Upregulated Th2/Treg Brain Gene Expression in Pups of Nematode-Infected Mice Associated with Enhanced Expression of Leukocyte Trans-endothelial Cell Migration Across the Blood Brain Barrier

Nawal El Ahdab

Institute of Parasitology

McGill University

Montreal, Canada

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Abstract

A maternal murine infection with an intestinal nematode parasite that induces a Th2/Treg response during pregnancy and lactation was previously reported to alter expression of over 5500 genes in the brain of their 7-day old male pups. To explore evidence that immune-related elements of the pup brain and transport of immune signals across the blood-brain barrier were altered, we mined the gene expression database for genes in relevant KEGG pathways regardless of whether or not the pathway itself was differentially regulated by maternal infection. Although the Th1/Th2 cell differentiation KEGG pathway was not differentially expressed in response to maternal infection, brain gene expression of the Th2 response cascade and its product, IL-4 was upregulated. Consistent with this, expression of several Th1 cytokines including IL-1β, as well as receptors and their related proteins were downregulated. Evidence of upregulation of the Treg response included upregulation of TGF-β family genes. The endocytosis pathway that transmits receptor-mediated cytokine and immunoglobulin signals to the pup brain was upregulated in neonates of nematode-infected dams, but genes associated with endosome formation component were downregulated suggesting impaired vesicle-mediated transport into the pup brain. The leukocyte trans-endothelial migration KEGG pathway was also upregulated, as was expression of integrin genes which mediate leukocyte attachment and paracellular diapedesis. This indicated that maternal infection may promote movement of leukocytes across the neonatal blood-brain barrier. Thus, we hypothesize that maternal infection may promote a Th2/Treg environment in the pup brain through migration of Th2/Treg cells across the blood brain barrier. Our study is the first to report the impact of a maternal *H. bakeri* infection on expression of immune related genes in the pup brain.

Résumé

Une infection maternelle murine par un parasite nématode intestinal qui induit une réponse Th2 / Treg pendant la grossesse et la lactation a précédemment été rapportée pour modifier l'expression de plus de 5500 gènes dans le cerveau de leurs petits de 7 jours. Pour explorer les preuves que les éléments immunitaires du cerveau des petits et le transport des signaux immunitaires à travers la barrière hémato-encéphalique ont été modifiés, nous avons exploité la base de données d'expression génique pour les gènes dans les voies KEGG pertinentes, que la voie elle-même ait été régulée différemment ou non par infection maternelle. Bien que la voie de différenciation des cellules Th1 / Th2 KEGG ne soit pas différentiellement exprimée en réponse à une infection maternelle, l'expression du gène cérébral de la cascade de réponse Th2 et de son produit, IL-4 a été régulée à la hausse. De plus, l'expression de plusieurs cytokines Th1, y compris l'IL-1β, ainsi que les récepteurs et leurs protéines apparentées ont été régulées à la baisse. Les preuves d'une régulation à la hausse de la réponse Treg comprenaient une régulation à la hausse des gènes de la famille TGF-β. La voie d'endocytose qui transmet les signaux de cytokine et d'immunoglobuline médiés par les récepteurs au cerveau des petits a été régulée à la hausse chez les nouveau-nés de mères infectées par des nématodes, mais les gènes associés à la formation d'endosomes ont été régulés à la baisse, suggérant une altération du transport médié par les vésicules dans le cerveau des petits. La voie KEGG de migration trans-endothéliale des leucocytes a également été régulée à la hausse, tout comme l'expression des gènes d'intégrine qui interviennent dans la fixation des leucocytes et la diapédèse paracellulaire. Cela indique que l'infection maternelle peut favoriser le mouvement des leucocytes à travers la barrière hémato-encéphalique néonatale. Ainsi, nous émettons l'hypothèse que l'infection maternelle peut favoriser un environnement Th2 / Treg dans le cerveau des petits grâce à la migration des cellules Th2 / Treg à travers la barrière hémato-encéphalique. Notre étude est la première à rapporter l'impact d'une infection maternelle à *H. bakeri* sur l'expression de gènes immunitaires dans le cerveau des petits.

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Contribution of Authors

This manuscript-based style thesis is written by Nawal El Ahdab as per the guidelines of McGill University, and relevant literature is listed after each chapter. It is co-authored with my supervisor Dr. Marilyn E. Scott. Being the primary author of this manuscript, I was responsible for the methodology, data analysis, interpretation, and manuscript writing. This thesis is a secondary data analysis from the published database, created by Dr. Manjurul Haque, including the excel sheet on the relative differentially expressed brain genes in pups of infected dams on postnatal day 7 available as supplementary information in Haque et al. (2019). Dr. Haque guided me on how to properly navigate the data. Dr. Marilyn E. Scott and Dr. Kristine G. Koski provided feedback and guidance at all stages of the research including hypothesis generation, study design, data interpretation and drafting of the manuscript to ensure proper quality and standards are met along with the soundness of argument logic.

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List of Abbreviations

APC	Antigen presenting cell
BAM	Binary alignment map
BBB	Blood brain barrier
CD	Cell surface marker
CNS	Central nervous system
CSF	Cerebrospinal fluid
E_n	Embryonic day n
GD	Gestation day
GI	Gastro-intestinal
Ig	Immunoglobulin
IGSF	Immunoglobulin superfamily
IL	Interleukin
INF	Interferon
ITG	Integrin
KEGG	Kyoto encyclopedia of genes and genomes
L_n	n stage of larvae
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MMP	matrix metallopeptidases
NFAT	Nuclear factor of activated T cells
NF-kb	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
PD	Protein deficient
P_n	Postpartum day n

RNA	Ribonucleic acid
SCFA	Short chain fatty acids
TGF	Transforming growth factor
Th	T helper
TNF	Tumor necrosis factor
Treg	Regulatory T cell

Chapter I - Introduction

Parasitic infections are endemic in developing countries where they induce morbidity and some are a major cause of mortality (Stepek et al., 2006). The endemicity is attributed to poor living conditions, lack of adequate sanitation and hygiene, and poor education and health care in these regions (Stepek et al., 2006). The two main groups of parasites are the unicellular protozoa, such as malaria, and the metazoan helminths including cestodes, trematodes and nematodes (Coban et al., 2018).

Gastrointestinal (GI) nematode infections infect around 50% of the human population, the majority of which are children and pregnant women (Stepek et al., 2006). Infections induce great morbidity such as anaemia, malnutrition, secondary infections, and mortality in severe cases (Stepek et al., 2006). For the past 50 years, the proportion of the population infected with GI nematodes has been increasing causing the loss of 39 million disability-adjusted life years (Stepek et al., 2006). However, due to the asymptomatic nature of most GI nematode infections, they remain neglected in terms of public recognition and research funding (Stepek et al., 2006). Furthermore, GI nematodes induce chronic infections in livestock which makes them a concern for their economic consequences (Vande Velde et al., 2018).

Murine models are used to study GI nematode infections and amongst these well-established models is *Heligmosomoides bakeri* which has the advantage of being a natural pathogen of mice (Behnke et al., 2009a) that induces a Th2/Treg immune response in the host (Maizels et al., 2012) similar to human and livestock GI nematodes. Thus, it provides a useful laboratory model to study immune responses to GI nematodes for developing drugs and vaccines to fight infections in humans and livestock (Valanparambil et al., 2017). This model has recently been shown to impede fetal and neonatal development and thus it has been of considerable interest for studies on the impact of a maternal GI infection on offspring (Haque et al., 2019; Haque et al., 2018; Kristan, 2002b; Kristan et al., 2001; Odiere et al., 2010a; Odiere et al., 2012; Odiere et al., 2010b).

Recent studies on murine *H. bakeri* infections have demonstrated impacts on offspring physical development with shorter fetal crown rumps (Odiere et al., 2010a) lower body mass

in week old pups, and impaired organ development in pups (Kristan, 2002b). Generally, inflammation induced by maternal infections has been associated with cognitive impairment (Hagberg et al., 2015; Rosenberg, 2018) however, to date little is known about the impact of maternal *H. bakeri* infection on offspring brain development.

Murine brain development starts during the second week of embryonic life with the differentiation of the neural progenitor cells and extends for several weeks after birth (Rosenberg, 2018). Numerous factors impact brain development ranging from the molecular events of gene expression to environmental factors. However, neither genes nor environmental factors are prescriptive or determinative of outcomes (Stiles et al., 2010). Maternal infection in general imparts an environmental impact on the developing fetal and/or neonatal brain which often leads to neurological disorders (Hagberg et al., 2015). This impairment in neural development has been associated with inflammatory cytokines due to their ability to cross the placenta (Dammann et al., 1997) or transfer via nursing postnatally (Field, 2005), following which some can even cross the BBB or simply signal across the BBB to the neuro-immune system (Pan et al., 2011). Most of the inflammatory cytokines tied to cognitive impairment are of Th1 type such as IL-1β and tumor necrosis factors (TNFs) (Menza et al., 2010). Pregnancy itself induces Th2 type cytokines (Shah et al., 2019; Sykes et al., 2012) as do *H. bakeri* infections (Maizels et al., 2012) thus, the fate of brain development in conditions of cumulative Th2 cytokine signaling remains under speculation. Despite the favorable nature of Th2 type inflammation in promoting learning and cognition (Derecki et al., 2010) upregulated inflammation signals to the brain are mostly detrimental (Ofek-Shlomai et al., 2014) especially as they have a damaging impact on BBB permeability (Kim et al., 2012). Besides its role as a protective barrier, the BBB is a selective gateway for leukocyte infiltration (Pachter et al., 2003) and cytokine passage and signaling (Pan et al., 2011) making it vulnerable to inflammation.

Two studies have explored the impact of maternal *H. bakeri* infection on fetal and neonatal brain development. Differential expression of 96 fetal brain genes was reported just prior to parturition (Haque et al., 2018). In the postpartum brain, virtually no differential expression was detected in 2-day old pups but over 5500 genes were differentially expressed in 7-day old male pups (Haque et al., 2019). The affected genes in both cases were involved

in various functions including metabolic, immune, and neurodevelopmental process (Haque et al., 2019). Haque et al. (2019) focused on the upregulation of genes related to long term potentiation (LTP). LTP is a process by which synaptic connections between neurons become stronger with frequent activation. LTP is thought to be a way in which the brain changes in response to experience and is a mechanism mediating learning and memory (Baudry, 2001). A follow-up study in our lab using a Barnes maze has shown enhanced spatial memory of pups of infected dams in finding the maze escape hole (Sophia et al. 2021, manuscript in preparation), providing some evidence of phenotypic consequences of upregulated LTP gene expression.

Enhanced cognitive performance is associated with a heightened Th2 immune response especially through its hallmark cytokine IL-4 (Derecki et al., 2010). In addition, the Th2 immune response is naturally predominant in the brain since it dampens the potentially harmful Th1 inflammation (Lazarski et al., 2013) that is linked to cognitive impairment (Derecki et al., 2010). Taken together these findings have triggered our interest to observe the impact of the maternal *H. bakeri* infection on immune-related brain genes from the database and KEGG pathway analysis of Haque et al. (2019) with a focus on the Th2 immune response related genes. Our hypothesis of neuro-immune system activation was encouraged also by the decreased lymphoid organ mass and increased eotaxin in serum of pups of infected dams (Odiere et al., 2012).

Rationale and Objectives

Thus, our secondary data analysis focuses on the brain gene expression database and KEGG pathway analysis by Haque et al. (2019) with the goal of determining whether maternal infection had an impact on expression of genes associated with the neuro-immune system by focusing on the Th2/Treg immune response and the two principal mechanisms of signal transport across the BBB, leukocyte trans-endothelial cell migration and endocytosis. We devised a manual approach for examining KEGG pathway maps of interest in order to better our understanding of the KEGG pathway analysis results and integrate them with the gene expression data. This manual approach allowed us to add a layer of logic to the numeric evaluation of expression of genes and pathways in order to predict the subsequent consequences of the altered expression. This approach is essential to our understanding of the impact of the maternal infection on the immune system as immune-related KEGG pathway maps do not include all types of immune responses and do not include all steps and components of an immune response.

The literature has demonstrated the impact of maternal *H. bakeri* infection on the perinatal immune system through the upregulation of eotaxin in pup serum and decreased lymphoid organ mass (Odiere et al., 2012) along with the influence of nursing on worm expulsion in infected pups pre and post weaning (Darby et al., 2019). Furthermore, 96 genes were differentially expressed in the fetal brain with some involved in the immune system as a result of maternal *H. bakeri* infection. In addition, P7 pup brain gene expression was altered with an emphasis in Haque et al, (2019) on LTP, which is normally associated with a heightened Th2 immune response (Haque et al. 2019). As the parasite induces a Th2/Treg immune response in the infected host, we speculated that, via nursing, signals from the maternal infection would shift the immune related brain gene expression to mirror that of the mother's immune response by upregulating a Th2/Treg immune response.

Therefore, our first goal was to examine the impact of the maternal infection on immune-related brain gene expression to verify our hypothesis that the immune response in the pup brain mirrors the maternal immune response. Our second goal was to identify whether this

signal was more likely a molecule that crossed the BBB through endocytosis or a cell that crossed the BBB through leukocyte trans-endothelial cell migration.

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Chapter II - Literature Review

NEONATAL BRAIN

Murine Brain Development

The complex process of brain development in mice involves around 40,000 genes responsible for integrating and constructing neural circuits encompassing approximately 75 million neurons and 25 million glial cells (Sutcliffe 1988). The neural tube is the precursor of the central nervous system, its structure encompasses the brain and spinal cord. Formation of the neural tube starts on embryonic day 7.5 (ED) in mice. Neurogenesis starts approximately mid-gestation to form the brain mass through extensive proliferation of progenitor cells (Götz et al. 2005; Zhong et al. 2008). These processes are controlled by precise expression of a variety of genes and microRNAs (miRNAs) including miRNA let-7b, which can modulate the function of the cell cycle through cell cycle regulator cyclin-D1 and TLX receptors, expressed in the forebrain. Overexpression of miRNA let7b was associated with reduced neural differentiation and increased neural proliferation (Zhao et al., 2010). The neural tube cephalic end starts to divide into the 3 primary brain vesicles prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon on ED 9 (Chen et al. 2017). Shortly thereafter, the prosencephalon subdivides into the telencephalon (cerebral hemispheres) and diencephalon (mainly the thalamus and hypothalamus) (Chen et al. 2017). Synaptogenesis begins in the first week of birth and peaks during the second week of birth whereby on postnatal day (PD) 18 the brain becomes fully functional (Auvin et al., 2013). Brain development then continues into the first few weeks of birth ending at approximately week 3 (Chen et al., 2017).

The Blood Brain Barrier

The BBB acts as a selective barrier for the passage of cells and molecules from the blood vessel, through the endothelial cells lining the vessels, into the extracellular fluid of the central nervous system. Diffusion of material through the barrier relies on the immediate demands of the CNS and is monitored by adjusting the vascular diameter, which is a local and direct response to changes in needs such as, oxygen and nutrients. The BBB consists of complex functional anatomic units called the neurovascular unit (NVU) linking the nervous system represented by the neurons and the vascular system represented by the endothelial cells lining the blood vessels. The two systems communicate with the aid of supporting cells including the glial cells, mural cells (pericytes and myocytes), and components of the extracellular matrix (Figley et al. 2011). Among the first studies to demonstrate the existence of a selective barrier between the blood and the brain was that by Ehrlich in 1885 using a blue dye injected intravenously into a rat where all organs stained except for the brain (Saunders et al., 2014). Goldmann, his graduate student, later in 1913 verified the lack of barrier between the brain and cerebrospinal fluid (Saunders et al., 2014).

Although most regions of the CNS are vascularized with selectively permeable capillaries, the circumventricular organs' vasculature is a region in the brain located around the third and fourth ventricles containing fenestrated vessels, with a high permeability to solutes of higher passive permeability (Ganong et al. 2000). It consists generally of 2 different cell types the secretory made of pineal gland, median eminence, neurohypophysis, and the subcommisural organ and the sensory made of the subfornical organ, area postrema and the organum vasculosum of the lamina terminalis (Kaur et al. 2017). The circumventricular organ (CVO) facilitates the efflux of polypeptide hypothalamic hormones and permits substances that do not cross the BBB to trigger changes in brain function. This high permeability is important for the CVOs functions either in sensing blood solute concentrations or secreting molecules into the blood (Daneman et al. 2015). Part of the CVO is the choroid plexus, a secretory tissue of the brain ventricles that produces the cerebrospinal fluid (CSF) (Lun et al. 2015). Trafficking pathways for the entry of immune cells into the CSF remain unknown to date (Daneman et al. 2015). Trafficking pathways from

the blood into the brain have been described including endocytosis and diapedesis. Endocytosis propagates signals of impermeable cytokines and immunoglobulins across the BBB, a mechanism highly specific to the molecule involved. Diapedesis involves the passage of leukocytes between endothelial cells lining the blood vessels, a mechanism that involves loosening of the junctions holding endothelial cells together which risks compromising BBB integrity.

