Malnutrition and the immune response: Understanding the biology of nutrition-related immune dysfunction

Caitlin Naylor

B.S with Honours, University of Edinburgh, 2010

A Thesis presented to the Graduate Faculty

of the University of Virginia in Candidacy for the Degree of

Doctor of Philosophy

Department of Microbiology, Immunology, and Cancer

University of Virginia

January 2015

Acknowledgements

Gaining a PhD is not something that is done alone, success depends on a multitude of people guiding you along the way. I would like to first thank Nicole, who oversaw my mentorship during my first rotation in the Petri lab and dealt with the foibles of a newly-minted graduate student with unfailing kindness and patience. I would also like to thank my fellow lab-mates; a large part of the success I have found is because the atmosphere at work is so wondeful, and that is because of you. Thanks especially to Nate for the entertaining conversations and life advice while we shared an office, to Rajat for being a wonderful science partner, to Shiuly for those late-night mouse infections, to Stacey for being the reason my immunological data actually makes sense and was done correctly, and to Erica and Carrie for your endless help in tissue harvests and listening to my rants. I would also like to thank the PROVIDE team, it has been an honor to work on this project with you.

I would also like to thank my friends from my grauate year, especially Katie and Jessica, your friendship has been a lifeline. Thank you to my committee, Alison Criss, Loren Erikson, Young Hahn and Jim Casanova. Your support and advice kept me going in the best direction. Thank you to my parents, Cheryl and Olen, your encouragement throughout my life is the reason I had the ability and confidence to undertake this journey.

I would finally like to acknowledge and thank my mentor, Dr. William Petri. I could not have asked for a better scientific education, and am becoming the scientist I want to be due to your mentorship. Your unwavering belief in my abilities has been the greatest gift a student could have.

Table of contents

Abstract

Malnutrition affects 20% of the world's children. As a result of malnutrition children experience reduced congition, motor and development skills and reduced productivity and economic growth as adults. They are also at increased risk for morbidity and mortalitiy due to infectious disease: a malnourished child can be up to 9 times more likely to die from a diarrheal infection. Susceptibility to disease is a result of malnutrition-induced immunosuppression. High burdens of infections tax the already weak immune system and result in increased inflammation when young. Investigating the effect of immune dysfuncition in malnutrition is an active area of research, but currently there is a gap in knowledge as to the biological mechanism for immune cell dysfunction, and an unclear understanding of the relationship between immune inflammation and nutrition.

Leptin is a hormone intimately connected with nutrition. Leptin signals adequate energy from food intake; malnourished children have significantly reduced circulating leptin levels. Leptin is a hormone with pleiotropic effects, including regulation of the immune system. Leptin is thought to signal energy availability and could play a critical role in controlling the functional capabilities of immune cells. A mutation in the leptin receptor, Q223R, was found to increase susceptibility to *Entamoeba histolytica* in a cohort of children in Dhaka, Bangladesh. Using a murine model expressing either wild-type Q223 receptor or mutant R223 receptor, we investigated the effect of this mutation on the host's ability to respond to an *E. histolytica* infection. We found that neutrophil influx to the site of the infection was

reduced early after infection in R223 mice, and that neutrophil influx was important for amebic clearance. Leptin expression was increased in the cecum after infection, and we further observed that R223 neutrophils did not chemotax towards leptin to the same extant as wild-type neutrophils. This suggests that a plausible mechanism of susceptibility is reduced neutrophil migration towards the leptin expressed at the site of inflammation due to the diminished signaling capability of the R223 leptin receptor, ultimately resulting in a smaller neutrophil population unable to clear *E. histolytica*. This result sheds light on the effect that reduced leptin could have in malnutrition, and as this is a common mutation could represent a common defense mechanism against infection.

Malnutrition in children cannot always be rescued by nutritional supplementation. It has been modelled that stunting would only be reduced by a third if all cases of malnutrition were treated with food therapy. Oral vaccines such as the oral polio vaccine also have reduced efficacy in malnourished populations. Environmental enteropathy, a condition of chronic enteric inflammation seen in developing countries, is thought to play a role in exacerbating or causing malnutrition. The causes of environmental enteropathy are not clear, but repeated enteric infections could be a source. We evaluated markers of enteric inflammation in a cohort of children in Dhaka, Bangladesh, to determine association with malnutrition and oral polio vaccine performance. We also measured enteric infections, nutrition, and markers of systemic inflammation. We found that up to 82% of the children had abnormally high levels of enteric inflammatory markers, and high enteric pathogen presence. Markers of both enteric and systemic inflammation correlated with

malnutrition, as well as with oral polio vaccine antibody response. We also found that diarrheal incidence did not correlate with enteric inflammation, but with systemic inflammation. This result suggested that environmental enteropathy could be the result of subclinical enteric infections, while overt disease drove a more systemic response. Furthermore, both pathways can result in malnutrition. These results have exciting implications for how we consider the interaction of nutrition with inflammation.

Chapter 1: Introduction

Malnutrition and the Immune system

1.1 The global scale of malnutrition

Malnutrition is a catch-all term for an imbalance of energy intake and nutrients, and includes both overnutrition and undernutrition. This dissertation will discuss only undernutrition, referred to as malnutrition. Malnutrition is when there is chronically or acutely insufficient food intake or poor absorption of nutrients. Common methods of measuring malnutrition are stunting (height-for-age z score of -2 standard deviations or lower), being underweight (weight-for-age z score of -2 standard deviations or lower), and wasting (weight-for-height z score of -2 standard deviations or lower)[1].

In 2013 the WHO, World Bank, and UNIFECF estimated there were 161 million stunted children, and 99 million underweight children[2]. These children are primarily located in Africa, Asia and Oceania. It is a condition of poverty: even within developing countries the lowest income population has the highest burden of malnutrition[3]. Malnutrition results in not only morbidity and mortality, but in loss of cognition, language and motor development, productivity, and eventual economic earning power, perpetuating the poverty that causes it[4–6].

1.2 The effect of malnutrition on disease susceptibility

Malnutrition can be identified as the cause of mortality in 45% of deaths in children under 5 years of age[7]. A large proportion of those deaths are as a result of increased susceptibility to infection, a well-known effect of malnutrition. The overall risk of death to a variety of infections increases with worsening nutrition[8]. Two meta-analyses each combining infection and nutrition results from 10 cohort studies

throughout Asia and Africa found that even mild malnutrition (WAZ -1 – -2) significantly increased mortality from many diseases[9,10]. The types of diseases exacerbated by malnutrition span a wide range[9–11]. Diarrheal and respiratory infections exhibit the greatest increase in morbidity and mortality: children with an HAZ <-3 have an odds ratio of 9.5 for mortality by diarrhea, and of 6.4 for respiratory pneumonia[8]. However, within these categories it appears that not all infections are affected equally. Studies investigating the causes of diarrhea in Bangladesh found that susceptibility to some pathogens such as ETEC, *E. histolytica*, and *Cryptosporidium* were associated with malnutrition, while susceptibility to other enteric pathogens such as *Shigella flexnerii* and *Campylobacter jejunii* was not associated[12,13].

1.3 Immune dysfunction as a result of malnutrition

Malnourished children in general have more severe infections, raising the question of how the immune system differs in malnourished versus well-nourished children. In a malnourished child a variety of cell types exhibit dysfunction. A powerful method to study the effect of malnutrition on immune function is to follow a cohort of children who are receiving nutritional rehabilitation for malnutrition. Comparison of immune parameters before and after the rehabilitation allows investigation into the effect of malnutrition specifically. This study design allows children to be their own controls and avoids some of the confounders of matched well-nourished and malnourished cohorts such as precise age matching and the possibility of differing infections and enteric enteropathy.

Impaired cellular function as a result of malnutrition

A consistent observation within cohorts has been impairment of immune function in malnourished children, and that function can be rescued by nutritional therapy. An early study by McMurray *et al* in Colombia investigated a large cohort of malnourished children that had received nutritional therapy, following them for a year after treatment [14]. Children were suffering from severe malnutrition, and were recruited when they presented at an outpatient hospital with gastrointestinal or respiratory disease, and received a diet with increased protein and energy for 4-5 weeks at the hospital. Measures of immune function were lymphocyte counts, serum immunoglobulin and complement concentrations, lymphocyte blastogenesis, and a PPD skin reaction test. Tests were performed at admission, then every 2 weeks until discharge; follow-up tests were performed at 1, 2, 6 and 12 months after discharge. A well-nourished cohort with no infections was also included for comparison at admission. Lymphocyte counts were normal, but blastogenesis was low in malnourished children. IgG and complement protein C3 were reduced in malnourished children while IgA was increased. After renutrition IgG and C3 increased, IgA decreased. Malnourished children exhibited no response to the PPD test on admission; after renutrition up to 90% of children responded to the test. Humoral and cellular immunity are clearly linked to the nutritional status of children, and it is striking that all measures see improvement after renutrition. At 12 months after discharge, however, there were differences. Immunoglobulin levels had fallen for all children, but were not below the well-nourished control group's levels. Lymphocyte blastogenesis, however,

fell back to near-admission levels, and the majority of children (65%) lost their sensitivity to PPD at one year. This would suggest that renutrition has a variety of effects, and that some factor is not sustained once the children leave the treatment center.

A study performed in Mexico found that neutrophil function was improved in a cohort of children diagnosed with severe protein-energy-malnutrition, determined by a WAZ or WHZ of 3 standard deviations below the norm, after nutritional rehabilitation[15]. Children free of infections were given a milk formula fortified with corn syrup and protein, as well as vitamins, folic acid and iron, for 4 weeks. Neutrophils were isolated from whole blood collected upon enrollment and discharge. The cells were tested for phagocytosis and microbicidal activity against *E. coli*, and chemotaxis towards human serum. Comparison of values before and after nutritional rehabilitation gave a percent change in function. All functional assays showed an improvement after renutrition. In addition to neutrophil function, lymphocyte proliferation was also assayed. Again, this increased after renutrition. This study found that neutrophil function is impaired as a result of malnutrition; this finding could have particular significance for enteric bacterial and protozoan infections. Diarrheal diseases are exacerbated by malnutrition, and it is possible that an underlying cause could be impairment in neutrophils, the first line of defense.

The effect of malnutrition on PBMC cytokine production has also been explored. A study in Chile investigated the effect of weight gain on cytokine production from stimulated PBMCs, and found that cytokine production was increased after weight gain[16]. This study contained a cohort of children with moderately severe PCM (WAZ -2

– -3). Blood was taken upon admission to a nutritional recovery center and after a 10% weight gain. Assays included serum leptin and IGF-1 concentration measurement, PBMC production of IL-1, TNFα, and IL-6 after LPS stimulation, PBMC production of IFNγ and IL-4 after PMA/ionomycin stimulation, and lymphoproliferation. Weight gain was accompanied by an increase in PBMC production of IL-1, TNFα, IL-6 and IFNγ and a decrease in IL-4 production. Lymphoproliferation was also increased after weight gain. Leptin was observed to increase after weight gain, but did not correlate with overall body fat percentages. This study found that weight gain correlated with an increase in cytokine production and systemic leptin. The increased leptin is suggested as an effector of the cellular recovery.

These studies form a picture of reduced immune cell function as a result of malnutrition. Renutrition can rescue this phenotype. While the studies utilize different assays and focus on different branches of the immune system, combined they reveal a common thread of functional impairment whether it be cytokine production, immunoglobulin production, or neutrophil response. The conversion from a negative PPD test to a positive test in children after renutrition reported by McMurray *et al* tallies well with the observation by Palacio *et al* that T cell cytokine production is markedly higher after weight gain in malnourished children. The addition of the work in neutrophil function, and that complement proteins are reduced in malnutrition indicates that all components of the immune system could experience dysfunction in a malnourished setting.

The long-term effect of renutrition on immune function is an interesting question. McMurray *et al* is the only study to follow their rehabilitated cohort for any

length of time, and found that the cell-mediated immunity was lost even though normal nutrition was maintained. The question is why this would occur. This study did not report WAZ, HAZ, or WHZ for the children after follow-up, and did target suffering from extreme malnutrition with symptoms such as edema and emaciation. It is possible that while the children maintained recovery from the overt physical symptoms of their malnutrition, they would still be underweight. This continued moderate malnutrition, combined with environmental burdens such as enteropathogen infection, could result in this loss of immune repair. It would be worthwhile to know if the nutritional rehabilitation efforts observed in PBMCs and neutrophils extend once a child returns to their previous environment and diet. This would speak to the differences between immune dysfunction due to poor nutrition, and those that are a result of chronic infection and inflammation.

Global malnutrition-induced dysfunction

A study by Bartz et al in Uganda employed metabolomics analysis to detect changes in hormones, growth factors, cytokines and metabolites before and after nutritional rehabilitation for children (6 months-5 years) with severe acute malnutrition (SAM). SAM was defined as a WHZ score <-3 and a mid upper-arm circumference of <110m[17]. Rehabilitation consisted of a milk formula supplemented with calories and protein. Blood samples were taken at enrollment, at discharge from the inpatient clinic, and after 4-10 weeks of RUTF (ready-to-use therapeutic food) treatment if applicable. While primarily a metabolite study, there were some interesting observations on inflammatory factors. Inflammatory factors including CRP, IL-6 and GM-CSF were elevated before rehabilitation. IL-6 and GM-CSF showed a

significant decline after rehabilitation, and IL-1β, IL-2 and IL-8 had a downward trend. A goal of this study was to identify mortality risk factors from the analysis panel; three factors that were associated with increased mortality were elevated IL-6, elevated TNFα, and hypoleptinemia.

The study by Bartz et al adds a new dimension to this research. The evidence for an improved immune (mostly inflammatory) response is sound, yet it is also observed that nutritional rehabilitation results in reduced inflammatory factors in the serum. The study in Uganda is unique from the others discussed here in that the children are followed passively; there is no stimulation of cell types to compare a specific response. The increased susceptibility to infection seen in children is likely to create an inflammatory environment regardless of an impaired cytokine response. The reduction in neutrophil function seen by Vasquez-garibay *et al* could lead to this as well; reduced efficacy of the first-line innate defense would result in further reliance on the acquired defense. There is also a potential role for environmental enteropathy. Environmental enteropathy is thought to occur as a result (or cause of) malnutrition, and will result in inflammation, cell damage and increased permeability of the intestine[18]. It is possible that some of the inflammatory factors measured by Bartz *et al* were a result of increased enteric infection, rather than systemic. In addition, the improved immune cell response resulting from nutritional rehabilitation would likely have the effect of reducing overall inflammation through the effective and rapid clearance of pathogens.

The next step in understanding immune dysfunction during malnutrition is investigating why cellular function is impaired. An interesting candidate for this is the

hormone leptin. Leptin concentrations are extremely reduced in malnutrition, and the hormone plays an important role in immune regulation and function[19–25]. The Palacio study saw an increase in leptin when the children in the cohort increased their weight that accompanied the improved PBMC cytokine response. Bartz *et al* also saw a large increase in leptin following the initial nutritional supplementation as well as the subsequent RUTF outpatient treatment. Hypoleptinemia was also a strong predictor for mortality in that study. The possible role of leptin in regulating immune function during malnutrition is supported by a study that found that CD4 and CD8 T cells incubated with leptin produced more IL-2, IFNγ, and IL-6 and had higher levels of the activation markers CD25 and CD69 after stimulation with PMA/ionomycin. An additional step forward in this area is research into the causes of malnutrition apart from nutritional deprivation. It has been theorized that continual infection and inflammation can lead to malnutrition[26]. If so, then investigation into the diseases most likely to lead to malnutrition as well as the identification of inflammatory risk factors could provide new avenues of treatment.

