MAGEE-WOMENS RESEARCH INSTITUTE & FOUNDATION

THE MAGEE-WOMENS RESEARCH INSTITUTE CLINICAL TRAINEE RESEARCH AWARD

FACE PAGE (must be typewritten)

APPLICANT INFORMATION		
NAME (Last, first, middle) [redacted]		[redacted] ARE YOU A RESIDENT OR FELLOW?: Fellow
POSITION TITLE: Fellow Physician		[redacted]
YEAR(S) IN TRAINING:5 YEAR(S) IN CURRENT PROGRAM:2		[redacted]
DEPARTMENT: [redacted]		
TEL: [redacted]	FAX:	E-MAIL ADDRESS: [redacted]

APPLICATION TITLE: Measuring Reactivity of Breastmilk Derived IgA from a Diverse Arkansas and Spain Population

HUMAN SUBJECTS RESEARCH ☐ No ☐ Yes		IRB APPROVAL DATE:
VERTEBRATE ANIMALS ☑ No ☐ Yes		IACUC APPROVAL DATE:
TOTAL FUNDS REQUESTED	$5000	

FACULTY SPONSOR	DEPARTMENT CHAIR OR DIRECTOR OF FELLOWSHIP/RESIDENCY PROGRAM
Name [redacted]	[redacted]
[redacted]	[redacted]
SIGNATUR [redacted]	SIGNATURE [redacted]

APPLICANT SIGNATURE	DATE
[signature]	[redacted]

PROPOSED BUDGET

Personnel – List **effort** for **all** personnel to be involved in carrying out the proposed research, whether or not salary is requested, **beginning with P.I.**

Name	Role	% Effort	Salary Requested	Fringe Benefits*	Total
[redacted]	[redacted]		[redacted]	[redacted]	[redacted]
[redacted]	[redacted]		[redacted]	[redacted]	[redacted]
[redacted]	[redacted]		[redacted]	[redacted]	[redacted]
[redacted]	[redacted]		[redacted]	[redacted]	[redacted]
		[redacted]	[redacted]	[redacted]	[redacted]

Equipment: n/a	Subtotal	
Supplies (list): Bacterial Media	**$500**	
Flow cytometry ($50/hour analysis and $150/hour sorting)	**$2500**	
Anti-Human IgA Antibodies ($450 per 500µg)	**$2000**	
	Subtotal	
Other Expenses-n/a	Subtotal	
	Total	$5000

*MWRIF Staff fringe benefit rate is 25.0%

BUDGET JUSTIFICATION

Senior/key personnel

[redacted]

Measuring Reactivity of Breastmilk Derived IgA from Diverse Arkansas and Spain Population

Abstract
Necrotizing enterocolitis (NEC) is a leading cause of morbidity and mortality in preterm infants, with unclear etiology. Bioactive components of maternal milk, such as IgA, shape the intestinal microbiota (Gopalakrishna et al 2019). Secretion of IgA into the intestine assists in the regulation of the microbiota and specifically controls the overgrowth of *Enterobacteriaceae*. Analysis of IgA binding to fecal bacteria showed an increase in the IgA unbound fraction of *Enterobacteriaceae* fraction prior to onset of NEC (Gopalakrishna et al 2019). Previous work done by Johnson-Hence and colleagues analyzed the anti-bacterial reactivity of breastmilk derived IgA (BrmIgA) using a relatively homogenous donor population of Caucasian women from Pittsburgh. In this cohort, there was considerable inter-donor heterogeneity in the anti-bacterial reactivity of breast milk-derived IgA, including reactivity to *Enterobacteriaceae*. Here, we propose to expand our cohort to include greater socioeconomic and ethnic diversity and correlate anti-bacterial IgA reactivity to maternal health data.