Leukocyte Trans-endothelial Cell Migration

The passage of leukocytes across the BBB is a multiple-step process known as trans-endothelial cell migration (TCM) or diapedesis (Collins et al., 2005; Muller, 2011). Leukocytes preferably cross into the brain from circulation at the CVO. Healthy individuals typically have 150,000 to 500,000 immune cells in the CSF (Schulz et al., 2005). In addition, the adhesion molecules ICAM-1 and VCAM-1, which are required for leukocyte entry into the CNS, are expressed on the choroid plexus epithelium (Steffenet al., 1996). Diapedesis is initiated by an inflammatory stimulus such as an infectious agent and inflammatory cytokines which increase surface expression of adhesion molecules such as selectins and cell adhesion molecules (CAMs). Circulating leukocytes recognize the site of infiltration through the selectins expressed on EC surface and upon interaction, leukocytes release chemokines to augment leukocyte surface integrin affinity to mediate adhesion to ECs (Muller et al., 2013). This activates adhesion and rolling of leukocytes onto ECs to establish firm adhesion onto the epithelium mediated by integrins and immunoglobulin super family proteins. Lastly, leukocytes squeeze in between ECs to migrate through the intercellular junctions of ECs into the brain via sequential interactions with several adhesive molecules including JAM-A/C (Woodfin et al., 2007; Woodfin et al., 2009)and PECAM (Muller et al., 1993). Junction integrity is therefore temporarily compromised to accommodate leukocyte migration (Muller, 2011).

Intercellular junctions at the BBB owe their protective selective characteristic to the “tight” junctions (TJs) that ensure their close adherence. Junctions are a collection of proteins that interact as a unit with the actin cytoskeleton of the adjacent cells to ensure controlled

permeability and adherence. TJ unit proteins include claudins, occludins, zona-occludens, and junction adhesion molecules (JAMs). Claudins are a class of over 25 members. Claudin knockout studies in mice have observed that claudin expression at epithelial barriers is tissue specific with cldn 1 at the epidermal barrier, cldn 16 in kidney epithelia, cldn11 in CNS myelin, and cldn 19 in peripheral myelin (Furuse et al., 2002; Gow et al., 1999; Hadj-Rabia et al., 2004; Hampson et al., 2008; Knohl et al., 2004; Miyamoto et al., 2005). Occludin is highly enriched in CNS ECs compared with ECs in non-neural tissue. However, occludin-deficient mice were shown to have a normal BBB function with observed calcification of the CNS. Therefore, occludins were suggested to regulate calcium flux across the BBB (Saitou et al., 2000). JAMs (JAM 1, 2 and 3) are members of the immunoglobulin subfamily that mediate interactions at tight junctions. JAMs are expressed on immune and non-immune cells and they regulate leukocyte extravasation and paracellular permeability (Johnson-Léger et al., 2002; Ludwig et al., 2005; Martìn-Padura et al., 1998). Notably, JAM4 was observed at the BBB in mice (Daneman et al., 2010). Zona-occludens (ZO-1, ZO-2, ZO-3) are cytoplasmic adaptors that link TJs to the actin cytoskeleton. TJs interact with aheren junctions (AJs) another junction that maintains BBB permeability. AJ units are calcium dependent intercellular junctions linked to the actin cytoskeleton. AJ units include caherins such as vascular endothelial (VE)-cadherin, and platelet EC adhesion molecules (PECAM)1 linked to the cytoskeleton by catenins (Daneman et al., 2015). The cadherin superfamily which includes cadherins, protocadherins, desmogleins, and desmocollins performs numerous functions in the CNS beyond barrier formation such as cell sorting, target recognition, synaptogenesis, and synapse function. There are over 100 cadherins, most of which are expressed in the developing vertebrate brain on immature cells, neurons, and glia (Letourneau et al., 2009). Protocadherins have over 20 genes in murines. Junction integrity is integral for maintenance of BBB integrity and function, expression of the various components is dynamic to ensure the possibility of self-repair (Cavey et al., 2009; Weber, 2012). Therefore, non-chronic infiltration causes temporary junction compromisation (Weber, 2012) whereas, chronic infiltration from a persistent infection as in encephalitis and meningitis could be detrimental if left untreated (Ellul et al., 2018; Hoffman et al., 2009).

Endocytosis

The highly selective permeability of the BBB is not only established through junctions but also through channels, transporters, vesicular transport, and lipid rafts. Transporters and channels mediate control of molecular flux. There are two main categories of transporters at the BBB. The first are efflux transporters, including MDR1, BCRP, and MRPs. These utilize the hydrolysis of ATP to transport their substrates opposite their concentration gradient. The second are highly specific nutrient transporters which can also remove waste products from the CNS into the blood (Daneman et al., 2015). As for vesicular transport it encompasses both entering the cell known as "endocytosis" and exiting the cell "exocytosis".

Endocytosis is important in our study as we are considering the influx of molecules. Endocytosis includes phagocytosis, pinocytosis, and receptor mediated endocytosis. Phagocytosis is the uptake of large particles such as microorganisms and dead cells by large endocytic vesicles called phagosomes. The two main phagosomes in mammals are the macrophage and the neutrophil both of which eliminate microorganisms from infected cells. Pinocytosis is the uptake of primarily lipid particles suspended in the extracellular fluid through the invagination of the cell membrane to form a vesicle that pinches off the membrane into the cell (Stillwell, 2013). Pinocytosis is thus, non-specific and consumes a lot of ATP making it less energetically favorable than receptor-mediated endocytosis (RME) (Stillwell, 2013). Generally, RME is initiated by the formation of a receptor-substrate complex where the membrane invaginates and a coating such as clathrin or caveloin may or may not form around the developing vesicle (Mayor et al., 2014). As the vesicle forms it buds off the membrane and sheds its coat to be recycled; thereafter an endosome merges with the vesicle to recycle the receptor after which the vesicle shuttles the substrate to its destination. As RME is highly specific, there are numerous variations of the mechanism depending on the substrate it is carrying. RME transports various molecules including metabolites, hormones, proteins, and even viruses. Clathrin-mediated endocytosis is utilized by many cell surface receptors such as G protein-coupled receptors, tyrosine kinase receptors (epidermal growth factor receptor, platelet-derived growth factor receptor, insulin receptor), and other non-kinase receptors (low-density lipoprotein receptor, transferring receptor)(Kaksonen et al.,

2018). On the other hand, caveolin dependent endocytosis utilizes lipid rafts formed of cholesterol and sphingolipids to carry out protein or lipid transport including cholera toxin, glycosylphosphatidylinositol (GPI)-anchored proteins, endothelin receptor and growth hormone receptor (Deurs et al., 2003; Nabi et al., 2003; Nichols et al., 2001). Therefore, lipid rafts are involved in specialised signaling such as T cell antigen-receptor signaling, B cell receptor signalling, and insulin-mediated translocation of glucose transporter GLUT4 (Alonso et al., 2001). Lipid rafts can also serve as signaling centers for nitric oxide, calcium, G protein-coupled receptors, protein tyrosine kinases, and even viral invasion among others (Anderson et al., 2002; Deurs et al., 2003).

In our study, we focus on the mechanism of endocytosis through the KEGG pathway map which is a general representation of the broad genetic components of the mechanism. Specifically, we are interested in endocytosis of TGF-β, immunoglobulins, and IL-4c. TGF-β utilizes clathrin dependent endocytosis where it initiates signaling by binding to its cell surface Ser/Thr kinase receptors, following receptor subunits are phosphorylated to facilitate docking of receptor-regulated Smad proteins (R-Smad) that mediate cell signaling to alter gene expression (Chen, 2009a). Immunoglobulin transport mechanism is highly specific to the immunoglobulin, utilizing both clathrin dependent and independent endocytosis mediated by various receptors including neonatal Fc receptor (FcRn), the low density lipoprotein receptor related protein (LRP), the transferrin receptor (TfR), and the insulin receptor (IR) (Xiao et al., 2013). IL-4 utilizes clathrin independent endocytosis and activates the JAK/STAT pathway where the internalization of IL-4R subunits utilizes Rac1 and Pak (Kurgonaite et al., 2015). When bound by IL-4, the IL-4Rα chain can recruit the IL-2Rγ chain to form type 1 IL-4R complex. Alternatively, the IL-4-bound IL-4Rα subunit can recruit the IL-13Rα1 chain to form type 2 complex, which is also induced when IL-13Rα1 bound by IL-13 recruits IL-4Rα (Junttila, 2018). Generally, type 1 IL-4R signalling is restricted to cells of hematopoietic origin, whereas type 2 signalling is more widely distributed (Kurgonaite et al., 2015).

Neuro-immune system

Innate vs Adaptive Immune System

The CNS environment is anti-inflammatory with high local concentrations of cytokines that suppress inflammation such as TGF-β and IL-10 (Burmeister et al., 2018), also gangliosides toxic to T cells are enriched in the CNS (Jales et al., 2011; Sandhoff et al., 2013). Therefore, the CNS relies on the innate immune system to play a critical role in recognizing and responding to pathogens. The adaptive immune system is activated upon encountering a signal to initiate an immune response. Major contributors to the innate immune system include epithelial cells, which prevent pathogen entry, phagocytes such as neutrophils and macrophages, the complement system, and pattern recognition receptors. In contrast to the innate immune system, the adaptive immune system provides highly specific responses to the invading pathogen. Although dendritic cells (DCs) play a major role in antigen presentation to T cells in the periphery they are not common in the CNS (D'Agostino et al., 2012). Nevertheless, in a state of inflammation, cells expressing a DC cell surface (e.g., CD11b, CD11c) have been detected in the meningeal coverings and choroid plexus (D'Agostino et al., 2012). Predominant cells involved in adaptive immunity are B and T lymphocytes.

The immune system plays various roles in the CNS besides protection against pathogens including neural development, axon guidance and migration, synaptic plasticity, long-term potentiation, sensory and motor learning, anxiety, sensory, and motor behaviors (Marin et al., 2013; Tanabe et al., 2018).

Immune Surveillance

To detect threats in the brain's environment, immune surveillance is continuous and plastic, which is necessary to maintain the health of the CNS environment. Immune cells such as microglia extend their feet throughout their environment almost like a spider's web (Jonas et al., 2012). These extensions allow microglia to monitor the brain environment. Hence, microglia are the predominant cell type comprising 80% of brain immune cells (Morimoto et al., 2019). Queues from the environment are then relayed to recruit immune cells. On the other hand, dendritic cells, T cells and circulating macrophages monitor the region at the BBB and CSF barrier. Lastly, in the CSF memory T cells circulate (Smolders et al., 2018).

Immune Response

Immune responses in the CNS are common and are initiated by resident microglia, astrocytes, and mast cells utilising pathogen-associated molecular patterns (PAMPs) by different classes of pathogen recognition receptors (PRRs) such as TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors, scavenger, complement, and mannose receptor to act as antigen presentation cells (Kigerl et al., 2014). Each of these PAMPs is specific to the pathogen and consequently releases a different set of inflammatory cytokines for immune cell recruitment. A common example is the activation of TLRs on microglia or astrocytes which would activate NF-κB signaling inside the cell, resulting in increased transcription of genes encoding IL-1 family cytokine (Kielian, 2006). Cytosolic inflammasome assembly activates nucleotide-binding domain leucine-rich repeat–containing receptors (NLRs) which activates caspase 1 leading to inflammatory cytokine release such as IL-1β, IL-6, CCL2, CXCL1, CCL20, CCL3, IFN-β, IFN-γ, and TNF (Jha et al., 2009). Thus, circulating immune cells such as T cells are recruited to the site of inflammation. Post-infiltration the recruited T cells are primed through direct interaction with MHCII on APCs to induce the required immune response for pathogen clearance (Geyer et al., 2019).

The specific combination of released cytokines by the APCs (microglia, astrocytes, mast cells) induces a unique immune response. Th1 activation is induced by APCs releasing IL-12 and

IL-23 to induce T cell maturation (Constantinescu et al., 2005) then together with IL-12, IFN-γ drives T-cell maturation toward a Th-1 phenotype. Other studies observed that IFN-γ-activated astrocytes are more efficient at stimulating Th2 rather than Th1 cells, which leads to IL-4 expression (Aloisi et al., 1998). In addition, expression of IL-4 and IL-10 by APCs, reduces Th1 cytokine expression and induces naïve T cells to differentiate into Th2 cells (Kaiko et al., 2008). However, studies have demonstrated the role of IFN-γ and TNF-treated astrocytes in activating Th1 or Th2 CD4+ T cells through upregulated expression of MHC-II, B7-1 and B7-2 for antigen presentation (Carpentier et al., 2005). As for Th17 it is activated by IL-23, in the presence of TGF-β and IL-6 (Bettelli et al., 2006; Mangan et al., 2006) where TGF-β upregulates IL-23R expression (Mangan et al., 2006). Although TGF-β is predominantly associated with suppression of Th1 and Th2 responses through the development of Treg cells (Bettelli et al., 2006), it also plays a crucial role in progression of the Th17 response.

Maternal Infection and Perinatal Brain Development

Maternal infections whether bacterial, viral, or parasitic impose a high risk of cognitive impairment on the fetal brain through inflammatory cytokines such as IL-1β, TNF- α, INF- γ, and IL-6 (Golan et al., 2005) which are mostly associated with Th1 type inflammation. It is important to take into consideration that these cytokines can originate from maternal, placental, and fetal or neonatal circulation to reach the developing perinatal brain. Neonatal intracerebral administration of IL-1β resulted in acute brain injury, as indicated by enlargement of ventricles bilaterally, apoptotic death of oligodendrocytes (OLs) and loss of OL immunoreactivity in the neonatal rat brain (Fan et al., 2009). As for TNF-α, previous studies have shown that TNF-α is involved in cellular differentiation, neurogenesis and programmed cell death during the development of the central nervous system (Babri et al., 2014). By binding to its receptor INF- γ, activates the JAK/STAT pathway and consequently downregulates neurogenesis, gliogenesis (Kulkarni et al., 2016). IL-6 has been observed to have potent influence on fetal brain development in response to maternal immune activation (Smith et al., 2007) affecting brain development, learning, and memory (Balschun et al.,

2004). Through STAT (signal transducers and activators of transcription) signaling IL-6 was observed to downregulate neurogenesis and gliogenesis (Todd et al., 2016). In addition, IL-6 triggers brain endothelial cell division and migration in normal physiological condition (Fan et al., 2008). Moreover, IL-6 regulates the expression of brain-derived neurotrophic factor (BDNF) that promotes neural development where higher levels of IL-6 were associated with lower levels of BDNF in embryos and in placentas of pregnant mice 24 hours post induction of inflammation (Gilmore et al., 2005). Therefore, due to the numerous functions IL-6 plays in neural development it has also been associated with cognitive impairment in offspring (Harry et al., 2006). A microarray-based study in mice observed that altered gene expression in the fetal brain due to maternal inflammation was reversed by eliminating maternal IL-6 (Smith et al., 2007).

On the other hand, helminth infection in pregnancy induces Th2/Treg cytokines including IL-4 and TGF- β (Reynolds et al., 2012) which promote neural development and are associated with enhanced cognitive performance. A study in our lab observed the upregulation of long-term potentiation (LTP) in brain of P7 pups which was associated with upregulated expression of IL-4 and foxp3 (Haque et al., 2019). IL-4 was associated with promoting learning and memory as observed through the impairment of cognitive performance in IL-4−/− mice compared to wild type mice while learning and performing a Morris water maze (Derecki et al., 2010). TGF-β promotes neural development by mediating axon specification where also TGF-β receptor signaling guides neuronal axon initiation in the brain (Yi et al., 2010). In addition, by activating Treg immune response TGF-β mediates dampening of Th1 inflammation (Littringer et al., 2018) to protect from the inevitable damage of neuro-inflammation (Kim et al., 2012). One study observed an association between maternal helminth infection and poor cognitive function in infants (Mireku et al., 2015). Studies in our lab recorded higher concentrations of interleukin (IL)-1β and TNF-α in maternal and fetal serum and of TNF-α in amniotic fluid in *H. bakeri* infected pregnant mice (Starr et al., 2014). Therefore, the impact of maternal helminth infection on fetal and neonatal brain development remains unknown.

EXPERIMENTAL MODEL

Our experimental model involves a murine *H. bakeri* infection in pregnant mice. *H. bakeri* is a GI helminth parasite that occurs naturally in the GI of mice. It was referred to initially as *Nematospiroides dubius*, then *Heligmosomoides polygyrus.* Most recently, the species name has changed to *H. bakeri* as the name for the strain common in laboratory mice (Behnke et al., 2009b). These changes in nomenclature are based on morphologic, metabolic, and genetic characteristics (Behnke et al., 2010). The discontinuation of the usage of the name *N. dubius* has succeeded (Behnke et al., 1991) however, *H. polygyrus* and *H. bakeri* continue to be used interchangeably in the literature.

Life Cycle

The life cycle of *H. bakeri* involves 4 larval stages followed by the reproductive adult worm stage, the third larval stage (L_3) being the infective stage (Rausch, 2010). The larvae grow to become infective within a week post egg hatching. Upon ingestion of L_3 larvae, the latter migrate to the small intestine and penetrate the submucosa, where they develop and then emerge from the submucosa and mature into adult worms within 7-9 days, resulting in an average prepatent period of 9-12 days prior to egg laying (Rausch, 2010). Adult male and female worms coil around the intestinal villi (Reynolds et al., 2012) where they mate with each female releasing 1200-1500 eggs per day (Rausch, 2010). The parasite remains confined in the intestine with no recorded potential for migration to other organs.