1.4 Leptin: a nutritional regulator of immune function

Leptin and leptin receptor expression

Leptin is a hormone produced primarily by adipocytes, but can be produced by other cell types including intestinal epithelial cells[22,27]. First identified as a satiety factor, it is an important signal of energy availability. It is often paired with ghrelin, presented as a counter to the increased hunger effected by ghrelin. Ghrelin, however, is much more dynamic, with peaks and dips reflecting an individual's hunger and

satiety[28,29]. Leptin levels do shift with the circadian rhythm, but exist at a steadier level overall than ghrelin[30]. Furthermore, when leptin is administered it does not actually cause satiety – the end of eating – though will reduce eating to normal levels in obese mice[19,31]. The instances when leptin is dramatically altered are when there is a dysfunction in nutrition: high levels are seen in obesity, and very low levels are seen in malnutrition[29,32].

Leptin receptor is ubiquitously expressed on many tissue types including immune cells and epithelial cells. There are six isoforms that arise from one coding gene, the three most common being the long form, the short form, and the soluble form. The long form and short forms of the receptor are the most commonly expressed form on the surface of cells[22,33]. The long form receptor contains an extracellular domain and an intracellular domain that has a Jak2 signaling site as well as 3 tyrosines that are phosphorylated for signaling cascades[34] (Figure 1.1). The short form receptor, containing the extracellular portion but only the JAK2 intracellular site (Figure 1.1), is also expressed in many cell types, and is the only leptin receptor form to be expressed in neutrophils[35]. The soluble form has only the extracellular domain; the function of this receptor is unclear, though it is thought that it could act as a sink for circulating leptin[36].

Leptin and the immune system

Perhaps unsurprisingly given the wide range of cells the receptor is expressed in, the role of leptin signaling can vary depending on the cell type in question. A common effect of leptin signaling is inhibition of apoptosis. Several studies using a

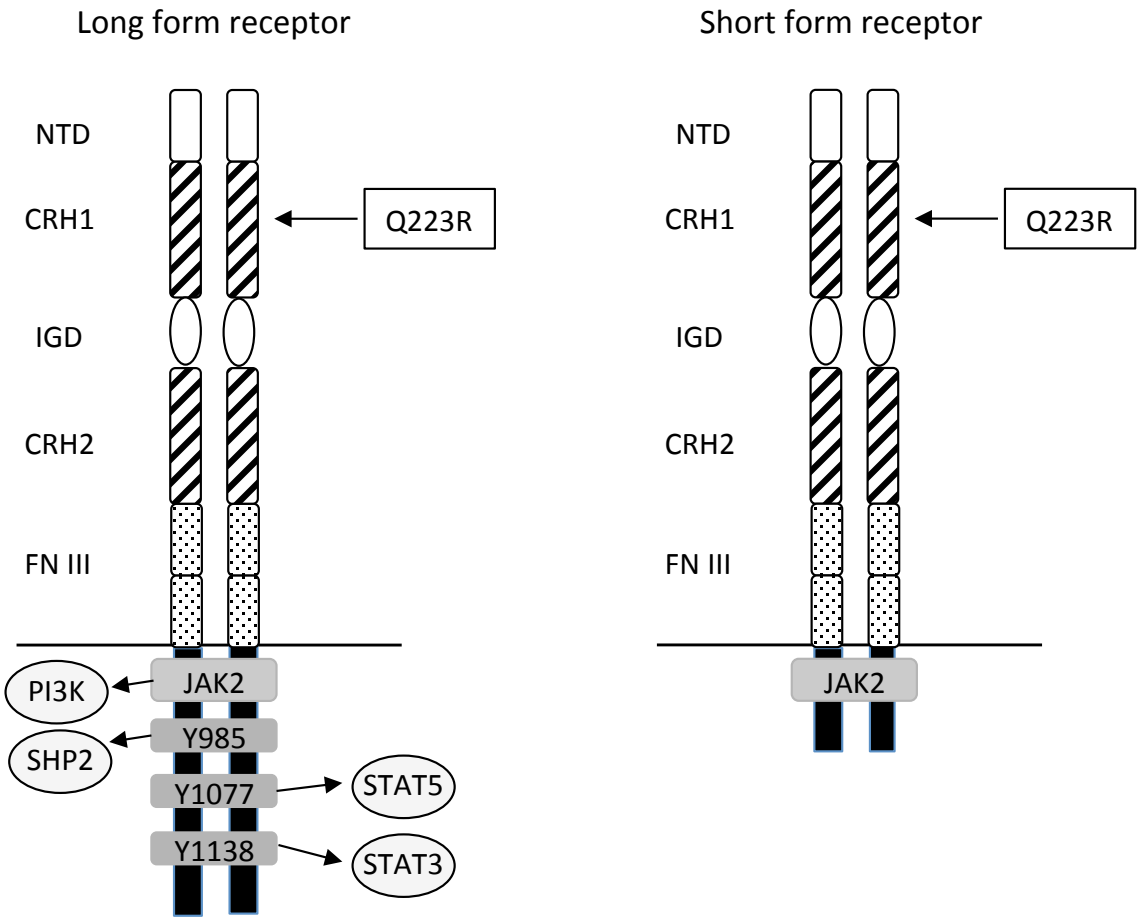
variety of cell types have found that leptin signaling inhibits natural and pathogen-mediated cell death[37–42]. Leptin has also been implicated in cell proliferation and cytokine secretion[22–25,43–45]. Leptin is increasingly being appreciated as an important mediator of immune function.

Adequate leptin signaling is required for functional immune responses. Malnutrition and obesity, both metabolic syndromes with altered leptin signaling, both result in increased susceptibility to disease[8,19,46]. The mouse equivalent, ob/ob leptin deficient mice and db/db leptin receptor deficient mice, also exhibit increased susceptibility to a variety of diseases[47,48]. This increased susceptibility to infection is due to reduced immune cell function. Ob/ob and db/db mice have atrophied thymuses, a phenomenon also seen in malnutrition, resulting in fewer mature T cells[49,50]. T cells from low leptin environments also have reduced cytokine release after stimulation. Supplementation of leptin to leptin-deficient individuals results in improved T cell counts and function[51]; *in vitro* culture of cells with leptin can improve proliferation and cytokine response in T cells from both normal and malnourished individuals, as well as from ob/ob mice[45,52]. One interesting potential mechanism that leptin could be regulating T cell function is through glucose metabolism. A study using T cells from fasted and ob/ob mice found that when the cells were incubated with leptin, glucose uptake from the media and glycolysis were increased compared to cells not incubated with leptin; this increase in glucose metabolism was accompanied by an increase in cytokine production and up-regulation of surface activation markers after stimulation[53]. Glucose metabolism is required for T cell function; therefore, leptin could

be acting as a signal of energy availability, reducing immune function when there is a deficit, such as during malnutrition.

In addition to enhancing immune cell function and acting as a regulation, leptin is also a signaling cytokine. Leptin levels are often increased after infection: increases have been measured in the lungs, serum, stomach, and peritoneal cavity after infections[54–57]. Leptin is also a chemoattractant for neutrophils. Neutrophils only express the short form of the leptin receptor, but *in vitro* incubation with leptin has been shown to enhance oxidative burst and surface presentation of CD11b[58–61]. In addition to serving as a chemotaxic agent *in vitro*, addition of leptin to the lungs of mice with bacterial pneumonia resulted in increased neutrophilia in the tissue and BAL fluid and improved *E. coli* and *Klebsiella pneumoniae* clearance[54].

Figure 1.1. Leptin receptor diagram of the long form and short form receptor. Modified from Peelman *et al*[62]. Extracellular portion is comprised of an N-terminus domain (NTD), cytokine region of homology 1 (CRH1), an immunoglobulin domain (IGD), cytokine region of homology 2 (CRH2), and two fibronectin III domains (FN III). Q223R mutation is shown in the CRH1 domain. Downstream signaling proteins are indicated in the grey circles.


Long form receptor
Short form receptor
NTD
CRH1
IGD
CRH2
FN III
Q223R
JAK2
PI3K
SHP2
Y985
Y1077
Y1138
STAT5
STAT3

1.5 Project goals

This projects aims to investigate the interaction of nutrition with immune function. We have approached this in two ways: 1) focus on leptin signaling in a murine model as an example of nutritional regulation and 2) evaluate markers of inflammation and their association with nutrition in a cohort of children.

Leptin signaling is regulated by nutritional status and has profound impacts on the immune response. The Q223R leptin receptor mutation is associated with increased *E. histolytica* infection, an infection also exacerbated by malnutrition[63,64]. Using this mutation in a murine model, we investigated the effect of altered leptin signaling on host response to *E. histolytica* infection. We hypothesized that the R223 leptin receptor mutation conferred susceptibility through an impaired innate immune response. This work using a focused, mechanistic approach has informed how leptin, a critical nutritional hormone, affects disease susceptibility.

Our second approach investigated the nutritional associations of a variety of inflammatory markers in a cohort of children suffering from a high rate of malnutrition and enteric infection. Children in the developing world suffer environmental enteropathy[18], that we hypothesized we can evaluate with enteric inflammatory markers. We further hypothesized that malnutrition within the cohort will correlate with increased inflammation. The broad array of biological and sociological markers employed in this study has increased understanding of the causes and effects of malnutrition.

Chapter 2:

Inhibition of neutrophil response to infection by the Q223R mutation

This work has been published as "Leptin receptor mutation results in defective neutrophil recruitment to the colon during *Entamoeba histolytica* infection"

Caitlin Naylor, Stacey Burgess, Rajat Madan, Erica Buonomo, Khadija Razzaq, Katherine Ralston, and William A. Petri. 2014 *mBio* 5(6):e02046-14

2.1 Introduction

Entamoeba histolytica: a parasite of poverty

Entamoeba histolytica is a protozoan parasite transmitted via the fecal-oral route. The cyst form is ingested in contaminated food or water; once it reaches the intestine it excysts to the trophozoite phase, damaging host tissue through amebic trogocytosis[65,66]. Infection is characterized by a large neutrophil influx and innate response[67–69]. Infection often is asymptomatic, though in 10% of cases active disease is evident. The majority of enteric disease is diarrhea and dysentery, with some more severe cases of amebic colitis. Re-infection is common, indicating a lack of lasting immune memory[65].

E. histolytica is an important pathogen of children in the developing world. It is significant cause of diarrhea and is endemic in many countries. It is a disease that is exacerbated by malnutrition: a child with a low HAZ score (height-for-age), has more symptomatic *E. histolytica* infections[64].

Leptin and E. histolytica infection

Nutrition plays an important part in *E. histolytica* susceptibility. Leptin is an important nutritional hormone powerfully affected by the nutrition status. It is well known that in malnutrition, circulating leptin levels are significantly lowered[16,32]. Produced primarily by adipocytes but also by intestinal epithelial cells[22,27], leptin is a signal of energy depravation and is thought to play an important role in how the body reacts to depleted energy stores. Leptin is a pleiotropic molecule, involved in many cellular systems including apoptosis inhibition and immune function.

The role of leptin during an *E. histolytica* infection has primarily been investigated using the mouse model of infection. These studies have found that leptin signaling is critical for an adequate host response to clear and control the amebae. Ob/Ob leptin-deficient mice had much higher rates of infection compared to wild-type controls as well as more severe tissue pathology. Db/Db leptin receptor mice had high rates of mortality, previously unheard of in this mouse model[38]. The use of leptin receptor tyrosine knockout mice further demonstrated the importance of leptin signaling. Mice with serine replacements at position 1138, responsible for STAT3 signaling, and at position 985, responsible for SHP2/MAPK/ERK, both have increased infection rates[38]. Multiple signaling pathways are important for protection against *E. histolytica*, demonstrating the critical function of leptin.

The Q223R leptin receptor mutation

A genetic approach to testing the importance of leptin signaling in malnutrition was taken in a cohort of 300 children in Dhaka who had been followed for 9 years. A candidate gene analysis comprising 100 genes including leptin and the leptin receptor was performed on the children. Common SNPs (at 5% in the population) were analyzed for increased susceptibility to *E. histolytica* infections. One of the strongest hits encoded a change in the extracellular domain sequence of the leptin receptor, a glutamine (Q) to arginine (R) SNP at position 223. Children homozygous for the R223 allele were four times more likely to have had an *E. histolytica* infection. These children were also infected earlier in life: a child with the R223 allele had their first infection on average at 1.51 years of age, while a Q223 child had their first infection on average at

2.56 years of age. The nutritional status of the children had no effect on these results and also did not change depending on genotype, indicating that it was the Q223 allele that was granting protection[63].

A mouse model of this SNP exists on a 129J/BL6 background. This mouse replicates the human phenotype, with an average Q223 mouse clearing an *E. histolytica* infection by 72 hours after infection while R223 mice still have a 40% infection rate[70]. The intestinal tissue of R223 mice at 72 hours after infection show greater intestinal disruption and epithelial disruption, as well as increased caspase-3 expression in the epithelial cells compared to the Q223 mice[38].

Hypothesis

The goal of this study was to better understand the effect of the Q223R mutation on the host's immune response to an *E. histolytica* infection. We had observed a rapid divergence in the ability to clear amoeba in the Q223 and R223 mice as well as increased amebic destruction in the R223 model. We hypothesized that the innate cellular response would be diminished in an R223 mouse.

2.2 Methods and Materials

Mice

Male 129/J mice homozygous for the Q223 or R223 allele (8-12 weeks) were maintained and bred at the University of Virginia under pathogen-free conditions. C57BL/J6 and BV.6-*Lepob*/J mice were obtained from Jackson.

Cecal lamina propria isolation.

Single cell suspension of cecal lamina propria was performed as described previously. In brief, cecal tissue was washed in HBSS containing DTT and EDTA for 15 minutes at 37°C to remove the epithelial layer. Tissue was then diced and incubated in RPMI containing collagenase D and DNase for 30 minutes at 37°. Digested tissue pieces were passed through 100 μm and 30 μm cell filters to obtain single cell suspension.

Histology

Cecal tissue sections were H&E stained. Scoring for submucosal edema and epithelial blunting was based on a scale of 0-3 for increasing severity. Samples were read blinded by two investigators and scores averaged for all sections; scores were then added for a final overall histology score between 0-6.

Murine infection with Entamoeba histolytica

Trophozoites for infections were originally derived from laboratory strain HM1:IMSS that were sequentially passaged *in vivo* through mouse cecum. Virulence has been maintained with periodic passaging. For all intracecal inoculations 2 x 10^6 trophozoites in 150 μl were injected intracecally after laparotomy. Mice were sacrificed 12 hours after infection and the contents collected and cultured in complete trypsin-yeast-iron (TYI-33) medium with supplemented with Diamond Vitamin (JRH Biosciences), 100 U/mL of penicillin and streptomycin and bovine serum (Sigma-Aldrich). The presence

of visible amebae 12-24 hours after sacrifice determined infection status. Murine infection results are representative of two independent experiments.

Neutrophil depletion

50 μg of anti-Ly6G monoclonal antibody or 50 μg of IgG isotype (BioXcell) control was injected into the peritoneal cavity 12 hours before infection. Depletion was confirmed with flow cytometric analysis of the lamina propria after infection.

