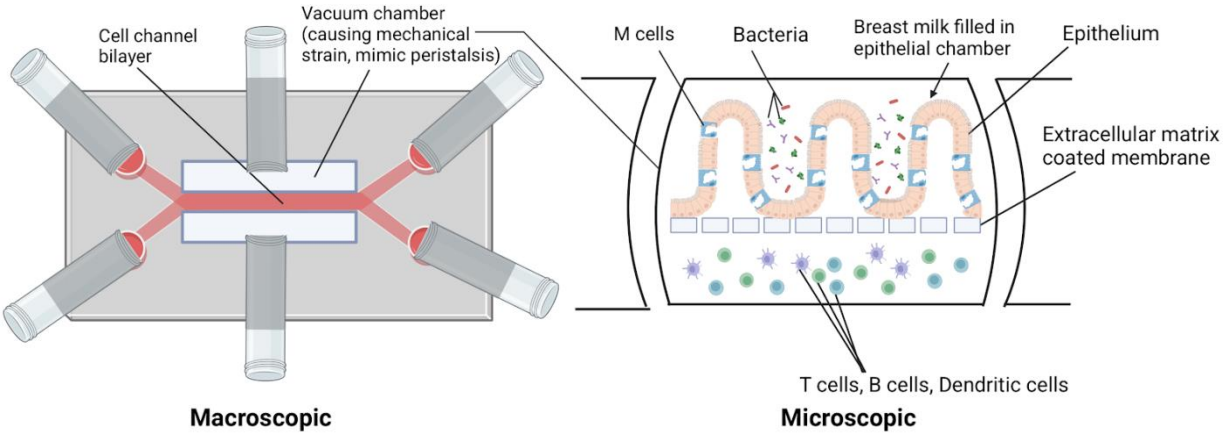

Figure 2. Macroscopic (top-down) and microscopic (cross-sectional) views of a microfluidic device model of a neonatal Peyer's patch. This device will allow the direct testing of the hypothesis through cross-culturing using two parallel microchannels with breast milk or control formula milk perfused into the upper channel. This model enables quantification of M cell differentiation and sIgA secretion in response to different conditions. Figure created using BioRender (https://www.biorender.com).

Bacteria will be cultivated in an upper microchannel in anoxic conditions simulating the intestinal lumen, separated from a lower microchannel containing PP immune cells (B, T, and dendritic cells) by an FAE membrane containing M cells [14]. In each trial, bacteria and milk will be introduced to the chip for 72 hours, allowing enough time to cause measurable genetic changes in cells without bacterial overgrowth [13]. Luminal sIgA levels and M cell maturation markers will be measured before and after this period of time. Based on prior research studies, 5 trials will be conducted for each group (breast milk or control formula and culturing with one of three bacteria species), totalling 30 trials. This will allow enough data to be gathered for accurate statistical analysis without being impractical, since each microfluidic device must be individually managed [16].

The two independent variables are the type of milk added to the lumen (breast milk or a control formula milk, which contains similar nutrients but does not contain immune factors) and the species of bacteria cultured (*Bifidobacterium infantis*, *Lactobacillus salivarius*, or *Staphylococcus epidermidis*; the predominant species of their genera in the neonatal gut microbiome) [3, 17, 18]. There is no control group without bacteria, since this would not reflect actual intestinal conditions; additionally, because there would be no interactions between bacteria and the immune system, there would be no production of sIgA.

The dependent variables are the changes in luminal sIgA levels and M cell maturation markers. sIgA will be measured using enzyme-linked immunosorbent assay (ELISA) [19]. To determine the amount of sIgA produced in the PP, the amount of sIgA in the breast milk must be subtracted from the total sIgA collected from those samples following the trial. The control does not require this step as formula milk contains no sIgA. The percentage of M cells expressing maturation markers SpiB, CCl9, and Gip2 will be measured using flow cytometry, since these markers have been used in previous literature to quantify PP development [8]. Dependent variables will be separately compared between all groups using statistical analyses such as two-way ANOVAs.

Breast milk will be sourced from the Rogers Hixon Ontario Human Milk Bank with informed donor consent, ideally using small quantities of milk from the same donor to limit confounding factors due to differing breast milk composition. Bacteria will be sourced from the Canadian Center for the Culture of Microorganisms. B, T, and dendritic cells are differentiated from hematopoietic stem cells, while M cells are differentiated from human intestinal epithelial stem cells, both of which can be obtained from

biobanks such as MilliporeSigma and cultured within one week [15, 20, 21].

Anticipated Results

Breast milk is anticipated to increase sIgA and M cell maturation in PPs, since maternal immunomodulatory factors and lymphocytes in breast milk increase the production of antibodies, which would increase sIgA production by the infant's immune system, as indicated by prior research [7, 9, 10].