Immune Response

H. bakeri is known to induce a Th2/Treg immune response in the host (Maizels et al., 2012). After larval inhabitation of the intestinal submucosa the resulting tissue damage is sensed by mucosal epithelial cells and keratinocytes and alarmins are produced such as interleukin 25 (IL-25), thymic stromal lymphopoietin (TSLP), and IL-33. Alarmins induce activation and differentiation of Th2 immune cells which release a number of cytokines including IL-4, IL-5, IL-9, and IL-13. IL-4 and IL-13 activate goblet cells to produce mucus while also triggering smooth muscle cell contractions to promote worm expulsion. IL-5 activates eosinophils (Finkelman et al., 1997; Lawrence, 2003) and the differentiation of AAMs (Reyes et al., 2007) and along with IL-9 activates basophils and mast cells (Faulkner et al., 1997; Faulkner et al., 1998) which exert anti-parasitic action using antibodies or complements. IL-25 is also produced by tuft cells in the gut epithelium leading to activation of Th2 cells and ILC2s, which promote the IL-13 response (von Moltke et al., 2016). IL-13 consequently amplifies the activation signal of tuft and goblet cells to promote an effective anti-helminthic mechanism (von Moltke et al., 2016). The Th2 response also drives immunoglobulin class switch recombination in B cells to produce IgE and IgG1 (Rausch, 2010). Granulomas form carrying neutrophils, macrophages, dendritic cells, eosinophils, AAMs, and Th2 cells form at the site of the invasion (Reynolds et al., 2012). Th2 effectors including serum IgE, eosinophils, mast cells, goblet cells promote mucous secretion, ion and fluid influx, RELM-β secretion, and smooth muscle contraction to facilitate intestinal worm expulsion (Hashimoto et al., 2009; Herbert et al., 2009; Tu et al., 2008). Helminth infection further induces the expansion of regulatory T cells (Tregs) and immunoregulatory monocytes along with higher levels of immunosuppressive cytokines IL-10 and TGF-β (McSorley et al., 2012). The TGF-β mimic produced by the parasite also exacerbates the Treg immune response (Johnston et al., 2017). *H. bakeri* infection also stimulates Foxp3 mRNA expression in T cells which activates T regulatory cells which release IL-10 and TGF-β (Rausch, 2010). In our case, pregnancy and infection coincide in the mothers which is a condition observed to exacerbate the Th2 response by further increasing IL-4 concentration in pregnant mice (Odiere et al., 2010a).

Infection Protocols and Value as an Experimental Model

Various infection protocols utilize this parasite each with a specific outcome. Primary infections involve a single oral dose of L3 larvae (Valanparambil et al., 2017). Primary infections are usually chronic due to the parasite's ability to induce diverse regulatory mechanisms including tolerogenic dendritic cells (DCs), alternatively activated macrophages (AAMs), regulatory T cells (Tregs), and regulatory B cells that mediate immune evasion and mediate tolerance to the parasite (Finney et al., 2007; Valanparambil et al., 2017). The outcome and length of the infection is strongly influenced by the genetic background of mice, as strains vary in susceptibility to chronic infection (Monroy et al., 1992). This protocol is ideal for studying chronic immune responses to the infection.

A challenge infection protocol is cleared more rapidly than a primary infection as the first oral dose of larvae is followed by drug treatment to remove the first infection and then a second "challenge" dose typically a week after treatment (Valanparambil et al., 2017). This allows sufficient time for the host to activate an adaptive immune response prior to treatment, but removes the adult worms that release immunosuppressive molecules, allowing the adaptive response to the challenge infection to efficiently clear the infection. The challenge protocol is ideal for studying the adaptive immune response and its elements specific to clearing the worms.

The trickle infection protocol is of particular value because it mimics the more natural transmission process whereby mice are repeatedly exposed to *H. bakeri* infection throughout their lives which allows them to acquire immunity (Reynolds et al., 2012). Trickle infection protocols involve repeated infections with 10 to several hundred larvae at a time that establish a persistent infection (Brailsford et al., 1992). Repeated exposure to larvae provides ongoing antigenic stimulation that results in the natural Th2/Treg response (Brailsford et al., 1992; Maizels et al., 2012).

It is difficult to extrapolate to nature from experiments utilizing a single dose infection (Brailsford et al., 1992). Therefore, the trickle infection used in our lab on CD1 outbred mice was designed to examine the impact maternal *H. bakeri* infection on neonatal and fetal development. This animal model is successful in producing results close to real-life

situations since the mice are outbred thus, they retain a level of genetic heterogeneity unlike inbred mice and the trickle infection protocol resembles the multiplicity of infections mice face throughout their lives (Tuttle et al., 2018).Therefore, there is a level of confidence in experimental observations as it is more natural rather than designed and manipulated. This model and its observations could help us better treat helminth infections in pregnant mothers and understand its impact on the offspring.

Impact of Maternal Infection on Fetal and Neonatal Development

The maternal *H. bakeri* infection has been observed to impact various aspects of fetal and neonatal development despite its localized residence in the maternal GI tract and lack of transfer of infection to the offspring. In contrast to maternal protozoan, viral and bacterial infections that can lead miscarriages (Depino, 2006) and/or cognitive and physical impairment in the neonates (Aguilar-Valles et al., 2020; Cordeiro et al., 2015; Mac Giollabhui et al., 2019). *H. bakeri* infection has been shown to be both detrimental and potentially beneficial for the neonate.

Impact on Growth and Organogenesis

Inflammation induced by maternal infections is known to disrupt fetal development, cause organ damage, pre-term birth, and even intra-uterine fetal death (Adams Waldorf et al., 2013). These implications of maternal inflammation are induced by either direct transfer of infection to the fetus, by the activated maternal T cells crossing to the fetus to release inflammatory cytokines, or by pathogenic molecules activating placental tissue to release inflammatory cytokines. Commonly detected inflammatory cytokines in the amniotic fluid are IL-1β, IL-6, IL-8, TNF-α, CXCL8, and CXCL10. Maternal *H. bakeri* infection does not cross-infect the fetus thus, the damage inflicted is often less severe. Nevertheless, maternal *H. bakeri* infection has been observed to reduce fetal crown-rump length perhaps associated with higher amniotic fluid IL-1β (Odiere et al., 2010a) and also reduce length and mass of pups up to 21-days old (Odiere et al., 2010b). Furthermore, maternal infection was observd

to alter placental gene expression which was associated with altered fetal development. One study observed the downregulation of 109 transcripts, including genes related to oxidative phosphorylation, and upregulation of 214 transcripts, including genes involved in ATP binding and hemopoiesis (Starr et al., 2016). Up-regulation of hemopoiesis genes was associated with increased placental mass previously reported in *H. bakeri* infected mice (Starr et al., 2016). In addition, postnatally the pups of infected mothers were reported to have a larger liver and stomach (Kristan, 2002b) but pup body composition and body mass at sexual maturity were unaffected (Kristan, 2004).

Impact of Maternal Infection on Fetal and Neonatal Immune System and Resistance to Infection

Maternal *H. bakeri* infection has been observed to impact the neonate immune system. One study associated the maternal infection with decreased lymphoid organ mass and increased eotaxin in serum of pups one to two weeks old, which is responsible for recruiting eosinophils into sites of inflammation (Odiere et al., 2012). A cross-fostering study demonstrated that infected pups, whether born to immune or naïve dams, cleared the infection better if nursed by immune dams than by naïve dams (Harris et al., 2006). This observation indicated that nursing imparted resistance to infection in pups which was associated with enhanced IgG concentrations in milk of immune mothers. Another study showed that post-weaning offspring from parasitized mothers were able to clear lower intensity infections of up to 120-140 worms but not higher intensity infections (Kristan, 2002a, 2004). Taken together, these studies encourage speculation of the role of nursing from infected mothers in the transfer of immune altering agents that impact the neonate immune system.

Brain Transcriptome

Alteration in brain gene expression is a newly observed phenomenon in studies on pups born to infected dams. Prenatally, one study noted the differential expression of 96 genes in the

fetal brain involved in metabolic processes, developmental processes and the immune system as a result of maternal *H. bakeri* infection (Haque et al., 2018). Postnatally, Haque et al. (2019) demonstrated the differential expression of over 5500 genes on P7 involved in long-term potentiation and various biological functions including metabolic, neurodevelopmental, and immune system. Taken together, these findings demonstrate the impact of maternal infection on the transcriptome starting prenatally and continuing postnatally. The impact of these alterations in transcriptome remains speculative until further validation through follow-up experimental studies.

Signal Sources that Could Alter Brain Gene Expression in the Pup

In order to understand the alteration in pup brain gene expression we must examine the possible channels that could relay signals to the pup brain. In the case of P7 neonates, the only source of signal communication between the pups and their mothers at that age is the breast milk. Molecules and cells in the milk could originate from the parasite directly or the activated immune system of the mother (Dauby et al., 2012; Harris et al., 2006; Kutty, 2014). These signals could be relayed in various manners. The first scenario is for cytokines, immunoglobulins or other molecules from the milk to cross from the GI tract into neonatal circulation and enter the brain via endocytosis. The second scenario is for these same immune modulators to activate the immune system of the neonate such as T cells and the latter would move through circulation to infiltrate the BBB. The third scenario is for maternal immune cells to cross the GI tract to infiltrate the BBB which would probably be the least likely scenario considering initially the difficulty of surviving the GI environment.

Another source for the signal to the pup brain could be through the altered microbiome colonization in the pup. At P7 *Lactobacillus* was the dominant genus in pups of infected dams (Haque et al. 2021, under review). The critical role of the microbiome in the gut-brain axis gives it influence over various functions including development, metabolism, homeostasis, motor and sensory function, and cognition especially through its product short chain fatty acids (SCFAs) (Dalile et al., 2019; Silva et al., 2020)which can alter gene expression (Astakhova et al., 2016). SCFAs are produced by *Lactobacillus* (Hati et al., 2019; Nagpal et al.,

2018). This critical role makes the altered microbiome a candidate for altered brain gene expression. In addition, the altered maternal microbiome in response to *H. bakeri* infection also would lead to altered production of metabolites by the microbiome (Haque et al. 2021, under review) which opens a wide array of possibilities including altered breast milk composition that would impact the pup.

Immune-related Constituents in Breast milk and their Function

Studies from the literature have demonstrated the impact of molecules contained in breast milk on neonate brain development (Anderson et al., 1999; Brown Belfort, 2017) and it remains a communication vehicle between the maternal infection and the pup brain making it our focus when speculating signal sources. Breast milk is a nutritional fluid for neonates rich with cytokines, immunoglobulins, soluble receptors, growth factors, short-chain fatty acids, vitamins, oligosaccharides, hormones, enzymes, lactoferrin, dietary antigens, and microorganisms (Dawod et al., 2019). These components are either derived locally from mammary gland epithelial cells or from the mother's circulation. The concentration of milk components changes throughout the different stages of breastfeeding from colostrum, the first fluid produced after delivery, to transitional milk, produced in the middle lactation period, to mature milk, which lasts until the end of the lactation period (Ballard et al., 2013). These components are implicated in neonatal system development including the nervous, endocrine, and immune system (Ballard et al., 2013; Cacho et al., 2017; Henrick et al., 2017). The immune modulating contents include TGF-β, IL-10, IL-6, IL-1β, TNF, IFN-γ, IL-4, IL-5, IL-12, IL-13, G-CSF, GM-CSF, M-CSF, IgA, IgG, and IgM. Our immune modulators of interest are TGF-β, IL-4, and IgG each of which plays a different essential function. TGF-β is the most abundant cytokine in milk with various functions in the neonate. TGF-β mediates the conversion of naïve T cells into antigen-specific T regulatory cells (Tregs) and inhibits Th17 differentiation (Coombes et al., 2007; Macpherson et al., 2006; van Vlasselaer et al., 1992). Tregs acts through their hallmark cytokine TGF-β to enforce tolerance to allergy via inhibition of allergen specific Th2 responses (Lim et al., 2005). IL-4 is less explored as a milk component however, it complements the anti-inflammatory function of breast milk as it is

a Th2 hallmark cytokine (Böttcher et al., 2000; Buescher, 1999; Goldman et al., 1986; Thai et al., 2020; Tomicić et al., 2010). Therefore, it has a prominent role in cognitive function, learning, and memory. Derecki et al. (2010) observed that IL-4 deficient mice, and mice injected with anti-VLA4 antibodies to inhibit T cell migration before and after mice were placed in a Morris water maze, required a longer acquisition time and had impaired memory. Immunoglobulins such as IgG, IgM, and IgA provide passive immunity for the neonate and mediate development of mucosal immunity in the GI (Mehra et al., 2006). In humans, IgGs cross the human placenta through neonatal Fc receptors expressed on syncytiotrophoblast cells as early as the 13th week of gestation (Palmeira et al., 2012; Pierzynowska et al., 2020).

Breastfeeding has been associated with enhanced cognitive performance where breastfed subjects reported a higher IQ, cognitive skills, and motor development (Horta et al., 2015; Kim et al., 2020; Tinius et al., 2020). These observations are related to the observed impact on neural connectivity, myelin formation, deep nuclear gray matter volume, and white matter development (Belfort et al., 2016; Blesa et al., 2019; Deoni et al., 2013). The enhanced cognitive performance can be linked to the role of the immune system in promoting neural development. Several types of cells have been observed in the neonatal brain. CD11c+ cells including monocytes, macrophages, dendritic cells, granulocytes, and NK cells which were observed along the ventricles and within the adjacent parenchyma on E16 and postnatal day 2 (Bulloch et al., 2008). One example of an immune cell with an active role in the CNS is the B-1a cells which accumulate in the neonatal brain where they mediate IgM secretion and promote the oligodendrocyte progenitor cell proliferation through the Fc receptors for IgM (Baumgarth, 2011). Antibody depletion of B-1a cells diminishes the number of oligodendrocytes and results in reduction of myelinated axons in neonatal mouse brains (Baumgarth, 2011; Tanabeet al., 2018).

Scientific Approach to Inferences from Brain Gene Expression Data

KEGG (Kyoto Encyclopedia of Genes and Genomes) was developed as an integrated database resource for interpretation of completely sequenced genomes generated by genome sequencing and other high-throughput experimental technologies via KEGG pathway

mapping (Kanehisa et al., 2016). It consists of 19 highly interconnected databases, containing genomic, chemical and phenotypic information. The pathways are manually curated by experts using literature.

The KEGG pathway analysis performed on the original database of over 5500 genes hence was a mapping process in search for the impacted biological functions. This mapping process is therefore more reliant on meeting a threshold for the number of up- or down-regulated expression of genes in order to report differential expression of the pathway in the KEGG analysis. One challenge of this numeric approach is provided by the example we previously reported where the insulin signaling pathway was both up- and down-regulated in response to maternal *H. bakeri* infection (Haque et al., 2019). A second example of how differential expression of a pathway may not be informative is the Th1 and th2 cell differentiation that involves the process of activating both Th1 and Th2 cell differentiation but Th1 and Th2 are considered a dichotomy with local responses being either Th1 or Th2 as the upregulation of one dampens the other. Therefore, the up or downregulation of this pathway is not enough to understand what is going on because it does not clarify which of the two response is being specifically up or downregulated. A third example is provided by some immune system related KEGG pathway maps that consider only the first step not the full process. Th1 and Th2 cell differentiation only activates cell differentiation and this KEGG pathway is decoupled from subsequent cascade of cytokines, B cell activation, and immunoglobulin release. Thus, reliance on differential regulation of KEGG pathways only may be insufficient, and a logical analysis of the sequence of expression of inhibitors, activators, or even biologically redundant molecules shared amongst many pathways can be helpful.

This challenge drove us to add a more structured manual approach for interpreting gene expression both in differentially and in non-differentially expressed KEGG pathways in order to understand the logical pattern of expression and its biological consequences.

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Chapter III - Upregulated Th2/Treg Brain Gene Expression in Pups of Nematode-Infected Mice through Enhanced Leukocyte Trans-Endothelial Cell Migration Across the Blood Brain Barrier

Nawal El Ahdab[1], Manjurul Haque[1], Kristine G. Koski[2] and Marilyn E. Scott[1]

Institute of Parasitology[1], School of Human Nutrition[2], McGill University (Macdonald Campus), Ste-Anne-de-Bellevue, Québec, H9X 3V9, Canada

Abstract

A murine maternal infection with the intestinal parasite *H. bakeri* that induces a Th2/Treg response during pregnancy and lactation was previously reported to alter expression of over 5500 genes in the brain of their 7-day old male pups. To explore evidence that immune-related elements and the transport of their signals across the blood-brain barrier of neonates was altered, we mined the gene expression database for genes in relevant KEGG pathways regardless of whether or not the pathway itself was differentially regulated by maternal infection. Although the Th1/Th2 cell differentiation KEGG pathway was not differentially expressed in response to maternal infection, brain gene expression of the Th2 response cascade and its product, IL-4 was upregulated. Evidence of upregulation of the Treg response included upregulation of TGF-β family genes. Consistent with this, expression of several Th1 cytokines including IL-1β, as well as receptors and their related proteins were downregulated. The endocytosis pathway that transmits receptor-mediated cytokine and immunoglobulin signals to the pup brain was upregulated in neonates of infected dams, but genes associated with endosome formation component were downregulated suggesting impaired vesicular transport into the pup brain. The leukocyte trans-endothelial migration KEGG pathway was also upregulated, as was expression of integrin genes which mediate leukocyte attachment and paracellular diapedesis. This indicated that maternal infection may promote movement of leukocytes across the neonatal blood-brain barrier. Thus, we hypothesize that maternal infection may promote a Th2/Treg environment in the pup brain through migration of Th2/Treg cells across the blood brain barrier.