Training plan: [redacted] has chosen to perform the research portion of his fellowship in my laboratory at the Rangos Research Building in the UPMC Children's Hospital of Pittsburgh. Our work focuses upon the interaction between the intestinal microbiota and the immune response, with a particular focus on how the phenomenon of immunological memory can be understood in the context of the microbiota. Immunological memory is so important in the intestine that mothers pass it onto their infants via the trans-placental and oral transmission of antibodies. We have demonstrated that the binding of maternally-derived IgA to the nascent microbiota of preterm infants can be important for preventing the development of NEC. More specifically, we have shown that a 'shift' in the level of IgA binding to the intestinal microbiota can precede the development of NEC. This discovery led us to investigate whether there may be heterogeneity in the reactivity of maternal IgA to various bacteria between different mothers. To test this question, we built a novel flow cytometric array to test the reactivity of maternal IgA, and with breast milk collected from a cohort of women residing in Pittsburgh, we demonstrated that maternal IgA reactivity to the most common bacteria found in preterm infants differed substantially between mothers. Dr. [redacted]s work in the laboratory will focus on testing whether similar results will be produced in cohorts that our collaborators in Arkansas and Spain have collected that will better reflect the diversity of the United States and Europe. Over the course of these studies Dr. [redacted] will be trained in a number of techniques that will serve him well in his development as a physician scientist. He will be extensively trained in flow cytometry, bacterial culture and protein isolation (antibodies from milk). Dr. [redacted] will also be trained in the rigorous use of controls, data collection methods and statistical analysis to ensure reproducibility. I have found that this work is perfectly suited to a Neonatology fellow as it can be completed in discrete pieces that fit around a clinical schedule. Our previous Neonatology fellow Dr. Johnson-Hence's work has been exceptionally well-received and is currently in revision at the *Journal of Experimental Medicine*. I have all the confidence in the world that Dr. [redacted] will complete a similar trajectory in my lab and find his training in my lab fruitful and useful.

Specific Aims Significance and Experimental Design

Several studies have identified microbial dysbiosis and subsequent immune dysregulation in the pathogenesis of NEC (Neu & Pammi 2018). NEC occurs when receptors in the intestine recognize antigens from microbes that activate pro-inflammatory cytokines and chemokines in the innate immune response (Neu & Pammi 2018). Therefore, it is important to understand the anti-bacterial binding of IgA because it provides the first source of antigen-specific immunity to the newborn and shapes gut microbiota (Rogier et al 2014).

Breastmilk is the recommended source of nutrition for all infants in the first 6 months, especially preterm infants. Formula is associated with an increased incidence of NEC (Meek & Noble 2022; Neu & Pammi 2018). There are multiple bioactive components that could affect the preterm microbiota and therefore the development of NEC, including IgA. The anti-bacterial reactivity of breastmilk-derived IgA reflects the maternal microbiome and GI infection history, since intestinal IgA+ B cells traffic to the mammary gland to secrete IgA antibody for the newborn (Gopalakrishna et al 2020). Secretory IgA (sIgA) modulates the microbiota by neutralization, enchained growth, increased uptake by Peyer's patches, tethering bacteria to mucous layer and modulation of bacteria expression but how it modulates the preterm microbiota is not clearly understood (Hand & Reboldi 2021).

Although there is no clear etiology of NEC, studies have found an association with decreased microbiota diversity and increased abundance of *Enterobacteriaceae* (Gopalakrishna & Hand 2019). We know that IgA-unbound *Enterobacteriaceae* is increased prior to the onset of NEC (Gopalakrishna & Hand 2019). Interestingly, the antibody specificity in breastmilk differs between mothers and is distinct to each host (Johnson-Hence et al, unpublished results). Specifically, Johnson-Hence et al. showed heterogeneity between donors with no two samples being identical with some donors being enriched for anti-*Enterobacteriaceae* reactivity while other donors lack this almost entirely. Interestingly, IgA reactivity was stable over sequential sibling births, suggesting that IgA+ B cells in mammary glands are stable over a long time. Lastly, the IgA anti-bacterial reactivity of preterm and term milk were indistinguishable. Therefore, if a mother lacks anti-*Enterobacteriaceae* reactivity in her breast milk, her infant may be at-risk for NEC throughout the maternal feeding period. It is critical to understand whether the anti-bacterial reactivity of IgA differs from mother to mother.