Amebic trogocytosis assay

Splenocytes were isolated by filtration through 100 μm cell filters followed by RBC lysis. Amebic trogocytosis and splenocyte death were quantified using imaging flow cytometry as described previously[66].

Determination of cellular populations in the lamina propria

Isolated lamina propria cells were surface stained for markers Ly6G, CD11b, CD45, Ly6C, and CXCR2 according to general flow cytometry protocol. All samples were run on a Beckman Coulter CyAn ADP LX and analyzed with FlowJo software (Treestar, Ashland OH).

Quantification of cecal proteins

Cecal tissue was homogenized in HBSS with 50mM HEPES, Triton X-100 and HALT protease inhibitor cocktail (Pierce). Lysate was aliquoted and stored at -70. Aliquots were not freeze-thawed more than twice. CXCL1, CXCL2, CCL3, CCL4 and G-CSF

luminex assays (Biorad) were run on a Biorad Bioplex 200. Leptin quantification was performed by ELISA assay (Peprotech). Results were normalized to total protein concentration as measured by BCA assay (Pierce) per aliquot used.

Neutrophil isolation and chemotaxis assay

Neutrophils were isolated from bone marrow of hind legs of R223 and Q223 mice. Bones were cleaned and ground in sterile HBSS, then cells were filtered through 100 μm and 40 μm cell filters. Anti-Ly6G MACS bead separation was performed according to protocol (miltenyi biotec) on the AutoMACS Pro. $4x10^4$ neutrophils were immediately added to the top of the 3 μm transwell filter. 600 μl of HBSS Ca+ Mg+ containing 10ng leptin, 50 ng leptin or 10 ng CXCL1 and CXCL2 (5 ng each) (Peprotech) was added to the bottom well. Chemotaxis was allowed to proceed for 1.5 hours at 37°. Filters were then removed from the plate and excess liquid removed from the top of the filter with a cotton-tipped applicator. Filters were cut out, fixed with methanol, then stained with crystal violet. Neutrophils were counted on one field of view with 20X magnification using an Olympus DP71.

Statistical analysis

Analysis performed on Prism 5.0 software. Student t-tests were performed for comparisons between two groups; Mann-Whitney test was performed for infection rate comparisons.

2.3 Results

R223 mice exhibited reduced inflammation and infiltration after infection with E. histolytica.

The mouse model of an *E. histolytica* cecal infection was used to determine the differences between Q223 and R223 mice early during an infection. Previous work had shown that by 12 hours after infection a difference could be observed in infection rates between Q223 and R223 mice, with complete eradication in Q223 but not R223 mice by 72 hours[70]. This 12 hour time point was therefore chosen to investigate the very early host processes that could account for the differences in phenotypes. Q223 and R223 allele-expressing mice were infected with 2 x 10^6 *E. histolytica* trophozoites for 12 hours, after which the cecum was harvested and evaluated for inflammation and infiltrating immune cells in the cecum. Histology scores reveal a reduction in inflammation in R223 mice, graded by submucosal edema and epithelial blunting (Fig 2.1 A, B). Epithelial destruction, not included in the histology measurements but commonly seen in previous histologic analysis of late amoebic infection, was not observed in either genotype at this early time point.

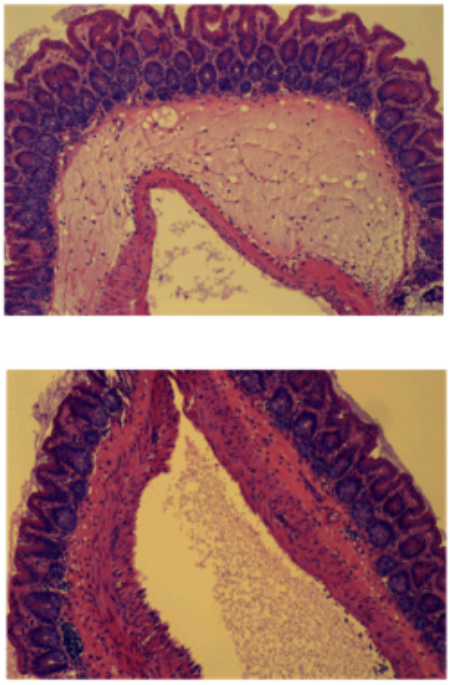
The 12-72 hour time to eradication of amebae in Q223 mice suggested an innate mechanism could be responsible. 12 hours after infection, cecal lamina propria tissue was reduced to single cell suspension and flow cytometry used to identify cell types. Neutrophils, identified as Ly6G and CD11b double positive cells (Fig 2.2 A), were observed to make up a smaller percentage of the cells in the R223 mice (Fig 2.2 B). Other innate cell types such as macrophages (CD11b+) and inflammatory monocytes (CD11b+ Ly6C+) were present at equivalent rates in both genotypes (data not shown).

Naïve Q223 and R223 mice had very low but similar amounts of neutrophils in the cecal lamina propria (Fig 2.2 C), and equivalent amounts of circulating blood neutrophils (Fig 2.2 D). The defect in neutrophil number therefore was not present at rest but only locally in the gut during an infection, suggesting a potential defect in infiltration or survival.

Figure 2.1 R223 mice exhibit reduced cecal inflammation.

Representative images of histology staining with H&E shows reduced inflammation in the cecum of R223 mice (A top: Q223 cecal section, A bottom: R223 cecal section). Histological scoring of epithelial blunting and submucosal edema was performed blinded (B). * P = .03 by two tailed t test

A



B

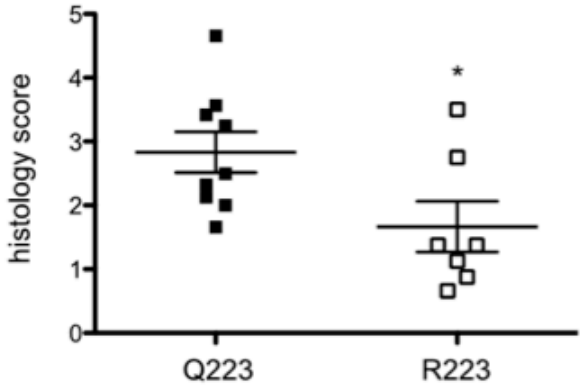
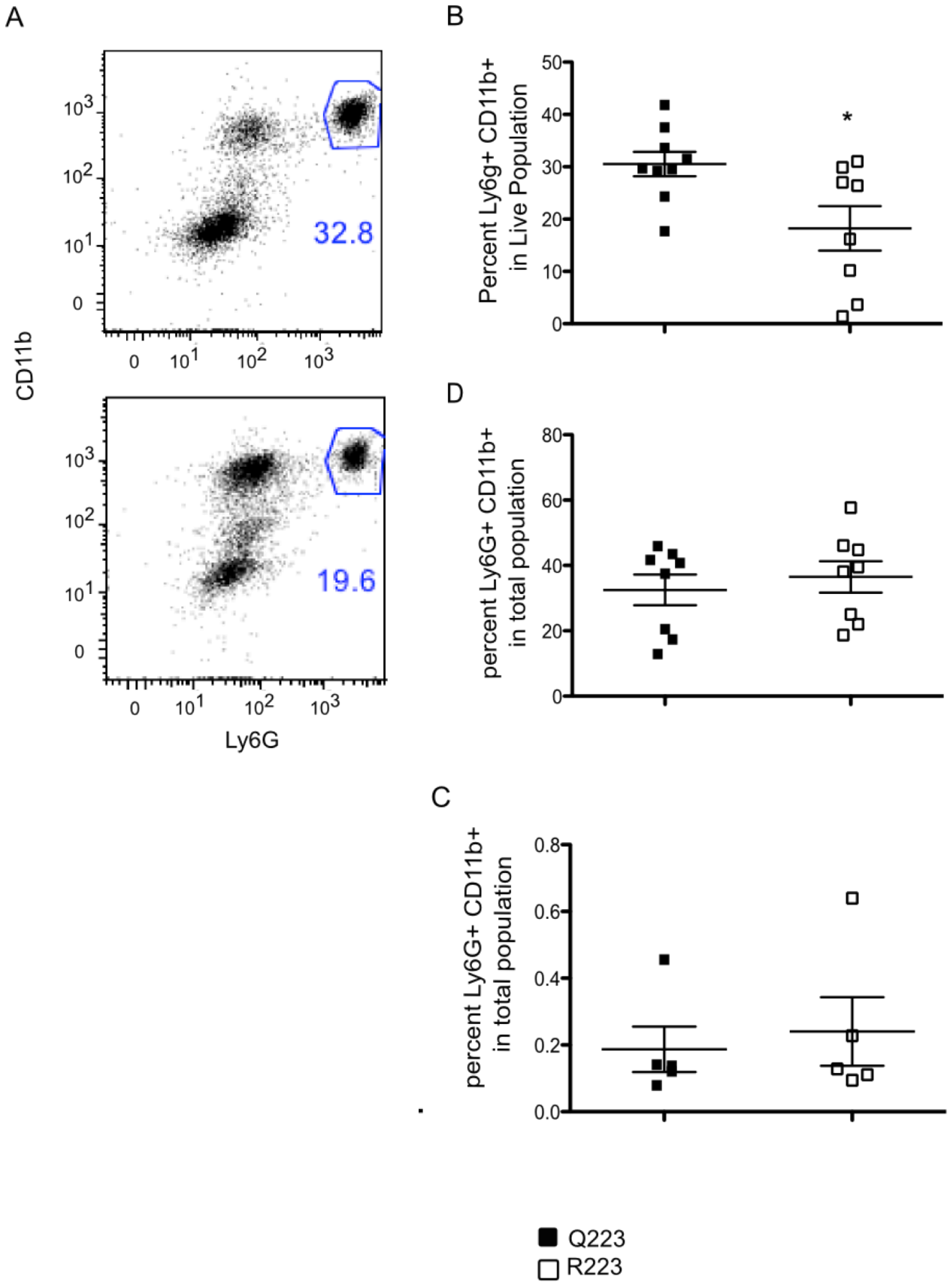


Figure 2.2 R223 mice had reduced neutrophil infiltration after infection

Neutrophils were identified in the lamina propria by double expression of Ly6G and CD11b (A top: Q223, bottom: R223). Analysis of cell populations in the cecal lamina propria 12 hours after infection revealed reduced neutrophil infiltration in R223 mice compared to Q223 mice (B). Neutrophil populations are present at equivalent frequencies in the cecum (C) and blood (D) of naïve mice. * P = .01 by two-tailed test.


A
B
C
D
CD11b
Ly6G
32.8
19.6
Percent Ly6g+ CD11b+ in Live Population
percent Ly6G+ CD11b+ in total population
percent Ly6G+ CD11b+ in total population
*
Q223
R223

Neutrophil infiltration was important for amoebic clearance

The importance of neutrophils specifically to clearance of an *E. histolytica* infection was unknown. To test if diminished neutrophil presence in the cecum was responsible for the susceptible phenotype of the R223 mice, neutrophils were depleted with anti-Ly6G monoclonal antibody in C57Bl/6 mice (Fig 2.3 A), an ameba-resistant strain of mouse[71]. Administration of anti-Ly6G MAb achieved significant depletion of neutrophils in the lamina propria (Fig 2.3 B). After 12 hours of infection, mice that had received the anti-Ly6G MAb had double the infection rate compared to mice that had received the isotype control. This suggests that a neutrophil response is critical for clearance of amebae early during infection, and that a reduction in this response is an important mechanism for the susceptibility observed in mice with the R223 allele.

Immune cell death and amebic trogocytosis were not enhanced by the R223 polymorphism

Previous work has shown that R223 cecal epithelial cells have increased caspase-3 activation and subsequent apoptosis after an *E. histolytica* infection, suggesting that leptin signaling could be responsible for cell survival in the face of amoebic challenge. The reduction in receptor signaling after leptin binding engendered by the R223 polymorphism could result in increased death of immune cells. Amebic trogocytosis has been identified as a killing mechanism of *E. histolytica* for several cell types, including Jurkat cells[66]. We tested if reduced leptin signaling could result in an increase in amebic trogocytosis and cell death. This was done by comparing the death and ingestion rates of splenocytes isolated from ob/ob (leptin-deficient) mice and control C57Bl/6 mice after incubation with *E. histolytica*. Cell death was increased

when splenocytes were incubated with amebae as expected, but there was no significant difference in cell death between the ob/ob and C57Bl/6 cells (Fig 2.4 A). Amebic trogocytosis, measured by the percentage of amebae that had ingested fluorescently-labeled fragments of splenocyte cell membrane, was also not significantly different between the two splenocyte populations (Fig 2.4 B). A similar experiment was conducted with Q223 and R223 neutrophils, which also revealed no significant differences (data not shown). Decreased leptin signaling with the 223R mutation therefore did not increase the susceptibility of immune cells to amebic killing.

Figure 2.3 Depletion of neutrophils increased susceptibility to amebiasis. C57BL/6 mice were treated with 50 μg of anti-Ly6G MAb or IgG isotype control 12 hours before infection with *E. histolytica*. Depletion of neutrophils was confirmed in the cecal lamina propria 12 hours after infection by flow cytometry (A). Infection was evaluated after 12 hours of infection by culture positivity on the day of sacrifice (B). * P = .03 by two tailed t-test + P = .04 by Mann-Whitney test

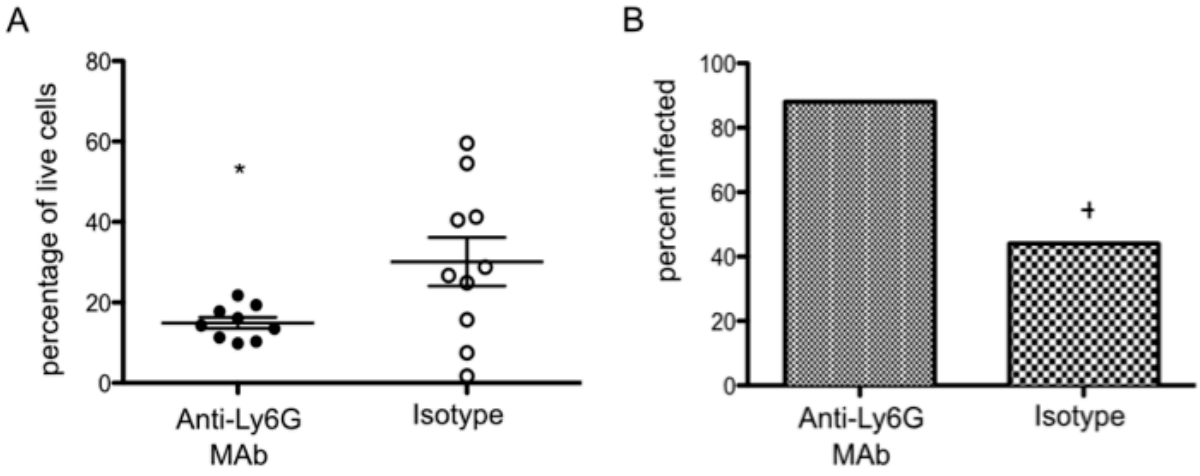
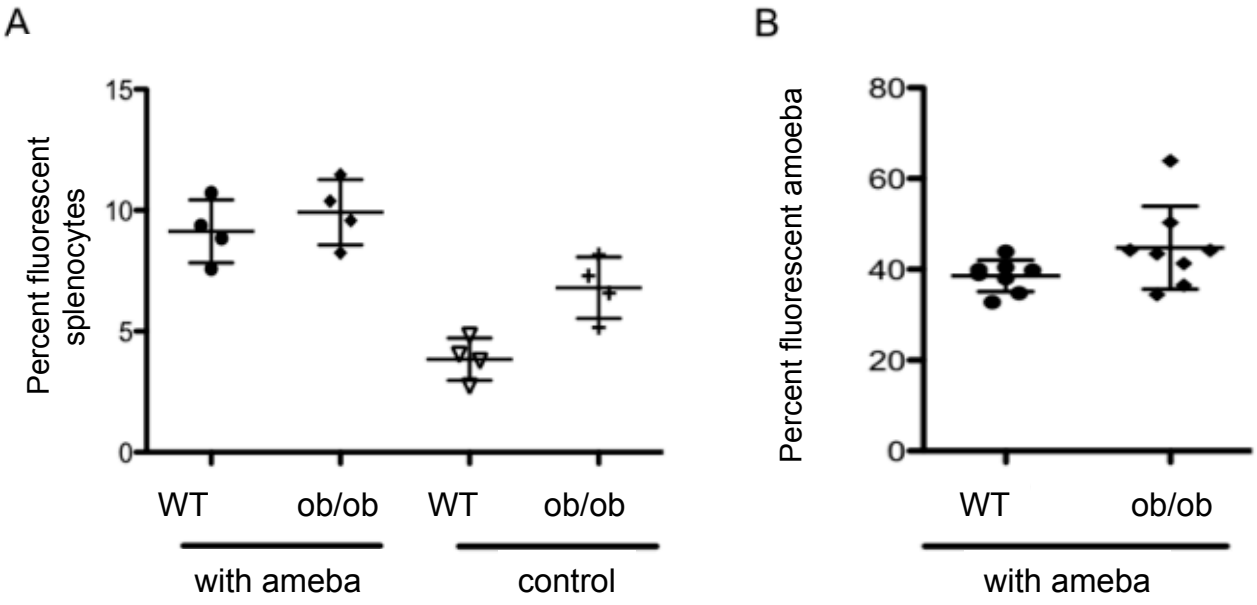

A
percentage of live cells
80
60
40
20
0
*
Anti-Ly6G MAb
Isotype
B
percent infected
100
80
60
40
20
0
+
Anti-Ly6G MAb
Isotype

Figure 2.4 Amebic trogocytosis of splenocytes is unaffected by reduced leptin signaling.