Introduction

Maternal gastrointestinal (GI) nematode infections have been observed to impact immune development of neonates (Odiere et al., 2012). GI nematodes have been shown to alter the concentration of cytokines and immunoglobulins in breast milk which in turn enhanced neonatal immune development (Darby et al., 2019; Harris et al., 2006; Hrdý et al., 2012; Le Doare et al., 2018). In addition, GI nematode infection during pregnancy has been shown to alter neonatal immune response to vaccines (Flügge et al., 2020) and allergens (Afifi et al., 2015; Lynch et al., 1987), and acquired immunity against conditions such as eczema (Mpairwe et al., 2014) and asthma (Masters et al., 1985; Ponte et al., 2014; Straubinger et al., 2014). Furthermore, the enhanced systemic immunity has been shown to promote long-lasting immunity to nematode infections in the offspring (Darby et al., 2019). Whether these systemic immune consequences are transmitted to vital organs such as the neonatal brain is unknown.

Breastfeeding is essential for neonatal immune development, in part because breast milk transports antigens, antibodies, immunoglobulins, cytokines, and leukocytes that enhance mucosal protection against inflammation and infection (Jackson et al., 2006; Slade et al., 1987). Both cellular and molecular components of breast milk can cross the blood brain barrier (BBB) through endocytosis (Smith et al., 2006) and trans-endothelial leukocyte migration (Carman, 2009), also known as diapedesis. Cytokines (Pan et al., 2011) and immunoglobulins (Filippi, 2016) are transported across the BBB by binding to surface receptors on endothelial cells. The resulting complex triggers an endocytosis signalling cascade that induces vesicle formation, budding, and intracellular transport (Gao et al., 2005). Immune cells not only can cross the BBB by endocytosis, but also through diapedesis of activated leukocytes between endothelial cells (Carman, 2009). Proteins such as integrins on the surface of leukocytes dock with cell adhesion molecules (*CAMs*) and other proteins on the surface of endothelial cells (Bishara, 2012). This leads to movement of the actin cytoskeleton (Schnoor, 2015) inside endothelial cells and loosening of tight junctions that allows leukocytes to squeeze between endothelial cells (Muller, 2011) and enter the brain. This process temporarily compromises junction integrity (Muller, 2011) after which

junction gene expression is upregulated to restore endothelial adherence and re-establish normal BBB integrity (Shen, 2012).

Two recent studies from our lab have shown that the neonatal brain is directly affected a murine intestinal nematode *Heligmosomoides bakeri* that remains restricted to the maternal intestine (Haque et al., 2019; Haque et al., 2018). In this model, pregnant mice received a repeated trickle infection with infective larvae during gestation and early lactation and gene expression of the late-term fetus and young male pups was explored. Whereas less than 100 genes were differentially expressed in the fetal brain (Haque et al., 2018), over 5500 genes were differentially expressed in brains of 7-day old (P7) pups. At P7, a key finding from KEGG pathway analysis was the upregulation of long-term potentiation and related pathways that promote synaptogenesis and spatial learning and memory (Haque et al., 2019). Upregulation of long-term potentiation is typically observed when the brain is exposed to Th2 conditions (Gadani et al., 2012) and IL4 knock-out mice have impaired neural development, learning, and memory (Derecki et al., 2010). Of note, *H. bakeri* infection induces Th2/Treg response in the infected host (Brosschot et al., 2018), raising the possibility that maternal infection may induce a Th2/Treg response in the pup brain.

The goal of this study was to explore the brain gene expression data collected by Haque et al (2019) for evidence that maternal *H. bakeri* infection altered the neonatal brain immune system at P7. The first specific objective was to determine whether expression of genes related to innate and/or adaptive immune responses were differentially expressed, with a focus on Th2/Treg genes. The second specific objective was to explore likely routes of transport of immune elements across the BBB. We considered endocytosis, leukocyte trans-endothelial cell migration and junction pathways and their related genes. Starting with the KEGG pathway analysis that had been performed by Haque et al (2019) and using more stringent cut-offs for differential expression, we devised a novel approach that manually inspected relevant genes in immune and blood brain barrier related KEGG pathway maps in order to find logical sequences of differential gene expression that would be expected to shift function.

Methodology

Source of Data

This study was a secondary analysis of immune-related and BBB neonatal brain genes that were differentially expressed in response to a maternal infection with the GI nematode *H. bakeri* (https://www.nature.com/articles/s41598-019-40729-w#Sec2) (Haque et al, 2019). Timed pregnant CD1 outbred mice had been given a repeated trickle infection of 100 ± 3 L3 larvae of *H. bakeri* or a sham infection of distilled water through oral gavage on embryonic days E7, E12, E17, and postpartum day 3 (P3). The male pups were sacrificed on P2 and P7 and total brain RNA from one male pup per litter was sequenced via Illumina HiSeq2000 sequencer. The paired end BAM files were uploaded to NetworkAnalyst and analysed to identify genes that were differentially expressed in the pup brain in response to maternal nematode infection with adjusted P value < 0.05 and fold change > 1. Exploration of the KEGG pathway database in NetworkAnalyst provided a list of the differentially expressed pathways with biological significance.

Procedures for Secondary Analysis of Differentially Expressed Genes in KEGG Pathway Maps

For our secondary analysis, we applied more stringent P value (<E-5) and log fold change (>1.2) cut-offs for differential gene expression than had been used by Haque et al (2019) to lower the false recovery rate (McCarthy et al., 2009).

We compared this more focussed gene expression database with genes in KEGG pathway maps (Fig 1). We focused on the ligand and receptor coding genes that activate the pathway and genes that code for final products rather than intermediates and signaling molecules that have high biological redundancy among pathways and less internal consistency in cascade expression. This allowed us to infer the implications of changes in gene expression for functions performed in the pathway independent of overall differential expression of the KEGG pathway.

Selection of Immune System Genes and Related KEGG Pathways

A list of immune system related genes was created using the more stringent cut offs in order to explore evidence that maternal infection altered expression of the different molecules and cells of the immune system (Table S1) based both on general categories of immune molecules and cells and on genes in immune KEGG pathways.

First, the database was mined for genes using the-general categories of molecules and cells involved in any immune response. Molecules explored included monocyte chemoattractant protein (*MCP*), colony stimulating factor (*CSF*), interferon (*INF*), interleukin (*IL*), chemokine, immunoglobulin (*Ig*), tumor necrosis factor (*TNF*), transforming growth factor (*TGF*), lymphotoxin, toll-like receptors, CD antigens, major histocompatibility complex (*MHC*), and selectin. Cells explored included myeloid cell lineage for innate immune cells (monocyte, macrophage, microglia, dendritic cell, granulocyte, neutrophil, basophil, eosinophil, mast cell) and lymphoid cell lineage for adaptive immune cells (NK cell, lymphoid cell, lymphocyte, T cell, B cell, plasma cell, and leukocyte). Differential expression of genes in these categories provided the first insight into possible alterations to the immune system in the pup brain in response to maternal infection.

Second, we prepared a list of differentially expressed genes related to the immune system from each of the 21 immune related KEGG pathways (Table 1) (https://www.kegg.jp/kegg/pathway.html) regardless of whether or not they had been reported as differentially regulated (Haque et al. 2019). The list included inducible proteins, linkers for activation, subunits, inhibitors, activators, receptors, domains, binding proteins, related proteins, "like" genes, other members of the family, and all other intermediate molecules in the pathway.

Selection of Blood Brain Barrier Genes and Related KEGG Pathways

In order to determine whether mechanisms known to transport immune cells, cytokines, and immunoglobulins across the BBB were influenced by maternal infection, we made a list of all relevant genes from the KEGG pathway maps for endocytosis and leukocyte trans-endothelial cell migration. This list included cell adhesion molecules, junction proteins, ligands and receptors, and vesicle formation genes.

Immune System

Immune related genes were examined to explore evidence that maternal infection may have altered expression of innate and adaptive immune systems, including the type of adaptive immune response.

Shift in Adaptive but not Innate System Gene Expression

Among the components of the innate immune system relevant to nematode immunity that could have been affected, we only observed evidence that pathogen recognition function was downregulated in response to maternal infection. Of the differentially expressed genes associated with the myeloid cell lineage, most CD cell surface markers and chemokine ligands were downregulated (Table 2). In addition, the RIG-1 like receptor signaling pathway was downregulated.

In contrast, we observed substantial differential expression of genes in the adaptive immune system, many of which were upregulated and many downregulated (Table 3), reflective of the complex feedback loops among arms of the adaptive response. The affected genes included those coding for CD cell surface markers, chemokines, leukocyte transcript (*LST1*), lymphocyte antigens (*LY86, LY6G6D*), cytotoxic T-lymphocyte associated proteins (e.g. *CTLA2α, CTLA2β*), T cell transcription factor (*TCF7L1*), T cell proliferation (*MTCP1*), T cell linkers of activation (*LAT2*), nuclear factors of activated T cells (e.g., *NFAT5, NFATC1, NFATC2, NFATC3*), proliferation, B cell development (e.g., *EBF3*), B cell receptor associated proteins (e.g., *BCAP29*), and immunoglobulin superfamily genes for immunoglobulin cell adhesion (*GM4926, IGSF3*) (Table 3). Additionally, KEGG pathway maps including T cell receptor signaling and B cell receptor signaling were upregulated (Haque et al., 2019) indicating the upregulated activity of the adaptive immune system.

Upregulation of Th2 and downregulation Th1 gene expression

Although the Th1 and Th2 cell differentiation pathway was not differentially regulated (Haque et al., 2019), in depth exploration of gene expression revealed several intriguing

results (Table 4). Evidence of a predominant Th2 immune response was seen in the relatively consistent pattern of upregulation from receptor (*Notch1/2*), to its intermediate complex *MAML1*, to its product hallmark Th2 cytokine gene, IL4. In addition, the intermediate complex *MAML1* and receptor *IL12Rβ2* which activate Th1 cell differentiation were upregulated. Although this suggests a heightened Th1 response, analysis of the downregulated cytokine-cytokine receptor interaction pathway showed the downregulation of four Th1 interleukins (*IL-1B, IL-15, IL-15RA, IL-18*). Furthermore, analysis of the non-differentially regulated toll-like receptor signaling pathway along with data mining revealed the downregulation of one TNF superfamily alpha inducible gene, two TNF superfamily receptors, four *INF* inducible proteins, and one INF stimulated protein (Table 4). Taken together, the data indicate the upregulation of Th2 immune response and downregulation of the Th1 immune response, consistent with the dampening effect of Th2 cytokines on Th1 cytokine expression.

Upregulation of Treg and downregulation of Th17 gene expression

Mining of our gene database showed upregulation of Treg-related gene expression (Table 4). TGF-β receptor 3 (*TGF-βR3*), TGF-βreceptor-associated protein (*TGFBRAP1*), and latent *TGF-β* binding proteins (*LTBP3, LTBP4*) were upregulated. Our data also suggests that *TGF-β2* expression was upregulated, based on the p-value. Analysis of the non-differentially expressed Th17 signalling pathway revealed downregulation of its product *IL-22*. Analysis of the non-differentially expressed IL-17 signaling pathway showed the downregulation of most autoimmune pathology genes including *CCL7, S100A8, S100A9, and MMP13* (Table 4). The upregulation of Treg genes suggests that maternal infection may play an important role in modulating inflammation, and also in downregulating Th17 auto-immune response.

Impact of maternal infection on genes involved in transport of immune signals across the BBB

Immune related transport across the BBB was examined to explore evidence that maternal infection may have altered either leukocyte infiltration and receptor-mediated endocytosis of cytokines or immunoglobulins into the pup brain.

Leukocyte trans-endothelial cell migration

The leukocyte trans-endothelial migration pathway capturing interactions between leukocytes and endothelial cells was upregulated (Haque et al., 2019). Furthermore, we observed upregulated expression of three integrin alpha genes (*ITGA3,4,11*) and integrin beta (*ITGB4*) that are important for docking and diapedesis. However, expression of the integrin beta 1 binding protein 1 (*ITGB1BP1*) was downregulated (Table 5). In addition, the regulation of actin cytoskeleton pathway that allows endothelial cells to expand and contract was upregulated; this is important for contracting the cells to make way for the leukocyte. Additionally, two matrix metallopeptidases (*MMPs*) involved in the formation of transcellular channels were differentially expressed; *MMMP15* was upregulated and *MMP13* was downregulated (Table 5).

Both adheren and tight junction pathways that are dynamically responsive to leukocyte migration across the BBB were upregulated (Table 5). Of particular relevance was the upregulated expression of nine cadherins and two catenins that are involved in adheren junctions. For tight junctions, expression of vinculin was upregulated whereas one occludin and one claudin were downregulated (Table 5). Additional differentially expressed genes related to cell adhesion, cell migration, and junction units are available in supplementary Tables 2 and 3. Taken together, this information provides evidence of heightened leukocyte migration between endothelial cells of the BBB in neonates of infected mothers.

Endocytosis

The endocytosis KEGG pathway important for passage through endothelial cells was upregulated (Hague et al. 2019). Genes involved in ligand receptor signaling at the cell surface that are necessary for the initialization and activation of cytokine and

immunoglobulin transport were upregulated, including genes related to TGF-β transport (*TGF-β*, *TGF-β* receptor, and *SMAD* signaling molecules) as well as genes critical for vesicle budding (dynamin and dynamin binding protein) (Table 6). However, we found evidence that intracellular trafficking was impaired based on downregulation of the genes involved in vesicular migration and endosome formation. The evidence included downregulation of several sorting nexin family genes (*SNXs*), vacuolar sorting proteins (*VSP29*), coiled-coil domain gene (*CCDC53*), and charged multivesicular body protein (*CHMP2A*), although the early endosome antigen 1(*EEA1*) gene was upregulated (Table 6). In addition, pathways for cytokine-cytokine receptor interaction and SNARE interactions for vesicular transport were downregulated (Haque et al. 2019). Therefore, despite the upregulation of the endocytosis pathway, the downregulation of vesicle formation genes suggests the impaired ability of the pathway to transport.

Discussion

Key Findings

Our study had three major findings based on our comprehensive interrogation of KEGG pathway maps using our database of differential gene expression. First, we found evidence of a slight dampening affect of maternal infection on innate immune gene expression in the pup brain but a substantial alteration in expression of components of the adaptive immune response. Second, within the adaptive immune response, we found that maternal nematode infection upregulated Th2 and Treg gene expression and downregulated Th1 and Th17 gene expression in the pup brain. Third, we found evidence of heightened leukocyte migration between endothelial cells of the BBB in response to maternal infection. The upregulated leukocyte trans-endothelial migration pathway and expression of diapedesis genes indicated enhanced migration of leukocytes which likely included Th2 and Treg cells. On the other hand, lowered expression of genes needed for vesicular transport indicated impaired endocytosis of cytokines and immunoglobulins.

Immune Environment: Innate and Adaptive Immune Regulation

Innate and adaptive immune response are important in the developing brain, with each having its own function. Given the highly protected environment of the CNS compared to the systemic immune system, the neuro-immune system is less likely to encounter lesions and pathogens that upregulate innate immune system activity, hence, the lower immune cell count (Aarli, 1983). The impact on the innate immune system in the pup brain was concentrated on pathogen recognition processes that impact the adaptive immune system (Liu et al., 2016). In our neonates, maternal infection downregulated the RIG-I-like receptor signaling pathway (Haque et al., 2019) and several chemokines and CD cell surface markers. This is logical since maternal infection signals in the form of immune regulators such as cells, cytokines, and immunoglobulins would influence the immune response itself with no direct impact on other homeostatic roles that the innate immune system mediates such as cell

debris clearance or activating the coagulation system; these functions require stimuli of their own irrelevant of maternal infection signals.

On the other hand, our analysis provided considerable evidence that maternal infection altered expression of the adaptive immune response in pup brain. In addition to the upregulation of B cell and the T cell receptor signaling KEGG pathways (Haque et al., 2019), we observed differential expression of numerous genes related to T and B cell related genes (Table 3) involved in differentiation, maturation, migration, activation, and also including those involved in Th1, Th2, Treg and Th17 responses such as receptors, ligands, and signalling molecules (Table 4). Together, these results clearly highlight the dramatic impact that this maternal infection had on the adaptive immune response in the brain of the uninfected neonate. This is logical given that nematode infections induce a strong adaptive immune response in their host (Maizels et al., 2012) and that this response has previously been shown to be influence the neonate immune system (Odiere et al., 2012). Parasite-specific IgG1 has been found in serum samples of uninfected pups of *H. bakeri* infected dams (Harris et al., 2006), and maternal infection with a related nematode, *Nippostrongylus brasiliensis,* has been shown to result in transfer of cytokines and antibodies to the pup and further to induce an adaptive immune response that offered lasting protection against direct infection of the pup (Darby et al., 2019). Our results extend these findings to involve the adaptive immune response in the pup brain.