Between bacteria, *Bifidobacteria* may lead to the greatest immune development, followed by *Lactobacilli* and then *Staphylococci*. Lower levels of intestinal *Bifidobacteria* in infants correlates to childhood allergies and asthma, indicating that Bifidobacteria are important to early immune development [22]. *Lactobacilli* are the first bacteria to colonize the neonatal microbiome, but are quickly replaced by *Bifidobacteria*, suggesting that they may not affect immune development as noticeably [4]. Opportunistic *Staphylococci* can cause severe disease under certain conditions, and their benefits to the host are unknown [23].

Certain combinations of milk and bacteria may cause greater immune development. The favourable health outcomes observed in breastfed infants may occur because breast milk encourages *Bifidobacteria* growth [3, 22]. Additionally, since formulas fermented by *Lactobacilli* increased sIgA production in human trials, samples from both milk groups may experience increased immune development when cultured with *Lactobacilli* [19].

Discussion

This study's use of two independent variables (type of milk and bacteria) allows for the effects of each of these factors on neonatal PP development to be determined separately while also accounting for possible synergistic effects. By comparing levels of M cell maturation markers and sIgA between the breast milk and formula groups, it can be determined whether breast milk contributes to greater PP development. This could provide evidence to encourage breastfeeding or possibly develop infant formulas which contain similar immune factors to breast milk, especially for preterm infants and those at high risk of developing autoimmune or inflammatory diseases. Additionally, differences in PP development between different types of bacteria may suggest the use of probiotics to encourage the colonization of bacteria which increase immune development in the neonatal gut microbiome. Combinations of breast milk and certain bacteria which cause greater effects than expected based on the rest of the data should be further studied to confirm their synergistic effects on neonatal PP development, which could lead to a variety of recommendations to improve neonatal immune health.

While our model uses a microfluidic model incorporating epithelial cells, immune cells, and bacteria to simulate the neonatal gut microbiome, it is important to recognize that it is a simplification of PPs. The model is limited because it disregards the dynamic nature of the neonatal gut which is influenced by other immune cells and factors, such as macrophages, regulatory T cells, and cytokines [5]. Furthermore, this study is limited by its usage of sIgA and M cell maturation markers to quantify PP development instead of measuring the number and activity of immune cells, which would allow for a more comprehensive understanding.

Conclusions

The main intent of this article is to address a critical gap in the understanding of how maternal immune factors and early colonization of the gut microbiome interact to shape neonatal immune development. The proposed *in vitro* method can study an under-researched aspect of neonatal immunity, underscoring the importance of breastfeeding and probiotics in preventing later-life inflammatory disease.

The results of this proposed study could inform more detailed *in vitro* or *in vivo* investigations about other aspects of intestinal immunity. Our study only considers PPs, since M cells are the primary immune cell involved in the production of sIgA by B cells, but other cell types like goblet cells appear to be vital to life as well [24]. Epithelial growth factors in breast milk have been found to inhibit antigen presentation to intestinal immune cells through goblet cell-associated pathways, so further studies could be more comprehensive, using multiple models to determine whether breast milk's opposing effects of encouraging PP maturation and inhibiting goblet cell-associated pathways have an overall benefit on neonatal immune development [25]. Researchers could also consider incorporating additional immune markers such as T cell activity to form a more holistic understanding of neonatal immunity. Furthermore, future studies could inform better clinical practices including determining the optimal duration of breast milk supplementation for best immune outcomes and the development of better formulas to encourage immune development. These steps will bring us closer to developing more informed nutritional and medicinal interventions that aim to promote immune tolerance, ultimately leading to healthy lives for the general public.

List of Abbreviations Used

ELISA: enzyme-linked immunosorbent assay
FAE: follicle associated epithelium
M cells: microfold cells
PP: peyer's patch
sIgA: secretory immunoglobulin a

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

This study uses breast milk, which will be sourced from banks such as the Rogers Hixon Ontario Human Milk Bank with informed donor consent. Bacteria will be sourced from

biobanks such as the Canadian Center for the Culture of Microorganisms. B, T, and dendritic cells will be differentiated from hematopoietic stem cells, while M cells will be differentiated from human intestinal epithelial stem cells, both of which can be obtained from biobanks such as Millipore Sigma. Since these stem cells are isolated from umbilical cord blood, there are no ethical concerns involved.

Authors' Contributions

AK: Contributed to the protocol design, reviewed current literature, drafted the manuscript, created figures, and gave final approval of the version to be published.
AN: Contributed to the protocol design, reviewed current literature, drafted the manuscript, created figures, and gave final approval of the version to be published.
LS: Contributed to the protocol design, reviewed current literature, drafted the manuscript, created figures, and gave final approval of the version to be published.

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