Splenocytes isolated from WT and Ob/Ob mice (4 mice per group) were incubated with *E. histolytica* trophozoites and compared for their ability to resist amebic trogocytosis and cell-killing using imaging flow cytometry. Cell death was assessed with fluorescent live/dead staining after incubation with amebae for 40 minutes, or as a control, incubation without amebae for 40 minutes (A). Amebic trogocytosis was assessed by the percent of amebae that had ingested fluorescently labeled splenocyte cell parts in 40 minutes (B). Four mice were analyzed per group and technical replicates from each mouse were collected; the mean values of the technical replicates are plotted. The mean values and standard deviations for each group are shown. There was no statistical difference observed between WT and Ob/Ob for cell death or ingestion.


A
Percent fluorescent splenocytes
15
10
5
0
WT
ob/ob
WT
ob/ob
with ameba
control
B
Percent fluorescent amoeba
80
60
40
20
0
WT
ob/ob
with ameba

Neutrophil attractant chemokines and leptin were present at equivalent levels in both genotypes

To determine if the host environment was responsible for the reduction in neutrophil infiltration we measured production of an array of neutrophil chemoattractants produced by epithelial cells and inflammatory cells. CXCL1, CXCL2, CCL3, CCL4 and G-CSF was measured in cecal tissue lysates from mice challenged for 6 and 12 hours using luminex bead assays and normalized to total protein content. We found that all chemokines tested were present equally in Q223 and R223 mice at both 6 hours (Fig 2.5 A) and 12 hours (Fig 2.5 B) after infection.

Leptin is also considered a neutrophil chemoattractant and is known to increase after many types of infection and to act as an inflammatory cytokine. To assess if there was any baseline difference between the genotypes that could cause the reduction of neutrophils observed, leptin was measured in the cecal tissue lysates and serum of naïve Q223 and R223 mice using an ELISA. Leptin levels in uninfected mice were revealed to be equivalent in both the serum (Fig 2.6 A) and the cecum (Fig 2.6 B) of both genotypes. Leptin was then measured in cecal lysates after 6 and 12 hours of *E. histolytica* infection to test if leptin production diminished or failed to increase in R223 mice (Fig 2.6 B). There was an increase in cecal production of leptin between 6 hours and 12 hours in both Q223 and R223 mice; this increase was significant in R223 mice ($p = .02$) and strongly trended in Q223 mice ($p = .06$). There was however no difference in cecal leptin levels between the genotypes at 6 hours or 12 hours. We concluded that leptin was a potential neutrophil chemoattractant to the gut and was capable of being produced as a response to infection in both genotypes.

Figure 2.5 Neutrophil chemoattractant expression in cecum after infection. Q223 samples are represented by black squares, R223 samples are represented by white squares. Protein levels of key neutrophil attractant chemokines after 6 hours (A) and 12 hours (B) after infection with *E. histolytica* trophozoites were equivalent between Q223 and R223 mice. All levels have been normalized to total protein and log transformed.

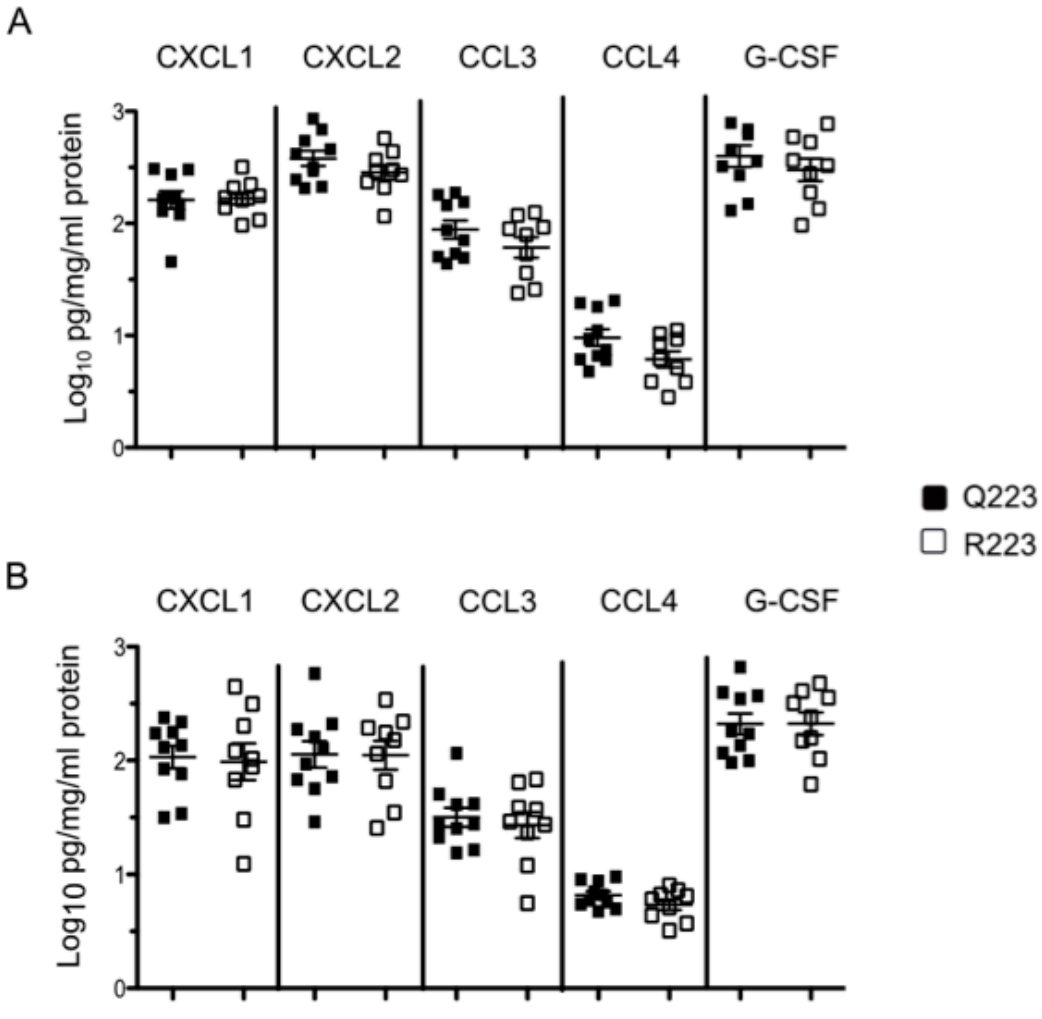
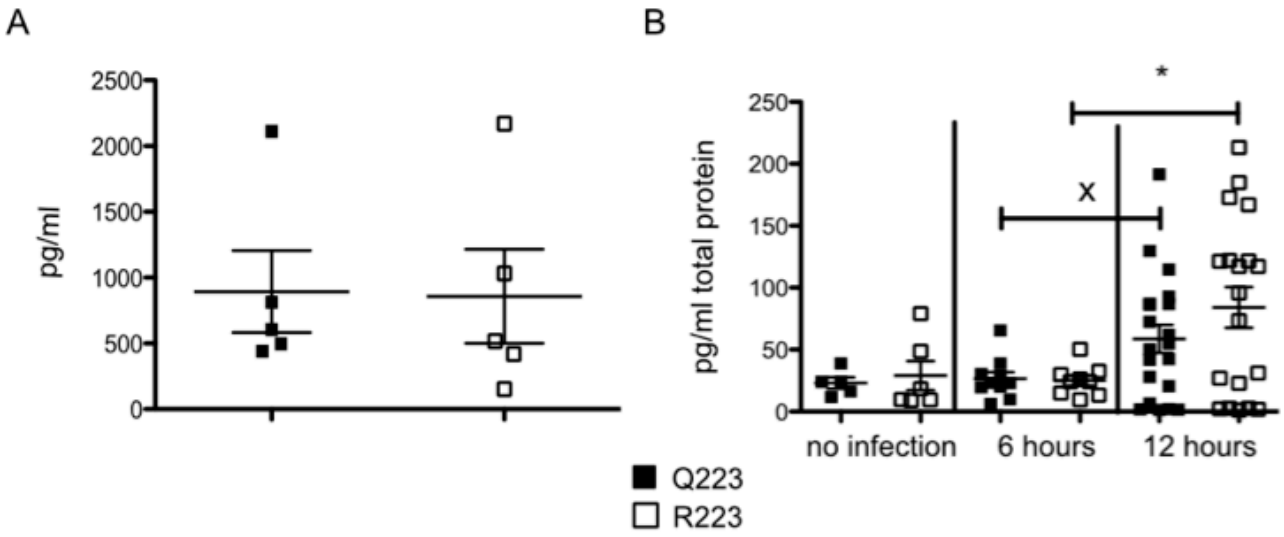

A
CXCL1
CXCL2
CCL3
CCL4
G-CSF
Log10 pg/mg/ml protein
0
1
2
3
Q223
R223
B
CXCL1
CXCL2
CCL3
CCL4
G-CSF
Log10 pg/mg/ml protein
0
1
2
3

Figure 2.6 Leptin is increased in the cecum during amebic colitis.

Leptin levels were measured in naïve Q223 and R223 mice in serum (A). Leptin was quantified in the cecal tissue lysate of mice with no infection and after 6 and 12 hours of infection (B). * P = .02; x P = .06 by two tailed T test

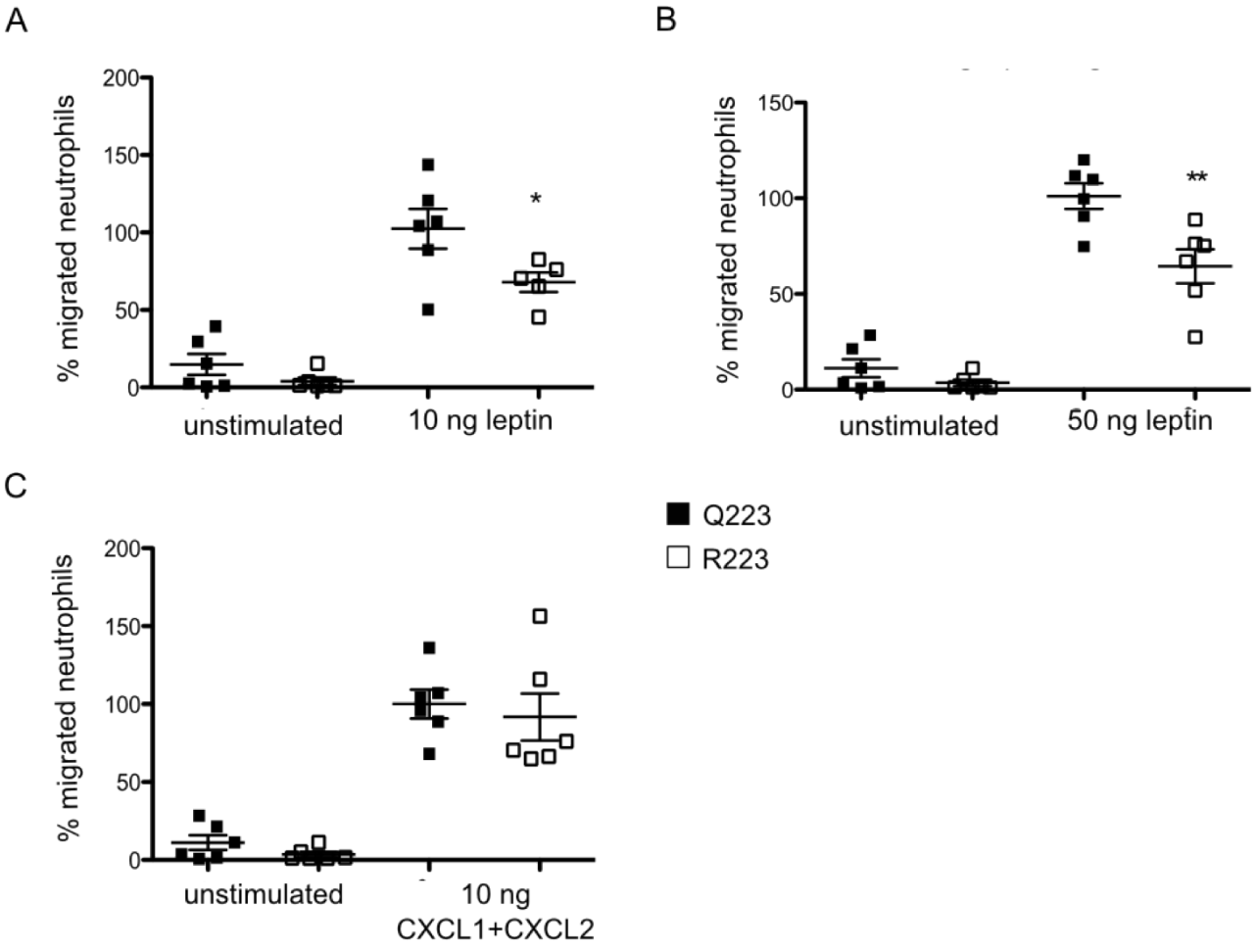

A
2500
2000
1500
1000
500
0
pg/ml
B
250
200
150
100
50
0
pg/ml total protein
*
X
no infection
6 hours
12 hours
Q223
R223

R223 neutrophils had an inhibited chemotactic response to leptin

The preceding experiments demonstrated the absence of an impact of the 223R mutation on neutrophil survival from amebic killing or in neutrophil chemoattractants in the gut. This suggested that reduced neutrophil infiltration in the R223 mouse was a result of an intrinsic defect of the neutrophils to respond to gut homing signals. As we had demonstrated that leptin markedly increased at 12 hours after infection compared to earlier time points, we hypothesized that leptin was a signal for gut recruitment, and additionally that R223 neutrophils would exhibit reduced chemotaxis towards leptin. To test this, bone marrow neutrophils were isolated using magnetic bead separation, and applied to a transwell filter plate that contained leptin or a combination of CXCL1 and CXCL2 in the lower well. Chemotaxis was allowed to proceed for 1.5 hours before the filter was fixed and stained. Leptin was able to induce a strong chemotactic response in both populations of neutrophils. R223 neutrophils demonstrated reduced chemotaxis compared to Q223 neutrophils towards 10 ng and 50 ng of leptin (Fig 2.7 A and B). Chemotaxis towards CXCL1 and CXCL2 was however equal in both neutrophil genotypes (Fig 2.7 C). We concluded that there was a leptin-specific defect in chemotaxis conferred by the 223R mutation.