Upregulation of Th2 and Treg Gene Expression

It is well established that *H. bakeri* infection induces a Th2/Treg response in the infected host (Maizels et al., 2012; Valanparambil et al., 2017) and we found evidence of this immune profile in the brains of the uninfected pups. The upregulation of the hallmark Th2 cytokine *IL-4* (Basha et al., 2014) together with genes in its signaling cascade were the first indication of a Th2 bias in the pup brain. IL-4 is an activator and recruiter of Th2 cells and its upregulated expression would precede the release of IL-13 (Bao et al., 2015), consistent with IL13, another hallmark Th2 cytokine, not being differentially expressed. In addition, the B cell receptor signaling pathway and the B cell development gene *EBF3* were upregulated

which would initiate a heightened Th2 cell response (Maddur et al., 2015). Upregulated expression of TGF family genes including receptors (*TGF- βR3*), binding proteins (*LTBP3, LTBP4*), and receptor associated proteins (*TGF-βRAP1*) (Table 4), and of *TGF-β* based on P value cut-off, all point to an upregulated Treg response.

Downregulation of Th1/Th17 Gene Expression

As expected when the Th2 response is upregulated (Lazarski et al., 2013), the Th1 response was downregulated as evidenced by the downregulated expression of Th1 type interleukins (*IL-1β, IL-15, IL-15RA, IL-18*), *TNF* related proteins, and *INF α* and *γ* related proteins (Table 4). This was expected given the dampening effect that *IL-4* and *TGF- β* have on Th1 cytokine production (Lazarski et al., 2013; Zhang et al., 2007). *H. bakeri* infection has been suggested to impair the neonatal systemic Th1 immune response to Th1 inducing agents (Cooper et al., 2011; Dauby et al., 2012). In addition, the autoimmune Th17 response was downregulated as indicated by the downregulation of its hallmark *IL-22* coding gene as well as most autoimmune pathology genes in the IL-17 signaling pathway. The downregulation of Th17 response may have been a result of Treg upregulation (Lee, 2018).

Source of Maternal Signal for Th2 and Treg Upregulation in the Pup Brain

The upregulation of Th2/Treg pup brain gene expression could have been initiated prenatally via fetal priming (Dauby et al., 2012) and/or postnatally via milk (Darby et al., 2019). As none of the 96 fetal brain genes differentially expressed in response to maternal *H. bakeri* infection at gestation day 18 (GD18) were related to a Th2/Treg immune response (Haque et al., 2018),it seems likely that Th2/Treg signals were transferred through breast milk rather than through fetal priming. Presence of Th2 cytokines in milk of mice exposed to a Th2-inducing allergen has been shown to induce higher *IL-4* concentrations in the colostrum indicating transfer of IL-4 through breast milk (Böttcher et al., 2000). In addition, Th2 cytokines in milk were shown to induce neonatal immunity given that control neonates cross-fostered by asthmatic dams showed signs of airway hyperresponsiveness and airway

inflammation (Leme et al., 2006) clearly indicating that maternal signals are transferred to the neonate through breast milk. We have extended these findings by showing that Th2/Treg immune signals in nursing pups were also upregulated in the neonatal brain in response to a Th2 maternal infection. This was evidenced by upregulated expression of genes involved in leukocyte infiltration, Th2/Treg signalling including IL-4 and TGF-β, indicating that infiltrating cells had a Th2 bias. Since the most likely route of communication of these signals between the maternal infection and the postpartum neonate brain is through breast milk, the milk is likely the source of maternal signals.

Transport of Immune Signals across the BBB

IL-4, *TGF-β*, and immunoglobulins can signal the brain via receptor-mediated endocytosis of the receptor-cytokine complexes through endothelial cells (Chen, 2009b; Iwasaki, 2017; Kurgonaite et al., 2015; Le Roy et al., 2005; Ruano-Salguero et al., 2020). The endocytosis pathway was upregulated (Haque et al., 2019) as was expression of ligands (*IL-4 and TGF-β*), receptor (*TGF-βR*), signaling molecules (*SMAD3, SMAD9*), and endosome formation scissors (*DNM1, DNM3, DNMBP*). However, our data indicated that endosome formation was impaired given the downregulation of SNARE interactions for vesicular transport pathway and several genes related to vesicle formation (*SNX1, SNX2, SNX5, SNX7, CCDC53, VPS29, CHMP2A*) (Table 6). This suggests that receptor mediated endocytosis of *H. bakeri* immune markers may not be functional despite it being a common pathway for immune signaling (Gleeson, 2014).

In contrast, our data strongly suggest that paracellular movement of leukocytes into the brain was enhanced as the leukocyte trans-endothelial migration pathway was upregulated. Furthermore, we observed upregulated expression of a variety of integrins that dock leukocytes to endothelial cells as well upregulated expression of the actin cytoskeleton pathway (Haque et al. 2019) that is needed to allow flexibility of the endothelial cells (Prasain et al., 2009). Given that *H. bakeri* induces a Th2/Treg response in infected mice (Maizels et al., 2012) and that the Th2/Treg gene expression is upregulated in the neonate brain, it is most likely that the migrating leukocytes are Th2/Treg cells.

At first glance the observed upregulation of junction unit pathways (tight, adhere, gap) would indicate tightening of the BBB. Tightening and loosening of junction units is a natural part of the dynamic process by which the BBB regulates movement of cells and molecules into the brain (Cavey et al., 2009; Weber, 2012). We suggest that, as a response to the junction loosening caused by leukocyte infiltration, junction expression may have been upregulated to restore the selective permeability that is critical for BBB integrity.

Taken together, our observations indicate that maternal signals more likely reached the brain through trans-endothelial migration of leukocytes than through endocytosis and that the upregulated expression of junction units may have been a response to increased diapedesis.

Potential Benefits for the Uninfected Pup

GI nematode infections, though usually considered to be detrimental, are now understood to also provide benefits not only to the infected host but also to the uninfected offspring (Arrais et al., 2020; Darby et al., 2019; Ponte et al., 2014; Straubinger et al., 2014). Maternal *H. bakeri* infection was shown to upregulate expression of long term potentiation and synaptogenesis-related pathways in 7-day old pup brains (Haque et al., 2019) that enhance learning and memory (Abraham et al., 2019; Martinez et al., 1996; Stuchlik, 2014). Our secondary analysis of the data from these pups also revealed an upregulated Th2 response and upregulated expression of *IL-4*, both of which are known to be necessary for memory and learning (Brynskikh et al., 2008). Furthermore, as Treg through *TGF-β* promotes neural development by mediating axon specification and *TGF-β* receptor signaling guides neuronal axon initiation in the brain (Yi et al., 2010), the observed upregulation of Treg responses would also have potentially positive impacts on learning and memory for the neonate. An upregulated Treg response also plays an important role in dampening Th1 inflammation (Littringer et al., 2018) which would limit neuro-inflammation that in turn compromises the integrity of the BBB (Kim et al., 2012).

Strengths and Limitations

A strength of this study was the identification of patterns of differential gene expression within KEGG pathway maps that is not revealed by KEGG pathway analysis alone, given that differential expression of pathways is not necessarily paralleled by its components including ligands, receptors, products, signaling intermediates, and internal pathways. Our more stringent choice of cut-offs for p-values and fold change allowed us to focus on a subset of the differentially expressed genes reported by Haque et al. (2019). Nevertheless, we acknowledge that we may have excluded important genes or included genes whose differential expression was of little functional importance. We also acknowledge the limitations associated with reliance only on gene expression data without confirmation by assaying protein concentrations or through functional assays to examine phenotypic effects.

Conclusion

Our thorough interrogation of pathways and genes associated with the immune response raise a number of hypotheses that if supported experimentally, could have important implications for neurodevelopment of offspring of nematode-infected mothers, at least in this murine model. Our data indicate that maternal *H. bakeri* infection promoted trans-endothelial migration of Th2/Treg cells across the BBB of the neonate, inducing a Th2/Treg response in the neonate brain. As this response may have potential benefits in reducing inflammation and promoting learning and memory, follow-up experimental studies to confirm the gene expression data and to explore behavioural response in the pups of infected dams would be important.

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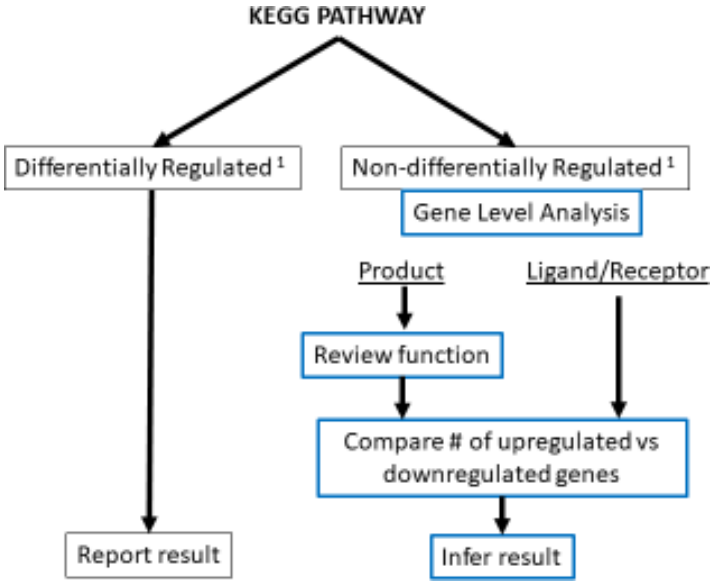
Figures

Figure 1. Schematic showing the approach for exploring KEGG Pathway maps using the database of differentially expressed genes. [1] Haque et al. (2019)



Tables

Table 1. List of immune related KEGG pathways considered in this study.

Classification	Pathway Name	Differential Expression[1]
Immune Related	Hematopoietic cell lineage	N/A
	Complement and coagulation cascade	N/A
	Platelet activation	N/A
	Toll-like receptor signaling	N/A
	Toll and Imd signaling	N/A
	NOD-like receptor signaling	N/A
	RIG-I-like receptor signaling	downregulated
	Cytosolic DNA sensing	N/A
	C-type lectin receptor signaling	N/A
	Natural killer cell mediated cytotoxicity	N/A
	Antigen processing and presentation	N/A
	T cell receptor signaling	upregulated
	Th1 and Th2 cell differentiation	N/A
	Th17 cell differentiation	N/A
	Il-17 signaling	N/A
	B cell receptor signaling	upregulated
	Fc epsilon RI signaling	upregulated
	Fc gamma R mediated phagocytosis	upregulated
	Intestinal immune network for IgA production	downregulated
	Chemokine signaling	upregulated
	Cytokine-cytokine receptor interaction	downregulated
BBB Related	Leukocyte trans endothelial migration	upregulated
	Tight Junction	upregulated
	Adheren Junction	upregulated
	Endocytosis	upregulated

[1] Differential regulation as reported by Haque et al (2019)

Table 2. List of innate immune system genes differentially expressed in the pup brain, in response to maternal *H. bakeri* infection.

Classification	Gene Name	Gene Symbol	Log 2 Fold Change	P-value
CD cell surface markers	CD302 antigen	CD302	-1.8988	7.17E-12
	CD40 antigen	CD40	-1.7484	4.33E-10
	CD83 antigen	CD83	-1.2487	3.03E-08
	CD300A antigen	CD300A	-1.4979	4.65E-08
	CD209f antigen	CD209F	-1.4813	9.01E-07
	CD93 antigen	CD93	1.3519	1.05E-05
	CD200 receptor 1	CD200R1	-1.5711	1.99E-05
	CD209g antigen	CD209G	-1.9848	3.77E-05
Chemokines	chemokine (C-C motif) ligand 9	Ccl9	-2.2198	8.11E-13
	chemokine (C-C motif) ligand 6	Ccl6	-1.9152	1.49E-10
	chemokine (C-C motif) receptor 1	Ccr1	-1.5864	6.32E-08
	chemokine (C-X-C motif) ligand 1	Cxcl1	-1.9013	5.24E-08
	chemokine (C-C motif) ligand 25	Ccl25	-1.6614	1.74E-08
	chemokine (C-C motif) ligand 12	Ccl12	-1.9695	7.17E-07
	chemokine-like factor	Cklf	-1.2909	3.37E-06
	chemokine (C-C motif) ligand 24	Ccl24	-1.2683	9.86E-05
	chemokine (C-C motif) ligand 7	Ccl7	-1.6164	3.00E-05

Table 3. List of adaptive immune system genes differentially expressed in the pup brain, in response to maternal *H. bakeri* infection.

Classification	Gene Name	Gene Symbol	P-value	Log 2 Fold change
Leukocytes	Leukocyte specific transcript 1	LST1	3.30E-08	-1.4504
Lymphocytes	Cytotoxic T lymphocyte-associated protein 2 alpha	CTLA2α	1.71E-11	-1.9618
	Lymphocyte protein tyrosine kinase	LCK	3.31E-10	-1.2848
	Cytotoxic T lymphocyte-associated protein 2 beta	CTLA2β	3.60E-09	-2.041
	Lymphocyte antigen 86	LY86	1.55E-08	-1.6468
	X-linked lymphocyte-regulated complex	XLR	1.26E-06	-1.7171
	Lymphocyte antigen 6 complex, locus G6D	LY6G6D	7.51E-05	-2.0459
T cells	Nuclear factor of activated T cells 5	NFAT5	1.68E-14	2.378
	Nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 3	NFATC3	6.84E-09	1.2199
	Nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1	NFATC1	9.49E-07	1.3363
	Mature T cell proliferation 1	MTCP1	7.32E-07	-1.3497
	Transcription factor 7 like 1 (T cell specific, HMG box)	TCF7L1	3.83E-07	1.5258
	Linker for activation of T cells family, member 2	LAT2	3.64E-06	-1.9848
	Nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2	NFATC2	1.11E-05	1.2136
	Linker for activation of T cells	LAT	2.73E-05	-1.9806
B cells	Early B cell factor 3	EBF3	3.53E-15	1.6944
	B cell receptor associated protein 29	BCAP29	3.70E-09	-1.4942

Immunoglobulin superfamily	Immunoglobulin superfamily, member 3	IGSF3	2.69E-08	1.7284
	T-cell immunoglobulin and mucin domain containing 2 pseudogene	GM4926	1.69E-06	-1.5855
CD cell surface markers	CD1d1 antigen	CD1D1	1.60E-05	-1.2071
	CD320 antigen	CD320	3.60E-11	-1.3617
	CD53 antigen	CD53	1.94E-10	-1.5542
	CD63 antigen	CD63	9.54E-08	-1.4596
	CD59a antigen	CD59A	4.72E-08	-1.7165
	CD84 antigen	CD84	6.02E-07	-1.4359
	CD48 antigen	CD48	5.13E-07	-1.8838
	CD86 antigen	CD86	2.09E-07	-1.4856
	CD52 antigen	CD52	1.50E-05	-1.7423
Chemokine	Chemokine (C-C motif) ligand 27A	Ccl27a	4.27E-09	-1.8182
	Chemokine (C-C motif) ligand 25	Ccl25	1.74E-08	-1.6614
	Chemokine-like factor	Cklf	3.37E-06	-1.2909
	Chemokine (C-C motif) ligand 24	Ccl24	9.86E-05	-1.2683
	Chemokine (C-X-C motif) receptor 5	Cxcr5	6.26E-05	1.7003
	Chemokine (C-X-C motif) ligand 11	Cxcl11	4.45E-05	-1.7877

Table 4. List of differentially expressed cytokine genes classified by immune response in the pup brain, in response to maternal *H. bakeri* infection

Immune Response	Classification	Gene Name	Gene Symbol	P-value	Log 2 Fold Change
Th1	Interferon	Interferon, alpha-inducible protein 27 like 2A	*IFI27L2A*	2.51E-09	-2.5516
		Interferon gamma inducible protein 47	*IFI47*	6.30E-07	-1.6666
		Interferon-stimulated protein	*ISG20*	7.81E-07	-1.442
		Interferon-induced protein 35	*IFI35*	2.15E-06	-1.3033
	Tumor Necrosis Factor	Tumor necrosis factor receptor superfamily, member 11a	*TNFRSF11A*	2.39E-08	1.2215
		Tumor necrosis factor, alpha-induced protein 8-like 2	*TNFαIP8L2*	5.60E-08	-1.6267
		Tumor necrosis factor receptor superfamily, member 12a	*TNFRSF12A*	6.00E-06	-1.2305
	Interleukin	Interleukin-1 receptor-associated kinase 1 binding protein 1	*IRAK1BP1*	3.38E-10	-1.5769
		Interleukin 18	*IL18*	4.49E-09	-1.4295
		Interleukin 1 beta	*IL1β*	1.33E-07	-3.1953
		Interleukin 15	*IL15*	6.30E-06	-1.662
		Interleukin 12 receptor, beta 2	*IL12Rβ2*	1.32E-06	2.0879
		Interleukin 15 receptor, alpha chain	*IL15Rα*	4.79E-05	-1.3734
Th2	Interleukin	Interleukin 13 receptor, alpha 2	*IL13Rα2*	1.62E-09	-1.9619
		Interleukin enhancer binding factor 2	*ILF2*	6.79E-08	-1.2226
		Interleukin 4	*IL4*	1.81E-07	1.2171
Treg	Transforming Growth Factor	Transforming growth factor, beta receptor III	*TGFβR3*	4.69E-08	1.2645
		Transforming growth factor, beta receptor associated protein 1	*TGFβRAP1*	8.92E-07	1.3063
		Latent transforming growth factor beta binding protein 3	*LTβP3*	1.35E-07	1.3637
		Latent transforming growth factor beta binding protein 4	*LTβP4*	3.43E-06	1.6984
		Transforming growth factor alpha	*TGFα*	1.30E-06	1.2669
	Interleukin	Interleukin 10-related T cell-derived inducible factor beta	*ILTIFβ*	3.20E-07	-2.3961
Th17	Interleukin	Interleukin 17 receptor D	*IL17RD*	4.41E-10	1.9374
		Interleukin 22	IL22	9.41E-06	-2.162
	Chemokine	chemokine (C-C motif) ligand 7	CCL7	3.00E-05	-1.6164
	Related Proteins	S100 calcium binding protein A8 (calgranulin A)	S100A8	3.07E-08	-2.4356
		S100 calcium binding protein A9 (calgranulin B)	S100A9	2.39E-07	-2.0865
		matrix metallopeptidase 13	MMP13	4.26E-05	-1.7194

Table 5. List of cell adhesion molecules and related genes involved in leukocyte trans-endothelial cell migration that are differentially expressed in the pup brain, in response to maternal *H. bakeri* infection.