The results from Johnson-Hence et al. reflect a homogenous cohort. We would like to compare them to a more diverse cohort from urban and rural Arkansas and a geographically different cohort from Valencia, Spain. Our long-term goal is to understand how IgA can be used to regulate the microbiota for the treatment or prevention of intestinal injury. Our specific objective is to examine maternal anti-bacterial IgA reactivity in a diverse set of donors and correlate these to maternal health data. Based on preliminary data from our laboratory, our central hypothesis is that diverse infection and microbiota-colonization histories in different mothers will induce diverse anti-bacterial reactivity signatures in maternal IgA. Whether our results are specific to the cohort studied, milk donors to the Mid-Atlantic Mother's Milk Bank, a relatively homogenous group compared to the rest of the United States and the world, is not clear. Here we will test the heterogeneity of anti-microbiota reactivity amongst milk donors from Arkansas and Valencia, Spain. We will test our hypothesis, in two specific aims:

1. Investigate the heterogeneity of anti-microbiota reactivity in donors from Arkansas and Valencia, Spain.

2. Using correlation analysis, test whether IgA reactivity clusters according to geographical location, socioeconomic background or other factors.

The rationale for this research is to understand the immunological reactivity of sIgA with regard to the microbiota in a diverse population. This knowledge is necessary to determine if supplementation of formula and human milk-fed infants in the NICU with recombinant IgA is necessary and feasible. It may help to understand why some human milk-fed infants can still develop NEC. The data from our study may provide more insight into the prevalence of NEC which is seen at a higher odds among non-Hispanic black infants (Jammeh et al 2018). By examining the reactivity pattern, we can also further advance the investigation into the factors affecting IgA efficiency.

Experimental design: include means by which data will be analyzed and interpreted

We will compare anti-bacterial reactivity from donors of different geographical locations Arkansas (urban and rural) and Valencia, Spain. Our goal is to identify if the heterogeneity that we detected amongst women in Pittsburgh is generalizable to donors from different parts of the world with varied environmental and immunological experiences.

The process of immunoglobulin A extraction and analysis of bacterial reactivity by flow cytometry is shown in **Figure 1**. For creation of the array, we identified strains within the University of Pittsburgh community and American Type Culture Collection (ATCC) that would be representative of the preterm infant microbiota. Bacteria were grown according to guidelines provided by ATCC or the providing investigator to stationary phase (~24-48 hours). Purified IgA from breast milk samples was thawed at 4°C and normalized to 0.1mg/ml before adding it to the arrayed bacteria. After washing the 96-well plate was stained with a secondary antibody staining mixture of Syto BC (DNA stain to separate bacteria from debris), an APC-conjugated Anti-Human IgA and blocking buffer of Normal Mouse Serum. Samples were then analyzed by flow cytometry. For each donor we run a separate plate that was stained only with the Syto BC/APC anti-human IgA mix (no milk-derived IgA). These control samples are used as background fluorescence controls to establish positive binding signals and normalize samples collected on different days.