Figure 2.7 R223 neutrophils exhibit reduced chemotaxis towards leptin but not CXCL1&2.

Chemotaxis of neutrophils from Q223 and R223 mice towards 10 ng leptin (A), 50 ng leptin (B) and a total mixture of 10 ng of CXCL1+CXCL2 (C) was compared. Neutrophils isolated from the bone marrow were added to the top of a 3 μm-pore filter and allowed to migrate for 1.5 hours. Migration counts are presented as percent of Q223 average neutrophil migration towards the chemoattractant (absolute values). * P = .02 ** P = .008 by one tailed t-test


A
% migrated neutrophils
200
150
100
50
0
*
unstimulated
10 ng leptin
B
% migrated neutrophils
150
100
50
0
**
unstimulated
50 ng leptin
C
% migrated neutrophils
200
150
100
50
0
unstimulated
10 ng
CXCL1+CXCL2
Q223
R223

2.4 Discussion

In this study we investigated the mechanism behind the increased susceptibility to amebiasis in mice that have the R223 leptin receptor mutation. We explored the differences in the innate immune response to amebic colitis in mice expressing the R223 mutation. The R223 mutation resulted in decreased neutrophil recruitment to the site of infection at 12 hours after infection, the time at which the increased clearance of infection by wild-type Q223 mice is manifest. Neutrophils with the R223 mutation had decreased chemotaxis to leptin *in vitro*. Finally, neutrophil depletion resulted in increased infection susceptibility, supporting the role of neutrophil recruitment to clear infection.

The Q223R leptin receptor mutation is common, expressed in up to 50% of Caucasian and Bangladeshi populations[63]. In addition to impacting *E. histolytica* susceptibility, the mutation has also been shown to affect host resistance to peritonitis and chronic bronchitis[72,73]. The studies of the impact of the mutation on host response to amebiasis may therefore suggest a common mechanism of susceptibility.

The rapid divergence of infection phenotypes observed, with full eradication of infection by 72 hours in mice with the ancestral Q223 leptin receptor allele[70], suggested that the innate immune system was responsible for the difference in infection clearance. R223 mice were observed to have a reduced neutrophil population in the lamina propria compared to Q223 mice 12 hours after infection. Depletion of neutrophils resulted in a doubled infection rate at 12 hours, confirming their importance for the early clearance of amebae. This echoes previous work that depleting all GR1+ cells resulted in increased amebic pathology[68]. Activated

neutrophils have also been shown to kill *E. histolytica*[74]. Together these data suggest that a vigorous neutrophil response is needed to achieve early elimination of *E. histolytica*, and could represent a critical crossroad in the host response that determines whether the pathogen is eradicated.

Previous work had found that leptin receptor presence on the epithelial barrier was critical for *E. histolytica* infection defense, as demonstrated by increased infection and disease severity with an intestinal epithelial knockout[38]. Bone marrow chimeras of wild-type and db/db mice revealed that the presence of leptin receptor on epithelial cells was critical for defense against *E. histolytica*[38]. Neutrophil infiltration to a site of infection is controlled by chemotactic signals; intestinal epithelial cells have the capacity to make a variety of these chemoattractants in response to a pathogen challenge including leptin[27,75–77], suggesting a possible mechanism for reduced neutrophil infiltration through decreased chemoattractant production. We found that all neutrophil-attractant chemokines tested for were detected in cecal lysate after infection, but there was no difference between Q223 and R223 mice in the concentration of these chemokines. Leptin was also produced equally between Q223 and R223 mice, both at baseline in serum, replicating the initial human cohort findings, and in the cecum before and after infection. We concluded there was no discernable difference between the ability of Q223 and R223 mice to make neutrophil attractant chemokines.

Immune cell survival after amebic challenge was also not affected by the R223 mutation. Leptin has known anti-apoptotic effects[42,44,78] and likely protects intestinal epithelial cells against ameba-mediated cell death. Previously transfection of HEK cells

with the Q223 receptor had been shown to provide leptin-dependent protection from cell death that was greater than that from the R223 leptin receptor[39], and the intestinal epithelial cells in R223 mice showed greater caspase-3 activation after infection[63]. In contrast to the importance of leptin signaling in epithelial cell defense, in immune cells we found no impact of the R223 mutation on increasing susceptibility to amebic killing. In the context of protection from amebic trogocytosis leptin signaling appears to have a different role in neutrophils than in epithelial cells.

Increased chemotaxis towards leptin was the most likely explanation for the increased neutrophil presence in the gut of Q223 mice. We observed between 6 hours and 12 hours after infection that there was a significant increase in leptin in the cecum. Other investigators have also observed an increase in leptin after infection. A human study of *H. pylori* infection also observed increased leptin expression in gastric epithelial cells after infection, which was subsequently reduced after eradication of infection[57]; LPS inhalation also resulted in increased BAL leptin levels[54]. In mice this elevation has been observed in peritonitis and pneumonia models; leptin was observed to rise significantly at 12 and 24 hours after cecal ligation and puncture, and both bacterial and viral pneumonia resulted in greater leptin concentrations in the BAL fluid[54–56]. Furthermore, these studies observed that increased leptin resulted in increased neutrophils at the sites of injury. Leptin thus appears to be part of the inflammatory response by epithelial cells under attack. The rise of leptin at 12 hours coincided with the observed neutrophil infiltration, suggesting a likely signal for the neutrophil influx apart from the traditionally described chemoattractants.

The leptin receptor mutation resulted in an intrinsic defect in neutrophil chemotaxis to leptin. Though only expressing the short form of the leptin receptor[35], neutrophils have been shown to migrate towards leptin[54,59,60]. JAK2 signaling from this short form receptor is known to activate PI3K and potentially p38; inhibitors of both signaling molecules can inhibit neutrophil chemotaxis towards leptin[22,26,28]. Little research has been done on the significance of leptin chemotaxis, and until now there has been no clear link between disease susceptibility and neutrophil migration towards leptin. We found that both Q223 and R223 neutrophils were able to chemotax in response to leptin in media, but R223 exhibited reduced chemotaxis. The lack of a difference in chemotaxis between the two genotypes towards a combination of CXCL1 and CXCL2, suggests that this chemotactic decline is restricted to leptin and does not interfere with other chemotaxis pathways. This data combined with our observation of increased leptin production at the site of infection suggests that neutrophil infiltration into the cecum is inhibited by the R223 mutation. However, it is also possible that bone marrow mobilization of neutrophils is affected. A variety of chemokines and cytokines have been shown to cause the neutrophil efflux from the bone marrow seen after infection[79]; leptin has not been identified as having a direct role in this migration as of yet, but as our experiments were performed with bone marrow-isolated neutrophils it is possible this could be happening. In addition, leptin has been shown to upregulate

oxidative burst[58]; upregulated leptin production could therefore be acting as a booster signal to more than just chemotaxis. Should the R223 receptor result in additional functional inhibitions, R223 neutrophils could be unable to efficiently kill the invading ameba and thus become more susceptible to the ameba themselves. The role of leptin has started to be appreciated in immune mediators such as T cells[19,45,80], and now leptin signaling appears to have a powerful role in neutrophil influx and thus response to infection in an *E. histolytica* infection.

This study has identified a novel leptin-mediated mechanism of innate defense, adding to the epithelial defense we had previously observed. While we are open to the possibility that this mutation has other yet to be discovered roles in inflammation and infection, the discovery that neutrophil chemotaxis is altered due to the R223 mutation is of significance as the mutation is common in human populations. This work may have relevance for obesity and malnutrition as both conditions result in an altered leptin environment. In conclusion, this study provided insight into host defense against *E. histolytica* infection, and to the role of leptin in disease and immunity, through the demonstration of leptin-mediated chemotaxis.

Chapter 3:

The effect of the Q223R leptin receptor mutation on receptor function[1]

Part of this chapter has been adapted from "Kinetics of leptin binding to the Q223R leptin receptor"

Hans Verkerke, Caitlin Naylor, Lennart Zabeau, Jan Tavernier, William Petri & Chelsea Marie. 2014. *PLoS One* 9 (4): e94843.

[1] Contribution to chapter: Oversaw work by Hans Verkerke that led to the manuscript "Kinetics of leptin binding to the Q223R leptin receptor"; performed all leptin receptor surface expression experiments

3.1: Introduction

The long form leptin receptor is composed of an intracellular portion containing three tyrosines and a JAK signaling site, and an extracellular portion containing 6 domains. The signaling sites on the intracellular portion activate several signaling proteins, including STAT3, STAT5, SHP2, PI3K and MAPK/ERK. The extracellular portion has 6 domains: an N-terminus, two cytokine regions of homology (CRH), an immunoglobulin domain, and two fibronectin type III domains (Figure 1.1). CRH2 contains the leptin binding site[34,62]. The receptor exists as a dimer on the surface of the cell. After leptin binding, a conformational change occurs that transmits the signal to the intracellular signaling sites[81].

The Q223R leptin receptor SNP is in the cytokine region of homology 1, and has unclear functional consequences. Previous work has found that HEK cells transfected with the R223 leptin receptor exhibited reduced receptor activation compared to HEK cells transfected with the Q223 leptin receptor. A STAT3-luciferase reporter was transfected in HEK293 cells, with a plasmid containing the Q223 and R223 receptors to report STAT3 activation. At basal levels and after leptin stimulation R223-expressing cells had reduced STAT3 activation[39].

The R223 mutation appears to reduce the signaling capabilities of the leptin receptor. Why this mutation has this effect is unknown. Here we test two potential possible functional impairments: 1) binding affinity for leptin is reduced in the R223 receptor, or 2) surface expression is down-regulated for the R223 receptor.

Hypothesis

The goal of this study was to test 2 potential effects of the Q223R leptin receptor mutation: impaired leptin binding to the receptor, and reduced surface expression of the receptor. We hypothesized that the mutation would not effect leptin binding or surface expression.

3.2 Methods

Tissue culture and transfection

HEK293T/17 cells were grown at 37°C with 5% CO_2. Cells were cultured in HEPES buffered DMEM-F12 (Gibco) supplemented with 10% fetal bovine serum. pMET7 constructs encoding the Q223 or R223 leptin receptor extracellular domain C-terminally fused to the Fc region of murine IgG1, a FLAG-tag and His tag were obtained from Jan Tavernier at the University of Ghent.

At 70-90% confluency, HEK293T/17 cells were transfected with Lipofectamine 2000 (Invitrogen) and the constructs, or an empty vector plasmid. 12 hours after transfection cell layers were washed and medium was replaced with serum-free Optimem (Gibco) supplemented with sodium butyrate.

48-72 hours after medium replacement supernatants were removed and cleared of cellular debris by centrifugation. Supernatants were then concentrated in 20x Amicon Ultra 15 mL centrifugal filter devices with a molecular weight limit of 100 kDa at 3000xg for 15 minutes. Before BiaCore analysis buffer was exchanged to 1x PBS.

Immunoblotting

Cell culture supernatants were heated to 95°C in 4X SDS-PAGE sample buffer and separated by molecular weight with SDS-PAGE in Mini-PROTEAN TGX Gels (4-20%). Replicate gels were stained with Coomasie blue or proteins were transferred to PVDF membranes. PVDF membranes were blocked with 5% milk in tris-buffered saline + 0.05% Tween 20 for 1 hour. Blots were probed with anti-murine LepR (R&D) and HRP-conjugated secondary antibody (Sigma Aldrich), or with HRP-conjugated anti-mouse IgG1 (Sigma Aldrich). Ab-specific HRP-conjugated anti-mouse IgG1 was used as non-specific binding control. Blots were washed 3 times in TBST between probing steps. Antibody-bound proteins were visualized with ECL reagents (Pierce). Stained gels were imaged using a typhoon fluorescent imager (GE).

Surface Plasmon Resonance

CM5 biosensor chips, N-hydroxysuccinimide (NHS), N-ethyl-N-(3-diethylaminopropyl)carbodimid (EDC), ethanolamine-HCl, and HBS-EP buffer were obtained from BiaCore AB (GE). Reagents included rabbit anti-mouse IgG1 (GE Healthcare) and recombinant murine leptin (R&D systems). Anti-mouse IgG1 was immobilized on two channels of a CM5 chip by amine coupling activated with NHS/EDC for 5 minutes. The antibody was injected at 200 µg/mL in sodium acetate buffer. 2000 resonance units (RU) of antibody were coupled in each channel. Activated surface was blocked with 1M ethanolamine.

Experiments were run on a BiaCore sensor T200 (GE) at 25°C. 100-200 µl of concentrated leptin receptor Fc chimera was injected for 100-300 RU of immobilized

receptor. Leptin concentrations (1.25 – 60 nM) were tested for each receptor chimera after surface equilibration. Association was monitored for 600 seconds, dissociation was monitored for 1000 seconds. Flow rate was set to 30 µl/min. Nonspecific binding was monitored with an antibody-coated channel lacking bound leptin receptor chimera. Sensorgrams were generated from reference-subtracted leptin binding data for each receptor.

BiaCore statistics and data analysis

Kinetic parameters were derived with BIA evaluation software 3.1 (Biacore AB). A non-linear least squares analysis model for 1:1 binding was applied to fit data. Means and SEM were calculated from two separate experiments. A student's t test was performed for association and dissociation rates.

Splenocyte isolation and staining

Spleens were removed from 8 week old Q223 or R223 129/J mice. Spleens were filtered through 100 µm cell filters with 1x PBS + 1% BSA to obtain a single cell suspension and RBCs were lysed. After centrifugation to remove lysed RBCs the cell suspension was filtered through a 40 µm cell filter. Splenocytes were either stained for surface receptors promptly or incubated with 10ng murine leptin (R&D) for 30 minutes, then stained.

Splenocytes were stained for leptin receptor using a rat anti-mouse monoclonal primary antibody (University of Ghent) followed by a goat anti-rat secondary antibody (BD biotechnology). Rat anti-mouse IgG was used as a nonspecific binding control.

Staining was then done for B220 and TCRβ. Cells were washed twice with 1x PBS + 1% BSA after each step. Cells were run on Beckman Coulter CyAn ADP LX and analyzed with FlowJo software (Treestar, Ashland OH)

Surface expression statistics and analysis

Student's t test was performed on mean MFI with PRISM 5 software.