Classification	Gene Name	Gene Symbol	P-value	Log 2 fold change
Cell Adhesion	Integrin beta 1 binding protein 1	ITGβ1BP1	6.09E-11	-1.9182
	Calcium and integrin binding 1 (calmyrin)	CIB1	1.04E-09	-1.4908
	Integrin beta 4	ITGβ4	1.71E-09	1.8016
	Calcium and integrin binding family member 2	CIB2	6.26E-09	-1.3909
	Integrin alpha 11	ITGα11	2.20E-08	1.7196
	Integrin alpha 4	ITGα4	3.81E-08	1.2737
	Integrin alpha 3	ITGα3	1.16E-06	1.2366
	Integrin alpha E, epithelial-associated	ITGαE	3.47E-06	-2.3048
	Matrix metallopeptidase 13	MMP13	4.26E-05	-1.7194
	Matrix metallopeptidase 15	MMP15	4.67E-05	1.295
Adheren Junctions	Cadherin, EGF LAG seven-pass G-type receptor 1	CELSR1	2.34E-11	2.5308
	Cadherin, EGF LAG seven-pass G-type receptor 3	CELSR3	5.52E-10	2.3788
	Cadherin 23 (otocadherin)	CDH23	8.92E-09	1.9526
	Cadherin 3	CDH3	1.01E-08	1.6134
	Cadherin, EGF LAG seven-pass G-type receptor 2	CELSR2	2.81E-08	2.8609
	Cadherin 6	CDH6	5.87E-07	1.6177
	Cadherin 4	CDH4	4.83E-06	1.3775
	Cadherin 5	CDH5	2.41E-05	1.2137
	Desmoglein 2	DSG2	2.09E-05	1.2256
	Catenin (cadherin associated protein), delta 2	CTNNδ2	7.83E-07	1.5838
	Catenin (cadherin associated protein), delta 1	CTNNδ1	2.55E-06	1.2385
Tight Junctions	Tight junction protein 1	TJP1	9.28E-09	1.234
	Claudin 10	Cldn10	3.11E-09	-1.5952
	Occludin/ELL domain containing 1	Ocel1	1.48E-09	-1.2976
	Cingulin-like 1	CGNL1	8.38E-11	1.9121
	Cingulin	CGN	1.11E-05	1.3057
	Vinculin	VCL	2.16E-07	1.7986

Table 6. List of genes involved in endocytosis pathway that are differentially expressed in the pup brain, in response to maternal *H. bakeri* infection.

Classification	Gene Name	Gene Symbol	P-value	Log 2 Fold Change
Initiation and Signaling	Caveolin 2	CAV2	6.89E-07	-1.2166
	Clathrin, light polypeptide (Lca)	CLTA	3.71E-08	-1.2065
	Dynamin 3	DNM3	4.95E-09	1.6835
	Dynamin binding protein	DNMBP	2.17E-05	1.5473
	EGF-like domain 7	EGFL7	5.75E-10	-1.5489
	Epidermal growth factor receptor	EGFR	1.53E-11	1.4725
	Transforming growth factor, beta receptor III	TGFβR3	4.69E-08	1.2645
	MAD homolog 3 (Drosophila)	SMAD3	6.35E-08	1.6784
	Adaptor protein complex AP-2, alpha 1 subunit	AP2α1	6.49E-05	1.3822
	Protein kinase C, alpha	PRKCα	3.03E-07	1.5849
	Rous sarcoma oncogene	SRC	5.11E-06	1.462
Vesicle Migration and Endosome Formation	Sorting nexin 5	SNX5	3.31E-07	-1.234
	Sorting nexin 1	SNX1	9.45E-08	-1.1603
	Sorting nexin 2	SNX2	2.29E-07	-1.1769
	Sorting nexin 7	SNX7	1.98E-08	-1.1221
	RAB7, member RAS oncogene family-like 1	RAB7L1	1.71E-09	-1.4527
	CDC42 binding protein kinase beta	CDC42BPβ	7.17E-06	1.8055
	Vacuolar protein sorting 29 (S. pombe)	VPS29	2.19E-11	-1.8892
	Coiled-coil domain containing 53	CCDC53	2.87E-09	-1.4254
	Charged multivesicular body protein2A	CHMP2A	5.63E-10	-1.6867
	Programmed cell death 6	PDCD6	2.68E-10	-1.7176
	Kinesin family member 5A	KIF5A	2.32E-07	1.6266
	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)	ARFGEF2	1.21E-09	1.9556
	Early endosome antigen 1	EEA1	1.71E-07	1.2971
	WAS protein family, member 2	WASF2	1.04E-07	1.5409

hapter IV - General Discussion

In our first objective we reported a shift in brain gene expression toward Th2/Treg immune response which is particularly intriguing as it indicates signaling from the maternal nematode infection to the developing pup CNS. In our second objective we obtained evidence for the upregulation of leukocyte infiltration for the recruitment of the immune response. These findings pave the way for a growing body of literature on the impact of the maternal *H. bakeri* infection on fetal and neonatal development. Our observation on the impact of maternal infection on neonate brain immune system establish a link between the previously observed influence of the maternal infection on neonatal systemic immune system (Odiere et al., 2012) to the impact on the neonatal brain neurodevelopment (Haque et al., 2019) thus, providing clues for experimental hypotheses to examine the origin of this signal and its consequences. One such experiment would compare the cytokine profile in the neonate brain, neonate serum, breast milk, and maternal serum in order to establish a possible connection between the source (maternal infection) transferring modulating agents to the effected body (neonate brain immune system) via the vehicle (breast milk, neonate serum).

A Novel Process for Mining Gene Expression Data

At the outset we started by devising a novel approach in order to utilize the existing gene expression data and the KEGG pathway analysis. Starting with a database of over 5500 differentially expressed genes, we applied more stringent cut-offs to narrow the list of genes and we focused on immune related genes based on the various categories of immune elements and immune related genes in KEGG pathway maps. Finally we used our list to examine the KEGG pathway maps. This novel approach was extremely helpful as it allowed us to make logical sense of the KEGG pathway analysis results and to create a coherent story between the observed gene expression data and the KEGG pathway analysis results from Haque et al. (2019) by revealing and relating patterns of differential gene expression beyond those indicated in the KEGG Pathway analysis alone.

As the KEGG pathway analysis relied more on numeric evaluation of gene expression rather than on a logical analysis based on gene functionality, the results of KEGG pathway analysis can provide contradictory information whereby a pathway could be both up and downregulated simultaneously. Our approach overcame this limitation and through detailed examination of gene expression in a pathway made conclusions based on logical sequence of immune events and their function. Therefore, our observations were not only internally consistent and coherent, but also consistent with observations previously made by Haque et. al (2019). Since our approach revealed potentially valuable findings that would have otherwise been overlooked, it helped us understand what was likely happening inside the pathway and what events would be expected to follow as a result.

Another reason for implementing our approach was a surprising challenge we faced of the lack of differential expression of KEGG immune response pathway maps despite the differential expression of hallmark cytokine genes and other key genes in these pathway maps. In many cases this was associated with the nature of these maps themselves that focus mainly on activation of cell differentiation or the initial activation of the response but not the subsequent steps involved in fully activating the response. Most pathway maps are divided roughly into 3 regions: pathway activation mediated by ligands and receptors, signaling molecules and intermediates of the pathway that propagate the signal, and products of the pathway that will perform the function of the pathway. Signaling molecules and intermediates were highly redundant and shared amongst many pathways. Therefore, in most cases, we focused on either ligands and receptors to assess pathway activation or products for assessing whether the pathway would perform its function. Accordingly, we assessed the likely direction and consequence of expression of the pathway.

Avoiding Bias

Secondary data analyses maximize the utility of large data sets (Tripathy, 2013). Interrogating such data sets with a new hypothesis introduces a different angle that can reveal hidden observations and produce new findings (Tripathy, 2013). However, secondary data analyses are subject to confirmation bias where the researcher may exaggerate a minor observation, emphasize a poorly validated link, or ignore counter

arguments and explanations in favor of the hypothesis (Kaptchuk, 2003). Therefore, it is essential to incorporate measures throughout the secondary analysis to maximize objectivity.

In this thesis, the primary study collected brain gene expression data and explored the impact of maternal H. bakeri infection on long-term potentiation in the 7-day old pup brain. We used the same gene expression database but explored the impact of maternal infection on the brain immune response and the mechanism by which immune elements cross the BBB. We used a subset of genes that were differentially expressed at more stringent P-value and log2-fold cut-offs, while taking into account the original KEGG pathway analysis.

To avoid bias throughout the study various measures were implemented in data collection and argument structure. To minimize design bias, our search strategy included all possible immune-related genes taken from KEGG pathway maps and from a list of immune-related categories and processes identified from the literature. To lower false discovery rate, more stringent cut-offs for P-value and log fold change were used. To minimize confirmation bias, we matched differentially expressed genes to KEGG pathway analysis. This allowed us to propose links that helped us predict possible consequences of the altered brain gene expression. These observations were then thoroughly discussed with an independent immunologist. To ensure compatibility and consistency in logic of observation, comparisons were made with the few analogous studies in drafting the thesis. Finally, from start to finish, numerous experts were involved in validating and assessing the logic and strength of the arguments.

Immune Gene Responses Evident in Pup Brain

It might have been expected that the innate immune system would have been strongly affected in the neonate whereas the still relatively immature adaptive system may have been less affected as there was no direct infection but only exposure to signals from the maternal infection. However, we found the opposite in the pup brain. In general, the innate immune

system is the more rapid and fast acting first line of defense and mediates ongoing functions such as debris clearance, maintenance of physical and chemical barriers and pathogen clearance that are not reliant on acquired or memory processes (Marshall et al., 2018). The innate immune system also supports adaptive immunity through its critical role in pathogen recognition and antigen presentation as part of the complex multi-step process of immune response activation (Marshall et al., 2018). The only observed impact of the maternal infection on the innate immune system was on its cooperative function with the adaptive immune system, as evidenced by downregulated expression of the RIG-1 like receptor signalling pathway and a few CD cell surface marker and chemokine ligand genes involved in pathogen recognition required for activation of an immune response. This is understandable as the other homeostatic functions of the innate immune system are essential and independent of signals of immune response regulation associated with the maternal infection. The downregulation of antigen presentation function elements can be explained by the well known immune-suppressive nature of molecules released by *H. bakeri* (Pritchard et al., 1994) especially upon primary exposure (Valanparambil et al., 2017). We suggest that immune-suppressive signals are likely getting through to the pup via breast milk along with the other immune modulating signals of the maternal infection.

In contrast to the innate system, the pup brain adaptive immune system was considerably affected. Several KEGG pathways were differentially expressed including B cell receptor signaling, T cell receptor signaling, chemokine signaling, and cytokine-cytokine receptor interaction. In addition, numerous genes controlling functions related to B and T lymphocytes were differentially expressed including those mediating transcription, development, maturation, activation, adhesion, migration, CD cell surface marker expression, cytokine expression, receptor expression, and signalling. Consequently, we observed upregulated Th2/Treg and downregulated Th1 and Th17 gene expression. This is consistent in that Th2 cytokines such as IL-4 can downregulate Th1 immune response (Lazarski et al., 2013). Also the parasite induces a Th2/Treg immune response in the infected host (Maizels et al., 2012) and signals from the parasite and from the mother's immune system are of a Th2/Treg nature. The Th2/Treg immune response also makes sense as the natural Th2 state of the neuro-immune system supports cognitive performance

(Derecki et al., 2010) whereas a Th1 immune response could be detrimental for cognitive performance (Derecki et al., 2010) and BBB permeability (Yarlagadda et al., 2009). Lastly, Treg immune response dampens Th1 and Th17 inflammation (Lee, 2018; Littringer et al., 2018). As prolonged inflammation can be detrimental to the brain (Ellul et al., 2018; Hoffman et al., 2009)the immune-suppressive role of Treg (Corthay, 2009) is key for avoiding the development of inflammation into a chronic state.

Since the maternal infection was the factor of influence on the pups and since it is well known to induce a Th2/Treg response (Maizels et al., 2012), the natural expectation is that the predominant immune response in the pup brain would be biased toward a Th2/Treg response. Yet the few studies that have examined cytokines in amniotic fluid show higher IL-1β concentration (Odiere et al., 2010a) whereas those that examined the neonate serum show higher eotaxin concentration (Odiere et al., 2012) in response to maternal *H. bakeri* infection. Thus, evidence from the literature shows a range of responses in the fetal and neonatal period but not a clear signal of an upregulated Th2/Treg response. Hence it was somewhat surprising, but very intriguing, to see the neuro-immune system of the pups almost mirroring the mother's systemic immune response.

Communication from Dam to Pup to Pup Brain

In the neonatal period, breast milk carries signals from the mother to the pup. Studies have observed variation in cytokine and immunoglobulin concentrations in milk under conditions of maternal infection (Harris et al., 2006; Verhasselt et al., 2008). Therefore, breast milk components may explain how a maternal infection in the GI tract results in altered gene expression in the brain of the uninfected pup. The line of logic emerging from the observed shifts in pup brain cytokine expression and altered expression of genes in the BBB is that the Th2/Treg biased cytokines, immunoglobulins, and cells move through breast milk into the neonate. Milk cytokines and cells could either cross the pup GI tract or could activate pup GI resident neonatal immune cells to activate Th2/Treg immune response elements that could cross into the pup circulation (Jakaitis et al., 2014).

In either case, the immune modulators would need to cross the pup BBB. Even though KEGG pathway maps related to both receptor-mediated endocytosis and diapedesis were upregulated, suggesting that both transport mechanisms were enhanced in response to maternal infection, our more thorough examination indicates that diapedesis of leukocytes is the more likely route by which immune signals cross the BBB. This conclusion emerged from the finding that the initial activation step of the endocytosis was upregulated but that the subsequent and important endosome formation step was downregulated. It was further supported by the upregulation of junction expression which is an expected consequence following disruption of junctions as leukocytes move between endothelial cells (Floris et al., 2004). The rapid dynamic nature of junctions requires that they re-establish their integrity which would involve upregulated expression of junction proteins (Chen et al., 2006). Thus, although we had thought it would be more likely that signals would reach the brain through endocytosis of cytokines rather than leukocyte trans-endothelial migration, as diapedesis risks impairment of BBB permeability (Floris et al., 2004), our analysis revealed a likely impairment in vesicle formation that would have prevented endocytosis. It is clear that cytokines and immunoglobulins associated with the maternal infection were actively upregulating chemokine signaling, Fc epsilon RI signaling, Fc gamma R mediated phagocytosis and downregulating cytokine-cytokine receptor interaction in the pup brain. However, follow-up experiments are needed to confirm our hypothesis that transport involves trans-endothelial migration of leukocytes more than endocytosis of cytokines.