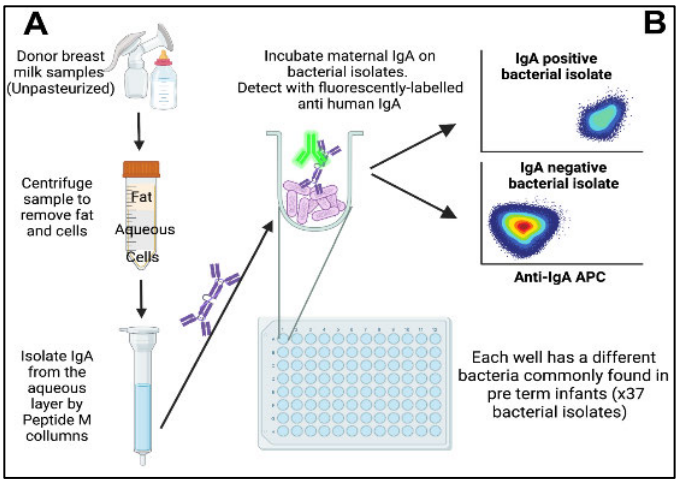

Figure 1. IgA Extraction from Breastmilk

Flow cytometric data from different donors is then normalized according to the background fluorescence of the 'unstained' plate and analyzed by hierarchical clustering according to the normalized geometric mean fluorescent intensity. For these experiments we will add additional components to our clustering analysis associated with maternal health (gestational age of the infant, BMI of the donor, rural vs. urban setting and drug use). We will compare donors from Arkansas and Spain to our previously analyzed samples from Pittsburgh.

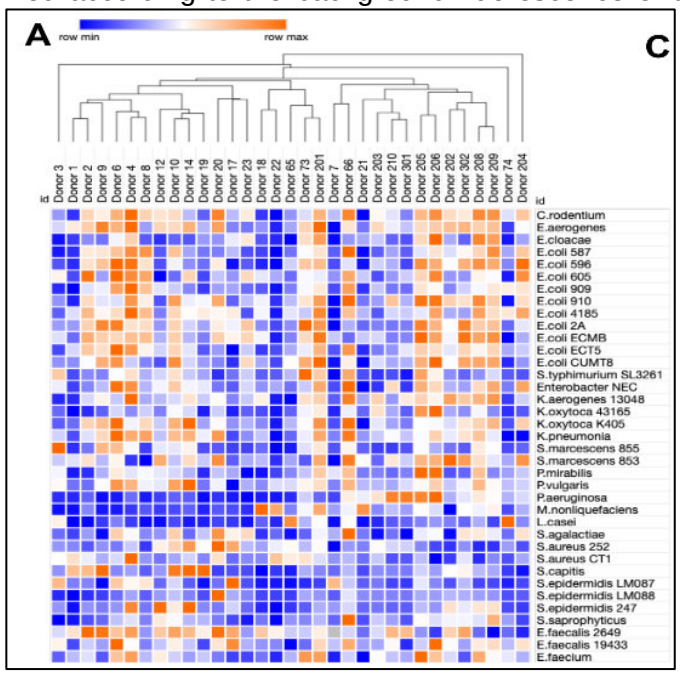

Figure 2. Heat map of normalized anti-bacterial IgA binding of 33 donors to 37 bacterial isolates.

Expected results: We expect that our previous result of substantial heterogeneity in the anti-bacterial reactivity of breast milk-derived IgA will be confirmed by these analyses. Meta-analysis including donor health data may yield differences. For example, we suspect that within the Arkansas samples urban and rural may cluster separately and perhaps the urban samples may cluster more closely to samples from Pittsburgh and Spain (Valencia) that are collected in more urban settings. Obesity has been shown to affect the composition of the microbiota and thus BMI may also affect clustering of the samples The widespread use of antibiotics, proton pump inhibitors and opioids are known to affect microbiota (Dowd & Rensen 2018) and these could also correlate to our clustering of the data.

Pitfalls and alternative approaches: Heterogeneity between donors may be highly individualized and we may not observe any clustering associated with location or any health data from the donors and. This would be an exciting finding and in the future, we could work to identify the sources that drive this phenomena, including donor microbiota and infection history.

This basic science project can be completed in the one-year time given per the funding guidelines. In addition, the overall time allocated for research provides sufficient additional time for corrections or revisions after the initial period. A power analysis performed in the lab based on our previous results indicates that we will need ~20-25 samples from each region to begin to see differences, well below the hundreds of samples available.

References

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