3.3 Results

Leptin binding kinetics are unaffected by R223 mutation

R223 and WT LepR-Fc chimeras were expressed in the supernatants of HEK293T/17 cells; HEK cells do not naturally express the leptin receptor, thus any leptin receptor collected from the supernatant will be the type specified by the transfected receptor. Figure 3.1 shows expression in the supernatant (A) and concentrates (B, C) for the preparation of the chimeras. To immobilize the leptin receptor on the Biacore chip anti-murine IgG1 was used as the capture molecule. Direct coupling could not be used as the concentrated preparation of receptor was not affinity purified. Murine leptin was diluted in Biacore running buffer to the required concentrations

Leptin-LepR binding was measured at 1.25, 2.5, 5, 6.25, 10, and 60 nM murine leptin. Kinetic parameters were calculated for a 1:1 binding ratio using global Langmuir fitting to sensorgrams. Figure 3.2 shows representative sensorgrams. The on rate (k_a), off rate (k_d) and dissociation constant (K_D) were derived from the global fit results from two independent experiments using WT and R223 chimera receptors that had been

prepared for each experiment. The kinetic constants are depicted in Table 3.1, and show no difference in any of the constants between the WT and R223 receptors. We concluded that the Q223R mutation does not affect the binding affinity of leptin to the receptor.

Figure 3.1. Expression of leptin receptor chimeras in cell culture supernatant. A) Expression of leptin receptor chimeras in HEK293T/17 cells. Supernatants from 24 and 48 hours post-transfection were subjected to SDS PAGE and western blotted with antibodies against murine IgG1 domains (Fc specific for lanes 1-5, Ab specific for lanes 6-8). Lane 1: mock transfected, 48 hours. Lane 2: Q223 transfected, 24 hours. Lane 3: Q223 transfected, 48 hours. Lane 4: R223 transfected, 24 hours. Lane 5: R223 transfected, 48 hours. Lane 6: mock transfected, 48 hours. Lane 7: Q223 transfected, 48 hours. Lane 8: R223 transfected, 48 hours. B) Concentration and buffer exchange of chimeras. Supernatants from replacement expression medium after 48 hours of growth. Supernatants, concentrates and filtrates subjected to SDS PAGE with coomassie staining (top) and western blotting with α-murine IgG1 (Fc region) (bottom). Lane 1: Expression medium only. Lane 2: Q223 supernatant. Lane 3: Q223 concentrate. Lane 4: Q223 filtrate. Lane 5: R223 supernatant. Lane 6: R223 concentrate. Lane 7: R223 filtrate. C) Supernatants, concentrates and filtrates (as in B) were transferred to PVDF after SDS PAGE and western blotted with α-murine leptin receptor. Lane 1: Expression medium only. Lane 2: Q223 supernatant. Lane 3: Q223 concentrate. Lane 4: Q223 filtrate. Lane 5: R223 supernatant. Lane 6: R223 concentrate. Lane 7: R223 filtrate.

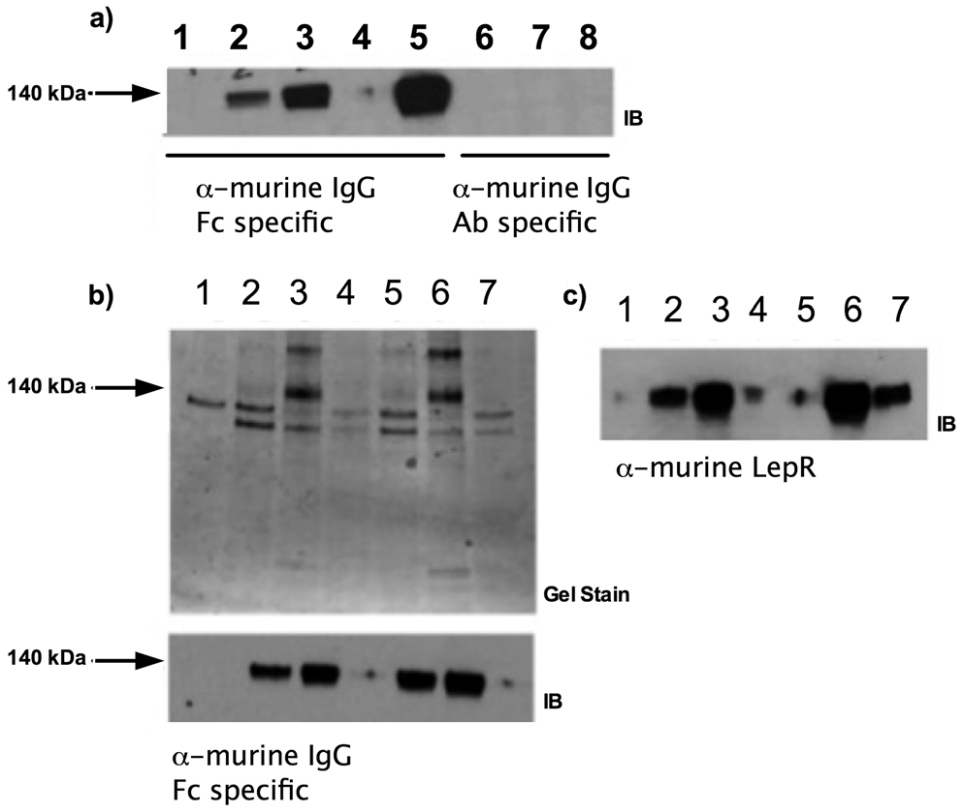

a)
1 2 3 4 5 6 7 8
140 kDa
IB
α–murine IgG
Fc specific
α–murine IgG
Ab specific
b)
1 2 3 4 5 6 7
140 kDa
Gel Stain
140 kDa
IB
α–murine IgG
Fc specific
c)
1 2 3 4 5 6 7
IB
α–murine LepR

Figure 3.2. Sensorgram analysis of leptin binding to Q223 and R223 receptor chimeras. A) Sensorgrams from surface plasmon resonance experiments with concentrated Q223 and R223 leptin receptor chimeras and murine leptin at concentrations from 1.25 – 60 nM. After receptor immobilization leptin was injected for an association time of 600 seconds and a dissociation time of 1000 seconds. B) Global analysis of kinetic parameters after background subtraction. Based on 1:1 binding kinetics using BiaCore T200 software. Fitted curves (thin black lines) are overlaid on sensorgrams to depict fitting to binding model.

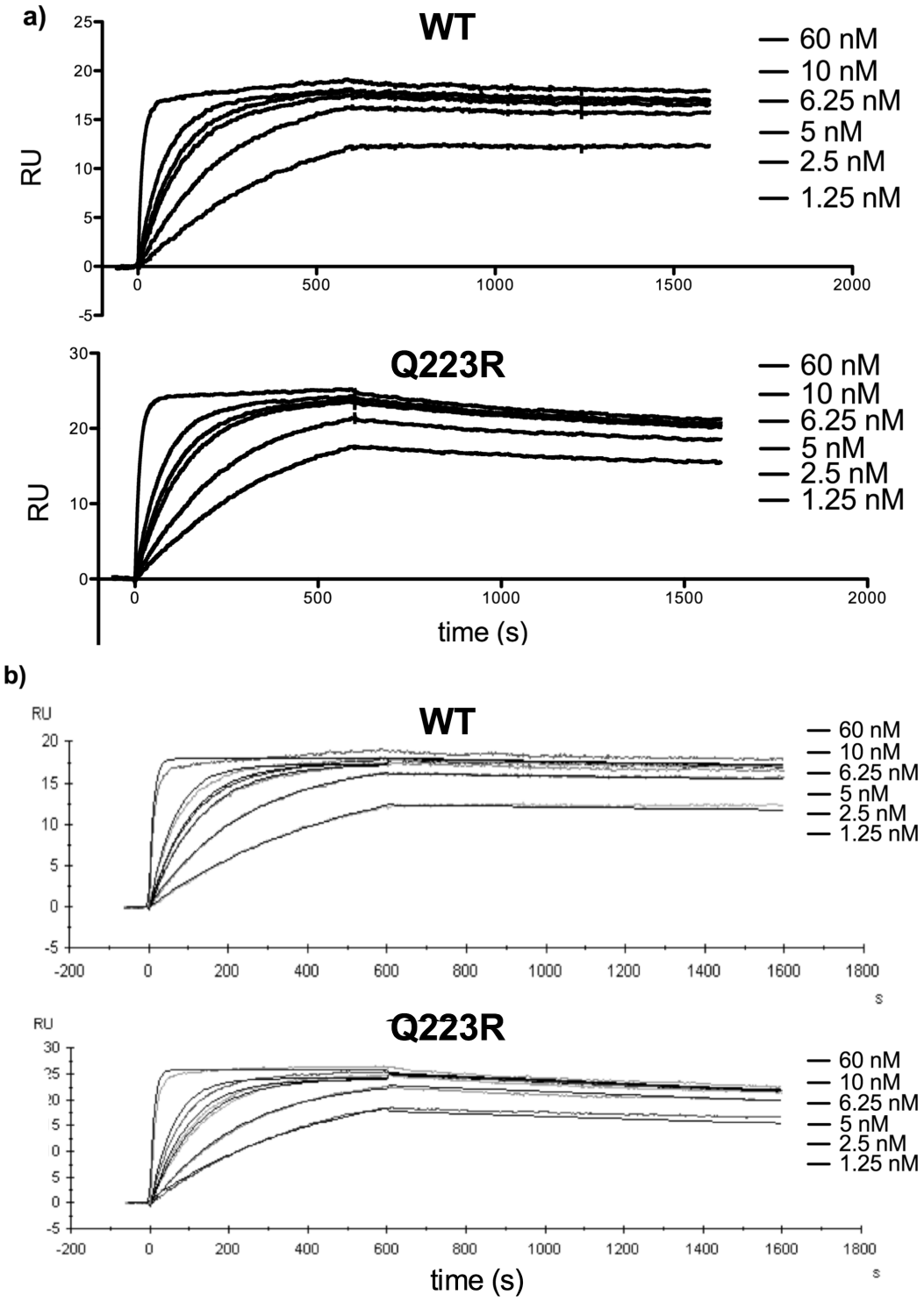

a)
WT
RU
— 60 nM
— 10 nM
— 6.25 nM
— 5 nM
— 2.5 nM
— 1.25 nM
Q223R
time (s)
b)
WT
Q223R
time (s)

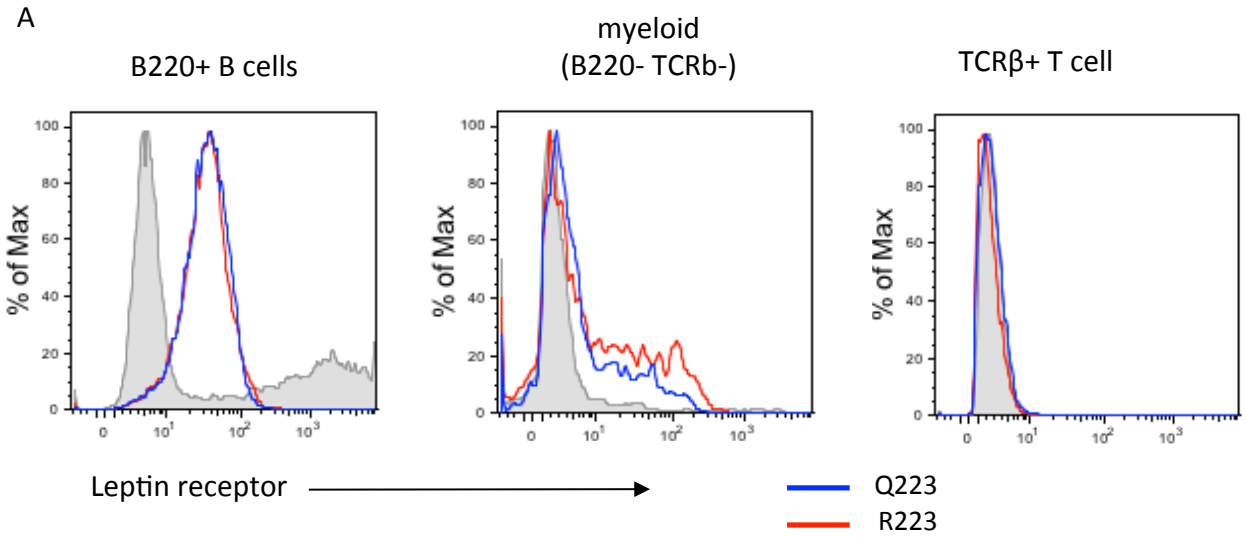
Table 3.1 Association and dissociation kinetics of leptin of Q223 and R223 receptor chimeras. Kinetic constants from two independent experiments for each receptor. Association (k_a) and dissociation (k_d) constants were measured with surface plasmon resonance for Q223 and R223 receptors. Affinity is depicted by K_D. X^2 is included to assess closeness of fit to model of 1:1 binding used in the analysis; < 2 is indicative of acceptable fit to the model.

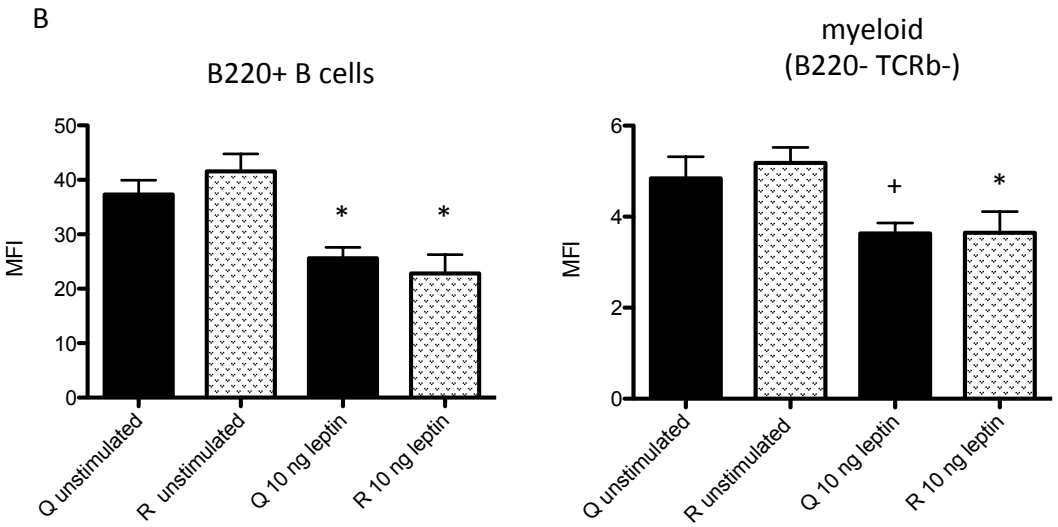
LepR type	k_a (10^6 M^{-1} s^{-1})	k_d (10^{-4} s^{-1})	K_D (10^{-11} M)	X^2
Q223	**1.76 ± 0.193**	**1.21 ± 0.707**	**6.47 ± 3.30**	**0.13-0.151**
R223	**1.75 ± 0.0245**	**1.47 ± 0.0505**	**8.43 ± 0.407**	**0.26-0.35**
P value	**0.931**	**0.744**	**0.615**	**----------**

The R223 mutation does not alter surface expression of the receptor

To investigate if receptor expression was altered by the mutation splenocytes were isolated from Q223 and R223 mice and stained with a leptin receptor monoclonal antibody (gifted by our collaborators at the University of Ghent). Splenocytes were also stained for surface markers to determine cell type. B cells (B220+) had the clearest and strongest detectable leptin receptor staining (Figure 3.3 A). Myeloid cells (B220- TCRβ-) had a small bimodal stain pattern (Figure 3.3 A); this likely due to expression on some cell types within this population, but not all. T cells (TCRβ+ B220-) had no detectable expression. There was no significant difference in the MFI of leptin receptor for Q223 or R223 B cells (37.33 $\pm$ 2.6 SEM vs. 41.55 $\pm$ 3.22 SEM) or myeloid cells (4.84 $\pm$ 0.47 SEM vs. 5.18 $\pm$ 0.33 SEM). There was a reduction in MFI observed for both cell populations after stimulation with 10 ng leptin, which was equal for both genotypes (Figure 3.3 C); down-regulation of the receptor reflects receptor internalization after leptin binding.