Four other lines of communication from the GI nematode in the dam to the pup brain also should be acknowledged. First, maternal cytokines may move into fetal circulation by crossing the placenta (Ratnayake et al., 2013). However, only modest differential brain gene expression was reported in the fetal brain at E18 (Haque et al., 2018) and there was a virtual absence of differential brain gene expression at P2 (Haque et al., 2019), suggesting limited placental transfer in this model. Second, *H. bakeri* excretory-secretory proteins including a TGF-β mimic that promotes a Treg response in the infected host (Johnston et al., 2017) may cross to the neonate. As we discussed previously, our findings support this communication mechanism as the Treg immune response and TGF-β expression were upregulated and their expected effect of dampening Th1 and Th17 gene expression was observed. Third, *H. bakeri*

releases micro RNAs (White et al., 2020) and Haque et al. (2018) reported differential expression of the micro RNA *let7* in the fetal brain of infected dams which can modulate neural proliferation (Zhao et al., 2010). Fourth, maternal *H. bakeri* infections alters both the maternal and neonatal microbiome (Haque et al. 2021, under review) which may polarize the pup systemic immune response toward a Th2 response (Nyangahu et al., 2020). The predicted increase in microbial production of short-chain fatty acids and vitamins (Haque et al. 2021, under review) may alter gut-brain cross-talk and neurodevelopment (González et al., 2016; Liu et al., 2019; Silva et al., 2020) in a way that contributes to our findings.

Broader Implications

The activation of the maternal immune system toward other infections has been associated with cognitive impairment in offspring, mostly associated with infections that induce a Th1 response (Aguilar-Valles et al., 2020; Mac Giollabhui et al., 2019; Vasistha et al., 2020). During fetal development, IL-1β concentration was higher in amniotic fluid of *H. bakeri* infected dams (Odiere et al., 2010a) hinting toward a fetal Th1 response which may have been associated with cognitive impairment (Donzis et al., 2014). However, given that *H. bakeri* normally induces a Th2/Treg response and that cognitive performance is enhanced by Th2 responses (Derecki et al., 2010), maternal infection may have a positive impact on cognitive performance in the 7-day old pup. The first evidence of this was the increased expression of genes associated with long-term potentiation in the P7 brain (Haque et al., 2019). A follow-up study in our lab used a Barnes maze with an escape hole to test the learning and memory capacity of weaned juveniles of *H. bakeri* infected dams and demonstrated that juveniles of infected dams had enhanced spatial memory compared with control pups (Noel et al., in preparation). These data indicate potential benefits of maternal *H. bakeri* infection for the uninfected pup.

Due to the long co-evolutionary history between *H. bakeri* and mice, we approached our observation with a positive outlook as there could be some evolutionary advantage. However, it is important to recall that this infection also has been shown to impact growth

and organogenesis (Kristan, 2002b) thus, causing developmental changes that may be detrimental.

Suggestions for Follow-up Studies

Our observations on the transcriptome and their implications have yet to be verified by experimental studies. In addition to qPCR to confirm gene expression data, it would be helpful to confirm the predominant immune response, cytokine, immunoglobulin concentrations and immune cell types and counts in pup brains. ELISA on pup serum and CSF could identify the cytokine and immunoglobulin profile. Similarly, a WBC count could indicate a general state of inflammation in the brain. Immunohistochemistry or flow cytometry could be performed on brain tissue to confirm whether T cell phenotypes are consistent with a heightened Th2/Treg expression. As for BBB permeability, a common test for permeability is the Evans Blue (EB) dye extravasation whereby the presence of EB in brain tissue indicates alterations in permeability as the dye is normally impermeable (Goldim et al., 2019). Also, a comparative study for transport of any common molecule or even a drug could be performed to test the normal functioning and transport across the BBB. Another option would be a comparative visualization of the expression of the various junction proteins through fluorescent labeling.

Conclusion

Taken together, we are confident and optimistic in the possibility that hypotheses emerging from our gene expression analysis can be tested in experiments and perhaps confirm that maternal *H. bakeri* infection induces a Th2/Treg response in the pup brain and that enhances diapedesis but impairs endocytosis.

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Appendix

Supplementary Tables

Table S1. List of immune related genes that meet the set cut-offs.

gene name	gene symbol	p-value	Log 2 fold change
myeloid/lymphoid or mixed-lineage leukemia 1	Mll1	6.45E-16	2.6558
myeloid/lymphoid or mixed-lineage leukemia 3	Mll3	9.73E-16	1.8857
CDK5 regulatory subunit associated protein 2	Cdk5rap2	2.91E-15	1.6542
myeloid/lymphoid or mixed-lineage leukemia 2	Mll2	5.27E-15	2.9443
cerebellar degeneration related antigen 1	Cdr1	1.36E-14	2.6173
RAR-related orphan receptor alpha	Rora	1.60E-14	1.6809
nuclear factor of activated T cells 5	Nfat5	1.68E-14	2.378
mitogen-activated protein kinase kinase kinase 1	Map3k1	3.18E-14	1.5322
Notch gene homolog 2 (Drosophila)	Notch2	4.88E-14	2.0131
triggering receptor expressed on myeloid cells 2	Trem2	5.35E-14	-1.9824
immunoglobulin superfamily, member 10	Igsf10	8.99E-14	2.0756
immunoglobulin superfamily, member 9B	Igsf9b	7.18E-13	2.8245
chemokine (C-C motif) ligand 9	Ccl9	8.11E-13	-2.2198
CREB binding protein	Crebbp	1.01E-12	1.8606
coiled-coil domain containing 107	Ccdc107	1.15E-12	-2.111
nuclear factor I/B	Nfib	2.50E-12	1.4485
cystatin B	Cstb	2.75E-12	-1.8201
cytochrome c oxidase subunit VIIb	Cox7b	2.99E-12	-2.2606
RAS-related C3 botulinum substrate 3	Rac3	3.28E-12	-1.6253
nuclear factor I/A	Nfia	3.48E-12	1.8779
hypoxia inducible factor 3, alpha subunit	Hif3a	3.65E-12	1.864
cathepsin H	Ctsh	4.90E-12	-1.6639

CD302 antigen	Cd302	7.17E-12	-1.8988
mastermind like 3 (Drosophila)	Maml3	7.88E-12	1.6855
ubiquitin specific peptidase 31	Usp31	1.06E-11	1.8424
mitogen-activated protein kinase kinase kinase 2	Map3k2	1.54E-11	1.368
cytochrome c oxidase subunit IV isoform 1	Cox4i1	1.58E-11	-2.0354
cytotoxic T lymphocyte-associated protein 2 alpha	Ctla2a	1.71E-11	-1.9618
cytochrome c oxidase, subunit VIIc	Cox7c	2.07E-11	-2.0345
coiled-coil domain containing 32	Ccdc32	3.27E-11	-1.6673
cytochrome c oxidase, subunit VIIa 2	Cox7a2	3.53E-11	-1.9814
CD320 antigen	Cd320	3.60E-11	-1.3617
S100 calcium binding protein A10 (calpactin)	S100a10	5.70E-11	-1.783
integrin beta 1 binding protein 1	Itgb1bp1	6.09E-11	-1.9182
guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1	Gnb2l1	6.33E-11	-1.8365
nuclear receptor co-repressor 2	Ncor2	6.36E-11	2.4817
coiled-coil domain containing 72	Ccdc72	6.73E-11	-1.824
late endosomal/lysosomal adaptor, MAPK and MTOR activator 3	Lamtor3	8.47E-11	-1.5656
cytochrome c oxidase, subunit VIb polypeptide 1	Cox6b1	8.85E-11	-1.8064
BCL2-associated agonist of cell death	Bad	9.98E-11	-1.5347
BCL2-like 11 (apoptosis facilitator)	Bcl2l11	1.10E-10	1.3264
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	Adamts1	1.12E-10	1.3939
coiled-coil domain containing 56	Ccdc56	1.19E-10	-1.8499
forkhead box N3	Foxn3	1.21E-10	1.7673
chemokine (C-C motif) ligand 6	Ccl6	1.49E-10	-1.9152
coiled-coil domain containing 153	Ccdc153	1.60E-10	-1.8643
RAS-like, family 11, member A	Rasl11a	1.76E-10	-1.535
cytochrome c oxidase, subunit VIc	Cox6c	1.89E-10	-1.9396

CD53 antigen	Cd53	1.94E-10	-1.5542
ubiquitin specific peptidase 34	Usp34	1.94E-10	1.5945
nuclear factor I/C	Nfic	1.94E-10	1.9803
mitogen-activated protein kinase kinase kinase 13	Map3k13	2.04E-10	2.2629
GATA zinc finger domain containing 2B	Gatad2b	2.28E-10	1.8123
natural killer tumor recognition sequence	Nktr	2.50E-10	1.2598
src homology 2 domain-containing transforming protein C3	Shc3	2.52E-10	2.036
CUGBP, Elav-like family member 2	Celf2	2.56E-10	1.8558
immunoglobulin superfamily, DCC subclass, member 4	Igdcc4	2.63E-10	1.6464
lymphocyte protein tyrosine kinase	Lck	3.31E-10	-1.2848
interleukin-1 receptor-associated kinase 1 binding protein 1	Irak1bp1	3.38E-10	-1.5769
BCL2/adenovirus E1B interacting protein 3	Bnip3	4.27E-10	-1.6971
CD40 antigen	Cd40	4.33E-10	-1.7484
anaphase promoting complex subunit 13	Anapc13	4.39E-10	-1.7916
interleukin 17 receptor D	Il17rd	4.41E-10	1.9374
B cell leukemia/lymphoma 2	Bcl2	4.84E-10	1.3446
mastermind like 2 (Drosophila)	Maml2	5.19E-10	1.4874
anaphase promoting complex subunit 11	Anapc11	5.24E-10	-1.6355
ras responsive element binding protein 1	Rreb1	5.37E-10	2.0917
protein kinase, interferon inducible double stranded RNA dependent activator	Prkra	6.72E-10	-1.4743
retinoic acid receptor responder (tazarotene induced) 2	Rarres2	6.81E-10	-1.5707
B cell leukemia/lymphoma 2 related protein A1b	Bcl2a1b	7.66E-10	-2.9945
histone deacetylase 4	Hdac4	8.23E-10	1.8345
splicing factor 3b, subunit 5	Sf3b5	8.72E-10	-1.5446
myeloid/lymphoid or mixed-lineage leukemia 5	Mll5	8.74E-10	1.9847

coiled-coil domain containing 90B	Ccdc90b	9.27E-10	-1.6183
BCL2/adenovirus E1B interacting protein 1	Bnip1	1.10E-09	-1.3242
coiled-coil domain containing 23	Ccdc23	1.14E-09	-1.3368
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 9	Adamts9	1.38E-09	1.321
interleukin 13 receptor, alpha 2	Il13ra2	1.62E-09	-1.9619
nuclear receptor co-repressor 1	Ncor1	1.66E-09	1.5845
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 12	Adamts12	1.68E-09	1.9621
RAB7, member RAS oncogene family-like 1	Rab7l1	1.71E-09	-1.4527
integrin beta 4	Itgb4	1.71E-09	1.8016
RAS related protein 1b	Rap1b	1.78E-09	-1.5287
forkhead box O3	Foxo3	1.87E-09	1.7157
TNF receptor-associated factor 3	Traf3	1.91E-09	2.0585
CD9 antigen	Cd9	2.11E-09	-1.6197
glycogen synthase kinase 3 beta	Gsk3b	2.22E-09	1.9234
interferon, alpha-inducible protein 27 like 2A	Ifi27l2a	2.51E-09	-2.5516
cytochrome c oxidase, subunit VI a, polypeptide 1	Cox6a1	2.67E-09	-1.4456
TRAF-interacting protein with forkhead-associated domain	Tifa	2.81E-09	-1.6203
coiled-coil domain containing 53	Ccdc53	2.87E-09	-1.4254
RAB26, member RAS oncogene family	Rab26	3.00E-09	-1.5025
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 18	Adamts18	3.13E-09	1.2523
MAD homolog 9 (Drosophila)	Smad9	3.38E-09	1.3487
cytochrome c oxidase, subunit Vb	Cox5b	3.57E-09	-1.5843
cytotoxic T lymphocyte-associated protein 2 beta	Ctla2b	3.60E-09	-2.041

COX16 cytochrome c oxidase assembly homolog (S. cerevisiae)	Cox16	4.15E-09	-1.7752
chemokine (C-C motif) ligand 27A	Ccl27a	4.27E-09	-1.8182
dedicator of cytokinesis 1	Dock1	4.35E-09	1.4669
coiled coil domain containing 28B	Ccdc28b	4.48E-09	-1.2477
interleukin 18	Il18	4.49E-09	-1.4295
CDC42 binding protein kinase alpha	Cdc42bpa	4.60E-09	2.2741
cytochrome c oxidase, subunit VIIIa	Cox8a	5.02E-09	-1.3628
phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	Pik3r1	5.40E-09	1.3438
malignant T cell amplified sequence 1	Mcts1	5.53E-09	-1.339
ataxin 1	Atxn1	5.65E-09	1.951
mitogen-activated protein kinase kinase kinase 9	Map3k9	6.19E-09	2.4578
BCL6 co-repressor-like 1	Bcorl1	6.45E-09	1.8165
coiled-coil domain containing 88C	Ccdc88c	6.79E-09	1.8684
nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 3	Nfatc3	6.84E-09	1.2199
BCL6 interacting corepressor	Bcor	6.95E-09	1.3748
myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4	Mllt4	7.29E-09	1.5566
ADAMTS-like 1	Adamtsl1	7.38E-09	1.6231
cystatin E/M	Cst6	7.67E-09	-1.6213
coiled-coil domain containing 115	Ccdc115	7.91E-09	-1.5257
MAP3K12 binding inhibitory protein 1	Mbip	8.55E-09	-1.5337
anaphase promoting complex subunit 10	Anapc10	9.23E-09	-1.5178
coiled-coil domain containing 84	Ccdc84	1.04E-08	-1.7195
T cell leukemia translocation altered gene	Tcta	1.05E-08	-1.4425
RAN, member RAS oncogene family	Ran	1.06E-08	-1.4659
adenylate cyclase 1	Adcy1	1.13E-08	2.386

ubiquitin specific peptidase 24	Usp24	1.15E-08	1.3304
PAK1 interacting protein 1	Pak1ip1	1.16E-08	-1.2902
BCL2/adenovirus E1B interacting protein 3-like	Bnip3l	1.18E-08	-1.3702
non-catalytic region of tyrosine kinase adaptor protein 1	Nck1	1.19E-08	-1.3549
sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	Sema4d	1.24E-08	1.3942
cytochrome c oxidase, subunit VIIa 1	Cox7a1	1.28E-08	-2.1301
Ras-like without CAAX 1	Rit1	1.35E-08	-1.3489
RAR-related orphan receptor beta	Rorb	1.36E-08	1.4579
Notch gene homolog 1 (Drosophila)	Notch1	1.47E-08	2.4114
a disintegrin and metallopeptidase domain 23	Adam23	1.50E-08	1.5141
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 17	Adamts17	1.54E-08	1.597
lymphocyte antigen 86	Ly86	1.55E-08	-1.6468
chemokine (C-C motif) ligand 25	Ccl25	1.74E-08	-1.6614
a disintegrin and metallopeptidase domain 12 (meltrin alpha)	Adam12	1.79E-08	1.5943
dedicator of cytokinesis 5	Dock5	1.88E-08	1.8291
ubiquitin specific peptidase 49	Usp49	2.02E-08	1.8333
cell division cycle 26	Cdc26	2.09E-08	-1.2625
cell division cycle 34 homolog (S. cerevisiae)	Cdc34	2.10E-08	-1.2529
ubiquitin specific peptidase 36	Usp36	2.13E-08	1.3243
Wiskott-Aldrich syndrome-like (human)	Wasl	2.16E-08	1.3013
myeloid leukemia factor 1	Mlf1	2.18E-08	-1.3263
integrin alpha 11	Itga11	2.20E-08	1.7196
Ras homolog enriched in brain	Rheb	2.25E-08	-1.281
S100 calcium binding protein A16	S100a16	2.28E-08	-1.4785
son of sevenless homolog 1 (Drosophila)	Sos1	2.33E-08	1.4037

tumor necrosis factor receptor superfamily, member 11a	Tnfrsf11a	2.39E-08	1.2215
CDC42 binding protein kinase gamma (DMPK-like)	Cdc42bpg	2.45E-08	1.5903
retinoid X receptor alpha	Rxra	2.64E-08	1.4956
RAB9, member RAS oncogene family	Rab9	2.66E-08	-1.4424
immunoglobulin superfamily, member 3	Igsf3	2.69E-08	1.7284
microtubule-associated protein 1 light chain 3 beta	Map1lc3b	2.73E-08	-1.2518
T-box 20	Tbx20	2.81E-08	2.5761
mechanistic target of rapamycin (serine/threonine kinase)	Mtor	2.95E-08	1.3607
CD83 antigen	Cd83	3.03E-08	-1.2487
S100 calcium binding protein A8 (calgranulin A)	S100a8	3.07E-08	-2.4356
anaphase promoting complex subunit 16	Anapc16	3.07E-08	-1.3616
leukocyte specific transcript 1	Lst1	3.30E-08	-1.4504
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 15	Adamts15	3.37E-08	1.4456
coiled-coil domain containing 58	Ccdc58	3.47E-08	-1.4715
integrin alpha 4	Itga4	3.81E-08	1.2737
mastermind-like domain containing 1	Mamld1	4.00E-08	1.4065
CD300A antigen	Cd300a	4.65E-08	-1.4979
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 3	Adamts3	4.67E-08	1.5286
S100 calcium binding protein A1	S100a1	4.68E-08	-1.6934
transforming growth factor, beta receptor III	Tgfbr3	4.69E-08	1.2645
CD59a antigen	Cd59a	4.72E-08	-1.7165
chemokine (C-X-C motif) ligand 1	Cxcl1	5.24E-08	-1.9013

myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 6	Mllt6	5.54E-08	1.4785
tumor necrosis factor, alpha-induced protein 8-like 2	Tnfaip8l2	5.60E-08	-1.6267
mastermind like 1 (Drosophila)	Maml1	5.77E-08	1.5213
forkhead box K1	Foxk1	5.90E-08	2.163
tolloid-like	Tll1	6.17E-08	1.4164
chemokine (C-C motif) receptor 1	Ccr1	6.32E-08	-1.5864
MAD homolog 3 (Drosophila)	Smad3	6.35E-08	1.6784
cytochrome c oxidase subunit III	COX3	6.77E-08	-1.3201
interleukin enhancer binding factor 2	Ilf2	6.79E-08	-1.2226
COX19 cytochrome c oxidase assembly homolog (S. cerevisiae)	Cox19	7.56E-08	-1.2507
Ras association (RalGDS/AF-6) domain family (N-terminal) member 7	Rassf7	7.57E-08	-1.2105
adenylate cyclase 9	Adcy9	7.78E-08	1.978
CD63 antigen	Cd63	9.54E-08	-1.4596
forkhead box O1	Foxo1	9.61E-08	1.7171
a disintegrin and metallopeptidase domain 19 (meltrin beta)	Adam19	9.92E-08	1.7922
CDC-like kinase 1	Clk1	1.03E-07	-1.389
JNK1/MAPK8-associated membrane protein	Jkamp	1.03E-07	-1.2537
arrestin, beta 1	Arrb1	1.09E-07	1.8425
runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	Runx1t1	1.14E-07	1.4627
dedicator of cyto-kinesis 3	Dock3	1.23E-07	1.9443
interleukin 1 beta	Il1b	1.33E-07	-3.1953
latent transforming growth factor beta binding protein 3	Ltbp3	1.35E-07	1.3637
adaptor protein complex AP-1, mu 2 subunit	Ap1m2	1.38E-07	-1.3653