Figure 3.3. Surface expression MFI of leptin receptor on splenocytes. Splenocytes were stained for leptin receptor, B220 and TCRβ and analyzed by flow cytometry for surface expression of leptin receptor. A) Histograms of unstimulated cell populations show a strong signal on B220+ B cells, and a mixed population with lower expression on B220- TCRβ- myeloid cells. T cells do not exhibit any expression. B) MFIs of B220+ and B220- TCRβ- myeloid cells without stimulation, and with 10 ng leptin stimulation. B cells and myeloid have reduced leptin receptor MFI after leptin stimulation. There is significant reduction in MFI in Q223 and R223 B cells after leptin stimulation ($p = .01$ and .007), and in R223 myeloid cells after leptin stimulation ($p = .03$). Q223 myeloid MFI reduction after leptin stimulation trends downward ($p = .06$).


A
B220+ B cells
myeloid
(B220- TCRb-)
TCRβ+ T cell
% of Max
Leptin receptor
Q223
R223


B
B220+ B cells
myeloid
(B220- TCRb-)
MFI
Q unstimulated
R unstimulated
Q 10 ng leptin
R 10 ng leptin

3.4 Discussion

The Q223R leptin receptor mutation is common, present at around 50% in Caucasian, African and Bangladeshi populations, and up to 90% in Asian populations[63]. Determining the functional consequence of this mutation would therefore be of broad relevance. This investigation found that neither ligand binding nor surface expression is altered by the R223 leptin receptor mutation.

The results from the surface plasmon resonance BiaCore assays revealed equal on and off rates and equal affinity. These experiments focused on the extracellular domain of the leptin receptor, observing the interaction of one receptor and one leptin ligand. This approach allowed focus, but potentially missed the effect of leptin receptor oligomerization. It has been shown that receptor activation is not dependent on receptor dimerization; the leptin receptor exists as a dimer, instead undergoing a conformational change upon ligand binding as an activation mechanism[81–83]. It had been thought that leptin receptor and leptin interactions occurred at a 2:2 or 2:4 stoichiometry ratio[82], reflecting the dimerization of the receptor, but more recent work suggests that the receptors actually exist in a tetramer rather than a dimer[84]. It is possible that the dimer or potential quaternary oligomerization is affected by the mutation, resulting in the attenuated STAT3 activation in cells expressing the R223 receptor[39].

We also found that surface expression of leptin receptor is not reduced as a result of the R223 mutation in splenocytes. B cells had the strongest and clearest MFI signal, while T cells did not express any detectable receptor. Previous work has found that T cells greatly upregulate leptin receptor after stimulation with anti-CD3[78]; our

studies did not detect leptin receptor on resting T cells, but did not stimulate the T cells, which could explain the difference in results. The bimodal pattern of expression observed in B220- TCRβ- myeloid cells is likely due to leptin receptor expression and absence on different subsets of this population. Considering the results shown in chapter 2, the leptin receptor positive population could be neutrophils. Leptin expression on other cell types such as epithelial cells could not be determined due to extremely high cellular death after isolation. Though we were not able to test leptin receptor expression on all cell types, our results, combined with the location of the mutation in an exon encoding an extracellular domain, indicates that surface expression is not altered by the mutation.

Our studies have eliminated two potential functional consequences of the R223 mutation. Neither ligand binding nor surface expression is affected in our assays. Possible avenues for future research include investigating oligomerization of dimers or tetramers, which could be accomplished through fluorescent microscopy. Labeling leptin receptor expressed in HEK cells and observing cell surface organization before and after leptin binding could reveal any defect in quaternary structure as a result of the mutation. Conformational change upon ligand binding could also be analyzed using FRET to detect any diminishment in conformational change.

Chapter 4:

Association of malnutrition and oral polio vaccine failure with enteric inflammation, systemic inflammation, and maternal health[2]

[2] Contribution: Performed all cytokine luminex assays on sera. Collaborated with Jennie Ma and Miao Lu to determine the best statistical method for result analysis, and performed all analysis of statistical results.

4.1 Introduction

Malnutrition and Vaccination

Malnutrition is a global issue, with the particular problem of malnutrition especially affecting children in the developing world. A common tactic to combat this problem is to provide nutritional supplementation to children and mothers who are considered undernourished. These interventions include promotion of breastfeeding, micronutrient supplementation of mothers and infants, hygiene interventions, complementary food support, food fortification and case management of severe malnutrition. However, it has been mathematically modeled that even if all children and mothers received interventions, only 1/3 of stunting cases would be cured[85]. It has also been observed that nutritional supplementation does not rescue the enteric enteropathy commonly seen in malnutrition in the developing world[86,87]. This suggests that there are other underlying causes and promoters of malnutrition than simply a lack of food.

Malnutrition also has an effect of the efficacy of some oral vaccines. Rotavirus and oral polio vaccines are live virus vaccines that induce a mucosal IgA response, important for enteric pathogens, while also activating the systemic immune response[88]. It has been noticed, however, that in some populations in the developing world these vaccines have lower immunogenicity and efficacy[89]. In a group of children in Dhaka, Bangladesh it was observed that children with a height-for-age standardized z score (HAZ) or weight-for-age standardized z score (WAZ) of at least two standard deviations below the norm (considered “stunted” and “underweight”, respectively) had reduced antibody titers towards all three Sabin strains of the oral polio vaccine (OPV) compared

to well-nourished children. This phenomena was not seen with the systemic vaccines for tetanus, diphtheria or measles[90]. This suggests that the effect of malnutrition on OPV response is specific for OPV and is centered around enteric health. We hypothesize that both malnutrition and OPV could have common pathologies stemming from poor intestinal health.

The PROVIDE study

A study funded by the Bill and Melinda Gates foundation headed by Dr. William Petri and involving the collaboration of 6 institutions is currently underway to investigate oral vaccine failure and malnutrition in the developing world. Known as the PROVIDE (Performance of Rotavirus and Oral Polio Vaccines In Developing Countries) project, the study involves 700 children from an urban slum in Dhaka, Bangladesh who have been recruited at birth and followed for 2 years. The study was designed as a randomized controlled clinical trial. Objectives of the study included determination of the efficacy of a 2-dose rotavirus vaccine, Rotarix, and the OPV efficacy when a single inactivated polio vaccine (IPV) replaced the 4th dose of the trivalent OPV. The prime objective was to investigate if environmental enteropathy was associated with reduced vaccine efficacy and malnutrition. Over the course of two years vaccinations have been administered and serum, stool and urine samples taken at key time points for measurement of biomarkers (Table 4.1). Anthropometry measurements have also been taken throughout all clinic visits. Figure 4.1 depicts a simplified timeline of vaccinations and sample collection for the study weeks addressed in this analysis. Table 4.2 depicts

the entire work plan of the PROVIDE study, including all vaccinations, sample collection time points, and assays.

Table 4.1. Markers measured in children included in analyses. Analytical groupings decided based on function of marker, and sample type. Description contains brief description of marker origin or function, Child Age refers to time at which sample was collected, Sample type is stool, serum, urine or questionnaire. An * indicates that the "sample" is counted as total days up until 18 weeks of age. FRA = field research assistant.

Biomarker	Description	Child Age (weeks)	Sample type	Analytical Grouping (Indicative of)
MPO	Myeloperoxidase; component of the neutrophil cytosol,	12	Stool	Enteric inflammation
calprotectin	Component of the neutrophil cytosol	12	Stool	Enteric inflammation
Neopterin	Produced by stimulated monocytes/macrophages	12	Stool	Enteric inflammation
Alpha-1 anti-trypsin	Protease inhibitor produced by cells to resist damage by neutrophil inflammation	12	Stool	Enteric inflammation
Mannitol	Sugar, easily absorbed by the intestinal tract	12 & 24	Stool	Enteric inflammation
Reg1β	Regeneration factor, produced by cells to repair damage	6 & 12	Stool	Enteric inflammation
Days of diarrhea	Number of days of diarrhea by 18 weeks of age	18*	FRA reporting	Enteric inflammation
IL-1β	Inflammatory cytokine produced by monocytes and macrophages	18	Serum	Systemic inflammation
IL-4	Induces differentiation to Th2 cells	18	Serum	Systemic regulatory
IL-5	Growth factor for B cells and eosinophils	18	Serum	Systemic inflammation
IL-6	Inflammatory cytokine produced during acute and chronic inflammation	18	Serum	Systemic inflammation
IL-7	B and T cell development and maturation	18	Serum	Systemic regulatory
IL-10	Down-regulates Th1 cytokines, enhances B cell survival and antibody production	18	Serum	Systemic regulatory
TNFα	Secreted by macrophages, regulates cell proliferation, apoptosis and differentiation	18	Serum	Systemic inflammation
MIP1β	Inflammatory cytokine produced by monocytes, chemotactic (CCL4)	18	Serum	Systemic inflammation
Ferritin (serum)	Acute phase protein, involved in iron storage; increased by IL-1b and TNFa	6 & 18	Serum	Systemic inflammation
CRP	Acute phase protein produced by the liver, interacts with immune effector systems	6 & 18	Serum	Systemic inflammation
sCD14	Soluble CD14; shed by activated monocytes, binds LPS	6 & 18	Serum	Systemic inflammation

LPS	Lipopolysaccharide; component of gram-positive bacterial membrane	6 & 18	Serum	Systemic inflammation
Activin	Growth regulation factor; associated with muscle wasting in cachexia	6	Serum	Systemic inflammation
Vitamin D	Absorption of calcium, iron, magnesium, phosphate and zinc	6 & 18	Serum	Nutritional
Zinc	Essential mineral, deficiency leads to growth inhibition and immune deficiency	6 & 18	Serum	Nutritional
Retinol binding protein	Transport protein for vitamin A	6 & 18	Serum	Nutritional
HAZ	Height-for-age Z score, stunting	18	Clinic measurement	Nutritional
WAZ	Weight-for-age Z score, underweight	18	Clinic measurement	Nutritional
WHZ	Weight-for-height Z score, wasting	18	Clinic measurement	Nutritional
Exclusive breastfeeding	Days when breast milk was the only food taken up to 18 weeks of age	18*	Questionnaire	Nutritional
Expenditure	Monthly expenditure for household	n/a	Questionnaire	Maternal health & SES
Income	Monthly income for household	n/a	Questionnaire	Maternal health & SES
Maternal education	Education considered a “yes” if primary school was attended	n/a	Questionnaire	Maternal health & SES
Maternal height	Centimeters	n/a	Questionnaire	Maternal health & SES
Maternal weight	kilograms	n/a	Questionnaire	Maternal health & SES

Figure 4.1. Timeline of study with vaccinations and sample collection, week 0 – 18. Study week refers to the age of the child. Trivalent OPV vaccinations were given at weeks 6, 10 and 14. Serum samples were taken at 6 and 18 weeks, stool and urine samples at 12 weeks.

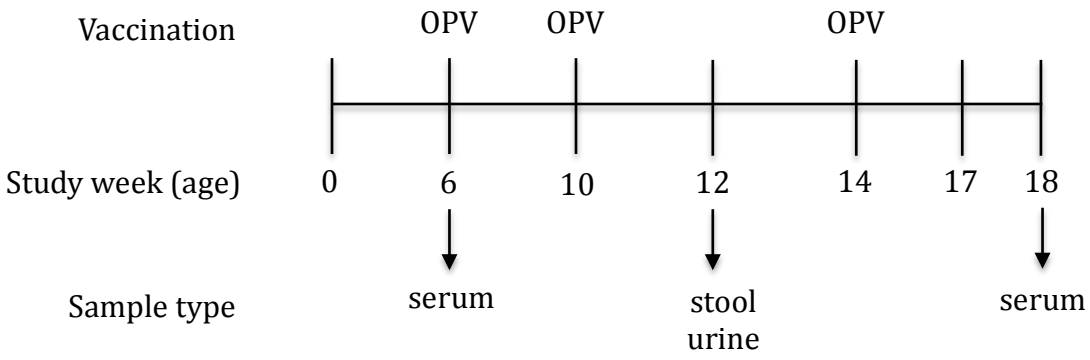

Vaccination
OPV
OPV
OPV
Study week (age)
0
6
10
12
14
17
18
Sample type
serum
stool
urine
serum

Table 4.2. PROVIDE study work plan. Clinic visits at defined study weeks (age in weeks) act to provide vaccinations (OPV = trivalent oral polio vaccine; Penta = diphtheria, tetanus, pertussis, hepatitis B, and Hib vaccines; Rota = rotarix rotavirus vaccine) and collect samples at specific time points (serum, stool, urine from children, serum and breast milk from mothers). *Table provided by Dr. William Petri.*

Table 1. Work Plan for the Study of 700 Children from Mirpur, Dhaka, Bangladesh

Clinic Visit # (child also home visited 2x/weekly)	1	2	3	4	5	6	7	8	9	10	11	12	13-15	16
Target visit schedule (age in weeks)	0	6 wks	10 wks	12 wks	14 wks	17 wks	18 wks	24 wks	39 wks	40 wks	52 wks	53 wks	65, 78, 91 wks	104 wks
EPI Vaccinations + Rotarix (-) Rotarix +/- IPV	BCG	Penta OPV	Penta OPV Rota Penta OPV		Penta OPV	Rota None			OPV IPV OPV IPV	Measles Rubella	OPV		Wk 65 Measles	
Infant blood draws		**2cc**					**2cc**	**HS**		**2cc**		**3cc**		**5cc**
Infant baseline stool sample	X													
Breast milk IgA: polio & rotavirus		X												
Maternal serum neutralizing antibody: polio		**5cc**												
Poliovirus Vaccine Measures														
Antibody in lymphocyte supernatant (ALS)		X								X				
Faecal IgA (all 3 virus types)		X				X				X		X		
Faecal excretion vaccine virus		XX			XXX						XXX			
Faecal cytokines		XX												
Serum neutralizing antibody		X					X			X		X		
Saliva swab for polio IgA												X		X
Rotavirus Vaccine Measures														
Rotavirus diarrhea	Continuous Diarrhea Surveillance and Testing for Rotavirus Diarrhea													
Antibody in lymphocyte supernatant (ALS)		X					X							
Faecal IgA		X					X							
Faecal excretion vaccine virus			XX			XXX								
Faecal cytokines			XX											
Serum IgA		X					X	X						
T Cell-mediated immunity:												X		
Phosphoflow		X					X			X				X
Tetramer-Flow Cytometry							X			X				X
ICS/CytoF*		X*					X*			X*				X
csSAM												X		
Systemic Vaccine Measure														
Serum tetanus IgG; saliva tetanus and measles IgA							X					X		X
Nutritional Measures														
Gestational Age Assessment	X													
Anthropometry (HAZ,WAZ)	X	X-c X-m	X	X	X	X	X	X	X	X	X	X	XXX	X
RBP, Vitamin D, zinc, ferritin		X					X							
Hematocrit								X						
Tropical Enteropathy Measures														
Lactulose-mannitol ratio urine				X				X		X				X
Serum α-LPS,CRP		X					X			X		X		X
Other Measures														
Diarrhea Surveillance (etiologic agents & severity) & Breast Feeding Duration	Household and Clinic Continuous Monitoring for Entire Study Duration													
Gut Microbiome (subset of responders/non-responders)	Specimens taken from scheduled stool samples and episodic diarrheal samples													
Child's HLA (and store mother/ child DNA for future study of genome polymorphisms)		X-c X-m												
Cognitive Function Assessment (infants)													Week 78	X
Maternal depression and intelligence (mothers)														X

Hypothesis

Environmental enteropathy due to infection will cause increased enteric inflammation. Malnutrition will be associated with enteric inflammatory biomarkers. Because we hypothesize that a common pathology leads to both malnutrition and poor oral vaccine response, OPV antibody response will also be negatively impacted by the same inflammatory biomarkers as malnutrition.