ADAMTS-like 3	Adamtsl3	1.38E-07	1.3184
guanine nucleotide binding protein, alpha q polypeptide	Gnaq	1.43E-07	1.5085
phosphoinositide-3-kinase, class 2, beta polypeptide	Pik3c2b	1.54E-07	1.7893
ubiquitin specific peptidase 13 (isopeptidase T-3)	Usp13	1.54E-07	1.2968
cystatin C	Cst3	1.55E-07	-1.4457
immunoglobulin-like domain containing receptor 2	Ildr2	1.57E-07	1.2626
forkhead box J2	Foxj2	1.61E-07	1.5213
Notch gene homolog 3 (Drosophila)	Notch3	1.62E-07	2.1223
discs, large (Drosophila) homolog-associated protein 1	Dlgap1	1.64E-07	1.5008
p21 protein (Cdc42/Rac)-activated kinase 4	Pak4	1.78E-07	1.4325
interleukin 4	Il4	1.81E-07	1.2171
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 7	Adamts7	1.87E-07	1.2347
SRC kinase signaling inhibitor 1	Srcin1	2.02E-07	1.8333
CD86 antigen	Cd86	2.09E-07	-1.4856
Duffy blood group, chemokine receptor	Darc	2.20E-07	1.8805
Snf2-related CREBBP activator protein	Srcap	2.21E-07	1.3655
S100 calcium binding protein A9 (calgranulin B)	S100a9	2.39E-07	-2.0865
Ras homolog enriched in brain like 1	Rhebl1	2.67E-07	-1.22
discs, large (Drosophila) homolog-associated protein 2	Dlgap2	2.69E-07	1.7469
dedicator of cytokinesis 9	Dock9	2.86E-07	1.5384
B cell leukemia/lymphoma 11B	Bcl11b	2.93E-07	2.0642
caspase 1	Casp1	3.03E-07	-1.323
protein kinase C, alpha	Prkca	3.03E-07	1.5849
dishevelled 3, dsh homolog (Drosophila)	Dvl3	3.09E-07	1.8678

CDC28 protein kinase regulatory subunit 2	Cks2	3.17E-07	-1.4964
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 2	Adamts2	3.18E-07	1.5077
interleukin 10-related T cell-derived inducible factor beta	Iltifb	3.20E-07	-2.3961
T cell lymphoma invasion and metastasis 1	Tiam1	3.23E-07	2.0286
discs, large homolog 2 (Drosophila)	Dlg2	3.24E-07	1.3519
cathepsin O	Ctso	3.43E-07	-1.2282
forkhead box J3	Foxj3	3.47E-07	1.2904
transcription factor 7 like 1 (T cell specific, HMG box)	Tcf7l1	3.83E-07	1.5258
coiled-coil domain containing 6	Ccdc6	3.94E-07	1.701
RAS-related protein-1a	Rap1a	4.09E-07	-1.3125
forkhead box M1	Foxm1	4.12E-07	1.3554
B cell CLL/lymphoma 9	Bcl9	4.40E-07	2.0256
forkhead box P1	Foxp1	4.55E-07	1.395
ELAV (embryonic lethal, abnormal vision, Drosophila)-like 3 (Hu antigen C)	Elavl3	4.64E-07	1.5461
Casitas B-lineage lymphoma b	Cblb	4.87E-07	1.4681
discs, large homolog 5 (Drosophila)	Dlg5	4.89E-07	1.7797
fos-like antigen 2	Fosl2	5.02E-07	1.6381
CD48 antigen	Cd48	5.13E-07	-1.8838
coiled-coil domain containing 160	Ccdc160	5.14E-07	-1.0062
CDC28 protein kinase 1b	Cks1b	5.41E-07	-1.305
CD84 antigen	Cd84	6.02E-07	-1.4359
caspase 12	Casp12	6.29E-07	-1.3853
interferon gamma inducible protein 47	Ifi47	6.30E-07	-1.6666
ubiquitin specific peptidase 42	Usp42	6.56E-07	1.3803
CUGBP, Elav-like family member 1	Celf1	6.56E-07	1.2228

phospholipase C, gamma 1	Plcg1	6.82E-07	1.248
chemokine (C-C motif) ligand 12	Ccl12	7.17E-07	-1.9695
mature T cell proliferation 1	Mtcp1	7.32E-07	-1.3497
B cell CLL/lymphoma 9-like	Bcl9l	7.61E-07	2.182
RAB32, member RAS oncogene family	Rab32	7.65E-07	-1.2409
interferon-stimulated protein	Isg20	7.81E-07	-1.442
mitogen-activated protein kinase binding protein 1	Mapkbp1	8.08E-07	1.5018
phospholipase C, beta 1	Plcb1	8.37E-07	1.2312
nuclear factor I/X	Nfix	8.62E-07	2.0714
a disintegrin and metallopeptidase domain 22	Adam22	8.70E-07	1.2892
TGF-beta activated kinase 1/MAP3K7 binding protein 3	Tab3	8.87E-07	1.2923
transforming growth factor, beta receptor associated protein 1	Tgfbrap1	8.92E-07	1.3063
CD209f antigen	Cd209f	9.01E-07	-1.4813
nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1	Nfatc1	9.49E-07	1.3363
NCK-associated protein 5	Nckap5	9.89E-07	1.4162
FAD-dependent oxidoreductase domain containing 2	Foxred2	1.02E-06	1.4511
cytochrome c oxidase subunit VIIb2	Cox7b2	1.04E-06	-1.7261
S100 calcium binding protein A13	S100a13	1.11E-06	-1.388
integrin alpha 3	Itga3	1.16E-06	1.2366
coiled-coil domain containing 28A	Ccdc28a	1.22E-06	-1.297
X-linked lymphocyte-regulated complex	Xlr	1.26E-06	-1.7171
transforming growth factor alpha	Tgfa	1.30E-06	1.2669
interleukin 12 receptor, beta 2	Il12rb2	1.32E-06	2.0879
hypoxia-inducible factor 1, alpha subunit inhibitor	Hif1an	1.33E-06	1.2766
adenylate cyclase 5	Adcy5	1.41E-06	1.7851

adenylate cyclase 6	Adcy6	1.42E-06	1.2083
GRB10 interacting GYF protein 1	Gigyf1	1.54E-06	1.5862
T-cell immunoglobulin and mucin domain containing 2 pseudogene	Gm4926	1.69E-06	-1.5855
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G	Sema3g	1.73E-06	1.424
immunoglobulin superfamily, DCC subclass, member 3	Igdcc3	2.05E-06	1.4696
squamous cell carcinoma antigen recognized by T cells 1	Sart1	2.06E-06	1.2246
interferon-induced protein 35	Ifi35	2.15E-06	-1.3033
sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4G	Sema4g	2.18E-06	1.5283
S100 calcium binding protein A5	S100a5	2.61E-06	-2.8835
NCK-associated protein 5-like	Nckap5l	2.71E-06	1.8654
RAB6B, member RAS oncogene family	Rab6b	2.86E-06	1.3191
mitogen-activated protein kinase 8 interacting protein 3	Mapk8ip3	2.91E-06	1.5485
mitogen-activated protein kinase kinase kinase 5	Map3k5	2.96E-06	1.269
protein kinase C and casein kinase substrate in neurons 1	Pacsin1	3.06E-06	1.4931
MAP/microtubule affinity-regulating kinase 4	Mark4	3.17E-06	1.4975
vav 2 oncogene	Vav2	3.25E-06	1.4015
chemokine-like factor	Cklf	3.37E-06	-1.2909
latent transforming growth factor beta binding protein 4	Ltbp4	3.43E-06	1.6984
linker for activation of T cells family, member 2	Lat2	3.64E-06	-1.9848
coiled-coil domain containing 103	Ccdc103	4.09E-06	-1.2257
microtubule-associated protein, RP/EB family, member 2	Mapre2	4.57E-06	1.3526
Rous sarcoma oncogene	Src	5.11E-06	1.462

splicing factor 1	Sf1	5.12E-06	1.3615
myeloid-associated differentiation marker	Myadm	5.14E-06	1.2936
discs, large (Drosophila) homolog-associated protein 3	Dlgap3	5.51E-06	1.4799
tumor necrosis factor receptor superfamily, member 12a	Tnfrsf12a	6.00E-06	-1.2305
MAP-kinase activating death domain	Madd	6.08E-06	1.2296
interleukin 15	Il15	6.30E-06	-1.662
CDC42 binding protein kinase beta	Cdc42bpb	7.17E-06	1.8055
guanine nucleotide binding protein, alpha O	Gnao1	7.35E-06	1.4771
MAP/microtubule affinity-regulating kinase 1	Mark1	7.88E-06	1.2378
CCR4-NOT transcription complex, subunit 3	Cnot3	8.09E-06	1.2313
interleukin 22	Il22	9.41E-06	-2.162
mitogen-activated protein kinase kinase kinase 8	Map3k8	1.01E-05	-1.4099
CD93 antigen	Cd93	1.05E-05	1.3519
nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2	Nfatc2	1.11E-05	1.2136
immunoglobulin superfamily, member 5	Igsf5	1.25E-05	-1.2568
Ras and Rab interactor 3	Rin3	1.27E-05	1.3482
immunoglobulin superfamily, member 6	Igsf6	1.29E-05	-1.3329
protein kinase C, epsilon	Prkce	1.29E-05	1.4301
p21 protein (Cdc42/Rac)-activated kinase 6	Pak6	1.37E-05	1.2262
CD52 antigen	Cd52	1.50E-05	-1.7423
delta-like 1 (Drosophila)	Dll1	1.53E-05	1.2633
splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	Sfpq	1.56E-05	1.2486
CD1d1 antigen	Cd1d1	1.60E-05	-1.2071
tolloid-like 2	Tll2	1.75E-05	1.5856
aryl hydrocarbon receptor nuclear translocator 2	Arnt2	1.84E-05	1.2532
deltex 3-like (Drosophila)	Dtx3l	1.86E-05	1.2808

CD200 receptor 1	Cd200r1	1.99E-05	-1.5711
phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1	Prex1	2.05E-05	1.2251
cytochrome c oxidase, subunit XVII assembly protein homolog (yeast)	Cox17	2.64E-05	-1.2697
linker for activation of T cells	Lat	2.73E-05	-1.9806
hairless	Hr	2.82E-05	1.4814
forkhead box D3	Foxd3	2.86E-05	1.4502
	Fas	2.91E-05	-1.6839
caspase 4, apoptosis-related cysteine peptidase	Casp4	2.97E-05	-1.6619
chemokine (C-C motif) ligand 7	Ccl7	3.00E-05	-1.6164
T-box 3	Tbx3	3.09E-05	1.2446
RAB38, member of RAS oncogene family	Rab38	3.15E-05	-1.2267
deltex 1 homolog (Drosophila)	Dtx1	3.64E-05	1.4082
CD209g antigen	Cd209g	3.77E-05	-1.9848
adenylate cyclase 3	Adcy3	3.83E-05	1.2505
matrix metallopeptidase 13	Mmp13	4.26E-05	-1.7194
chemokine (C-X-C motif) ligand 11	Cxcl11	4.45E-05	-1.7877
matrix metallopeptidase 15	Mmp15	4.67E-05	1.295
interleukin 15 receptor, alpha chain	Il15ra	4.79E-05	-1.3734
CUGBP, Elav-like family member 5	Celf5	4.90E-05	1.3492
chemokine (C-X-C motif) receptor 5	Cxcr5	6.26E-05	1.7003
retinoic acid receptor, alpha	Rara	6.41E-05	1.2092
adaptor protein complex AP-2, alpha 1 subunit	Ap2a1	6.49E-05	1.3822
RAB17, member RAS oncogene family	Rab17	7.18E-05	-2.0473
lymphocyte antigen 6 complex, locus G6D	Ly6g6d	7.51E-05	-2.0459
cytochrome c oxidase, subunit VI a, polypeptide 2	Cox6a2	8.74E-05	-1.2031
forkhead box P4	Foxp4	9.68E-05	1.2832
chemokine (C-C motif) ligand 24	Ccl24	9.86E-05	-1.2683

Table S2. Additional cell adhesion molecules (CAMs) and related proteins involved in leukocyte migration that were differentially expressed in the pup brain, in response to maternal *H. bakeri* infection.

Classification	Gene Name	Gene Symbol	P-value	Log 2 Fold change
CAMs	L1 cell adhesion molecule	L1CAM	8.70E-06	1.8125
	Down syndrome cell adhesion molecule	DSCAM	1.48E-08	2.0801
	Down syndrome cell adhesion molecule like 1	DSCAML1	3.01E-07	2.0985
Immunoglobulin superfamily	Immunoglobulin superfamily, member 5	IGSF5	1.25E-05	-1.2568
Fibronectin	Fibronectin 1	FN1	4.85E-10	1.9769
	Leucine rich repeat and fibronectin type III domain containing 1	LRFN1	9.51E-05	1.3025
	Ankyrin-repeat and fibronectin type III domain containing 1	ANKFN1	9.94E-10	1.8309
	Fibronectin leucine rich transmembrane protein 1	FLRT1	2.65E-09	1.84
	Fibronectin type III domain containing 1	FNDC1	1.94E-08	1.5198
	Fibronectin type III domain containing 3B	FNDC3B	4.09E-07	1.2979
	Leucine rich repeat and fibronectin type III, extracellular 2	ELFN2	3.04E-06	2.1185
Contactin	Contactin associated protein-like 1	CNTNAP1	1.30E-13	2.1963
	Contactin 2	CNTN2	1.86E-11	1.6599
	Contactin associated protein-like 5B	CNTNAP5B	2.44E-10	1.2308
	Contactin associated protein-like 5A	CNTNAP5A	6.06E-08	1.3932

Table S3. List of additional adheren junction proteins differentially expressed in the pup brain, in response to maternal *H. bakeri* infection.

Classification	Gene Name	Gene Symbol	P-value	Log 2 Fold Change
Protocadherin	Protocadherin 15	PCDH15	1.26E-10	1.508
	Protocadherin 17	PCDH17	1.03E-08	1.4387
	Protocadherin beta 18	PCDHβ18	2.10E-08	1.4765
	Protocadherin beta 22	PCDHβ22	4.06E-07	1.2856
	Protocadherin 9	PCDH9	7.17E-07	1.212
	Protocadherin alpha 11	PCDHα11	9.13E-07	1.7565
	Protocadherin beta 21	PCDHβ21	9.25E-07	1.3665
	Protocadherin beta 15	PCDHβ15	1.98E-06	1.2953
	Protocadherin 1	PCDH1	4.12E-06	1.8629
	Protocadherin beta 2	PCDHβ2	8.88E-06	1.2351
	Protocadherin gamma subfamily B, 6	PCDHγB6	7.12E-05	1.4781
Plakoglobin	Junction plakoglobin	JUP	3.72E-05	1.2995