4.2 Methods

*Study site and subjects***[3]*

The study subjects were drawn from Mirpur, Dhaka, Bangladesh, a densely populated area with a range of socioeconomic statuses (Table 4.3). Families recruited from this study were from the northern region of Mirpur, an area with predominantly low-income families. A community census to identify pregnant women was performed by trained female Bangladeshi field research assistants (FRAs). Children were enrolled within seven days of birth, after eligibility screening and study consenting. At enrollment the FRA completed a socioeconomic questionnaire with the mother and measured the infant's weight and height. There was a fixed enrollment of 700 children; withdrawals before study completion were not replaced.

[3] ** adapted from manuscript in preparation "The Performance of Rotavirus and Oral Polio Vaccines In Developing Countries (PROVIDE) Study: Description of Methods of an Interventional Study Designed to Explore Complex Biologic Problems". Beth D Kirkpatrick, E Ross Colgate, Josyf C Mychaleckyj, Rashidul Haque, Dorothy M Dickson, Marya P Carmolli, Uma Nayak, Mami Taniuchi, Caitlin Naylor, Firdausi Qadri, Jennie Z Ma, Masud Alam, Mary Claire Walsh, Sean A Diehl, the PROVIDE study teams, William A Petri Jr.

*Clinic methods***

Fifteen scheduled clinic visits were scheduled based on child age. Comprehensive primary care of offered to the families.

*Diarrheal surveillance***

FRAs visited homes twice weekly to perform diarrheal surveillance. A questionnaire including frequency of diarrhea, temperature, feeding history and use or antibiotics or oral rehydration therapy was used to record episodes. Diarrhea was defined as 3 or more loose stools in 24 hours, and independent episodes separated by at least 72 hours. Children who had diarrhea at the time of the FRA visit were referred to the clinic were severity was determined with a Vesikari scale.

*Nutritional status***

Anthropometry measurements were taken at every clinic visit using a calibrated digital scale and standardized supine length measurement. Malnourished children 3 or more SD below the mean were treated or referred to nutrition centers.

*OPV serum neutralizing antibodies***

Poliovirus Sabin types 1, 2, and 3 serum neutralizing antibody assays were performed at the Centers for Disease Control and Prevention (Atlanta, Georgia), using WHO-standardized assays.

Enteric inflammatory biomarkers

Markers of enteric inflammation included myeloperoxidase, calprotectin, and neopterin. ELISAs were used to measure MPO (American Laboratory Products Co, Salem NH), calprotectin (American Laboratory Products Co, Salem NH), neopterin (Genway Biotech, San Diego CA) and alpha-1 anti-trypsin (American Laboratory Products Co, Salem NH) in stool samples collected at 12 weeks. Reg1β was also measured as a measure of intestinal epithelial health. Reg1β was measured by ELISA (Techlab, Blacksburg VA) in stool samples collected at 6 and 12 weeks of age. All assays were performed at the International Centers for Diarrheal Disease, Bangladesh (ICDDR,B).

Systemic inflammatory biomarkers

Markers of systemic inflammation included endotoxin, C-reactive protein and sCD14. These markers were measured with ELISAs using serum collected at 6 and 18 weeks of age at the ICDDR,B. Inflammatory and regulatory cytokines were also measured in week 18 serum using a custom bioplex assay (Biorad) and included IL-1b, IL-3, IL-5, IL-6, IL-7, IL-10, TNFα, and MIP1β at the University of Virginia.

Cytokine data are categorized into bottom 50th percentile, 50-75th percentile, and 75-100th percentile, due to a high percentage of out of range values (30-50%). Out of range values were imputed with the lowest measured value for each plate. All other markers are analyzed continuously with no percentile grouping.

Micronutrients

Micronutrients were measured in week 6 and 18 serum samples. Retinol binding protein (as a biological indicator of Vitamin A), Vitamin D and ferritin were measured with ELISA (Quantikine; Immunodiagnostic Systems Ltd). Zinc was measured with atomic absorption spectrophotometry.

Mannitol

A lactulose/mannitol solution containing 250 mg/mL of lactulose and 50 mg/mL of mannitol. Children were given the solution at weeks 12, 24, 40 and 104 at 2 mL/kg of weight up to 20 mLs. Urine was collected for 2 hours following the lactulose/mannitol dose using pediatric urine collection bags. Analysis of samples was performed using high performance ion chromatography (HPIC).

Enteropathogen detection

The Taqman Array Card (TAC) system was performed by the method of Liu et al[91,92]. In brief, RNA and DNA were extracted from surveillance stools collected at 6 and 10 weeks, reverse transcribed, and then subjected to TAC amplification for >30 different enteropathogens.

Statistical Methods[93–98]

The univariate and multivariable analyses of biomarker association were performed for OPV titers at 18 weeks of age and nutritional growth from 0 to 1 year of age. All

statistical analyses were performed using the R Package 3.1 (www.r-project.org) and SAS 9.3 (SAS Institute; Cary, NC).

Univariate analysis

Linear regression for continuous responses was performed to evaluate the association of individual biomarker with the response one at a time. Based on univariate p values, False Discovery Rate (FDR) was calculated to correct for multiple comparisons and identify significant biomarkers at FDR 20% cut-off. The goal of univariate analysis with FDR correction was to find significant biomarkers for each response individually.

Multivariable analysis

Smoothly Clipped Absolute Deviation (SCAD) was applied for the multivariable analysis to identify a small subset of truly informative biomarkers. SCAD was used over other penalized methods in our biomarker data analysis because it can select important predictors consistently, and produce parameter estimators as efficient as if the true model were known. The optimal tuning parameter for SCAD was determined with 10-fold cross validation.

Clustering analysis

Hierarchical clustering of biomarkers was performed to classify them into relatively homogeneous clusters, i.e., biomarkers in same clusters are strongly correlated to each other.

4.3 Results

Cohort characteristics

The cohort families had a median income of US$166 (range US$39-1001) and a maternal illiteracy rate of 30%. Full characteristics of the cohort can be found in Table 4.3. The children had high burdens of infections. Table 4.4 depicts the detection rates of the most common enteric pathogens in the surveillance stool of children at 6 and 10 weeks of age. The children had on average 1.7 $\pm$ 1.4 detectable pathogens at 6 weeks, and 2.5 $\pm$ 1.4 at 10 weeks. Unsurprisingly, given the high burden of infection, these children exhibited elevated enteric inflammation. Myeloperoxidase and calprotectin, markers of neutrophil inflammation and common biomarkers of inflammatory diseases such as inflammatory bowel disease (IBS) and inflammatory colitis were abnormal (as defined by high income country standards) in 82% and 94% of the children respectively (Fig 4.2 A ,B, D). There were also elevated amounts of fecal alpha-1 anti-trypsin in 82% of the children (Fig 4.2 C, D), a protein normally found in the cytosol of cells, indicating a break in the normal barrier function of the gut[99].

Malnutrition and OPV failure in cohort children

The children were given trivalent OPV, which contains all three Sabin types. OPV response is evaluated by serum neutralizing antibody titer. A titer of less than 1/8 ($\log_2$=3) is defined as a failure[100]. Using this measurement, 11% of children failed to produce antibody to OPV1 or OPV3, and 3.4% failed to produce antibody to OPV2 (Table 4.5) at 18 weeks of age. The cohort also had a high rate of malnutrition, particularly stunting. Most of the children started out healthy, with normal HAZ at

birth. Over the first year of life the percentages of children with stunting (an HAZ score of less than -2 standard deviations below the norm) increased from 9.5% at enrollment to nearly 30% at one year of age (Figure 4.3). 20% of the children were born underweight (WAZ < -2), but the population then had an improvement in weight gain with only 11% underweight by week 10. However, this did not last and by one year 20% of the children were again underweight (Figure 4.3).

Table 4.3. Baseline Characteristics of PROVIDE Study Population**.

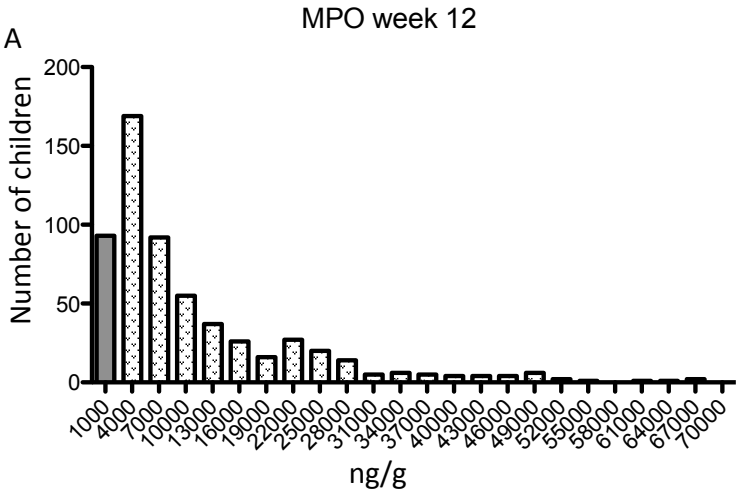
Baseline Characteristics	Mean ± SD	Range
Neonatal		
Age at enrollment (days)	4.9 ± 1.69	1--7
Female gender (%)	47.4	
Weight at enrollment (kg)	2.8 ± 0.37	1.7--4.1
Length at enrollment (cm)	48.69 ± 1.75	43.1--55.4
HAZ at enrollment	-0.90 ± 0.90	-3.67--2.88
WAZ at enrollment	-1.29 ± 0.84	-4.00--1.24
Gestational Age ≤ 36 weeks (%)	32	
Exclusive breastfeeding at birth (%)	94.6	
Home Birth (%)	25.9	
BCG given at birth (%)	2.3	
Maternal		
Age at enrollment (years)	24.65 ± 4.6	18--41
Age at 1st pregnancy (years)	18.8 ± 2.9	12--35
Total live births	2.1 ± 1.2	1--10
Vaginal delivery (%)	77.1	
Height (cm)	150.33 ± 5.5	134--187
Postpartum weight (kg)	49.31 ± 9.41	30--80
Postpartum BMI	21.76 ± 3.7	14.23--36.57
Children under 5 years of age	0.3 ± 0.5	0--2
Mother illiterate (%)	28.9	
Mother homemaker (%)	85.9	
Household / Socio-economic Status		
Total monthly income (Taka)*	12762.39 ± 9409.9	3000--77,000
Piped municipal water source (%)	96.9	
Toilet (septic tank) (%)	52.4	
Dwelling size equals 1 room (%)	72.4	
Household members	5.2 ± 2.2	1--18
** 1 Bangladeshi Taka = $0.013 during the study period*		

Table 4.4. Enteropathogens detected in the stools of children. Surveillance stool collected at 6 weeks and 10 weeks was tested for enteropathogen presence using the TAC system. Enteropathogens present in 10% or greater of the children are listed. EAEC = enteroaggregative *E. coli*, ETEC = enterotoxigenic *E. coli*, EPEC = enteropathogenic *E. coli*. *E. coli* types were called based on presence of virulence genes: EAEC – *aaiC* or *aatA*; EPEC – *eae* and *bfbA* (typical) or only *eae* (atypical); ETEC – *LT* only (LT ETEC), *Sth* or *STP* with or without *LT* (ST ETEC); EIEC – *ipaH*; STEC – *Stx1* and/or *Stx2*. Adeno 40/41 refers to adenovirus types 40 and 41; these are the adenoviruses that have been found to cause gastrointestinal disease but not respiratory virus. Panenterovirus refers to all enteroviruses tested, including OPV Sabin strains.

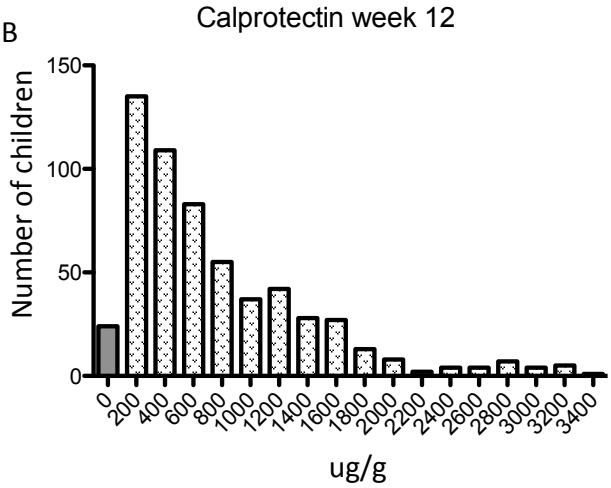
Organism	Children infected week 6 % (N = 357)	Children infected week 10 % (N = 354)
EAEC	37.0	49.2
Pan-enterovirus	35.3	84.2
ETEC	11.8	15.0
Rotavirus	10.1	15.3
adeno 40/41	10.1	11.0
EPEC	9.5	9.9

Figure 4.2. Distribution of enteric inflammatory markers. Inflammatory markers MPO (A), calprotectin (B), and alpha-1 anti-trypsin (C) were measured in stool samples taken at 12 weeks using ELISA. Gray columns represent normal values, white patterned columns represent values considered above normal. Normal values were assigned based on the ELISA kit recommendations (D).

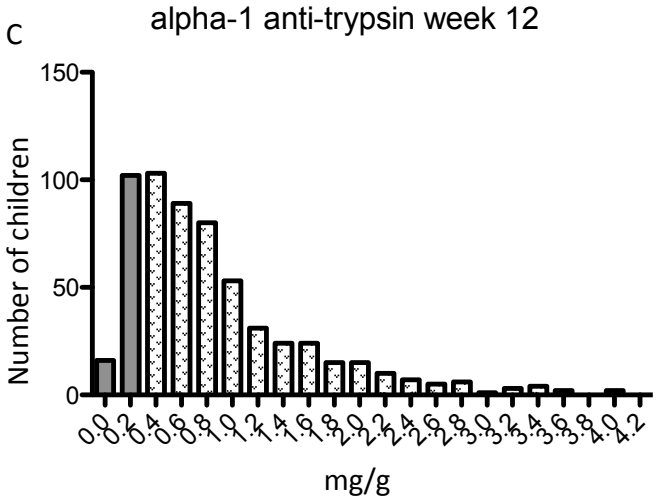
A



B



C



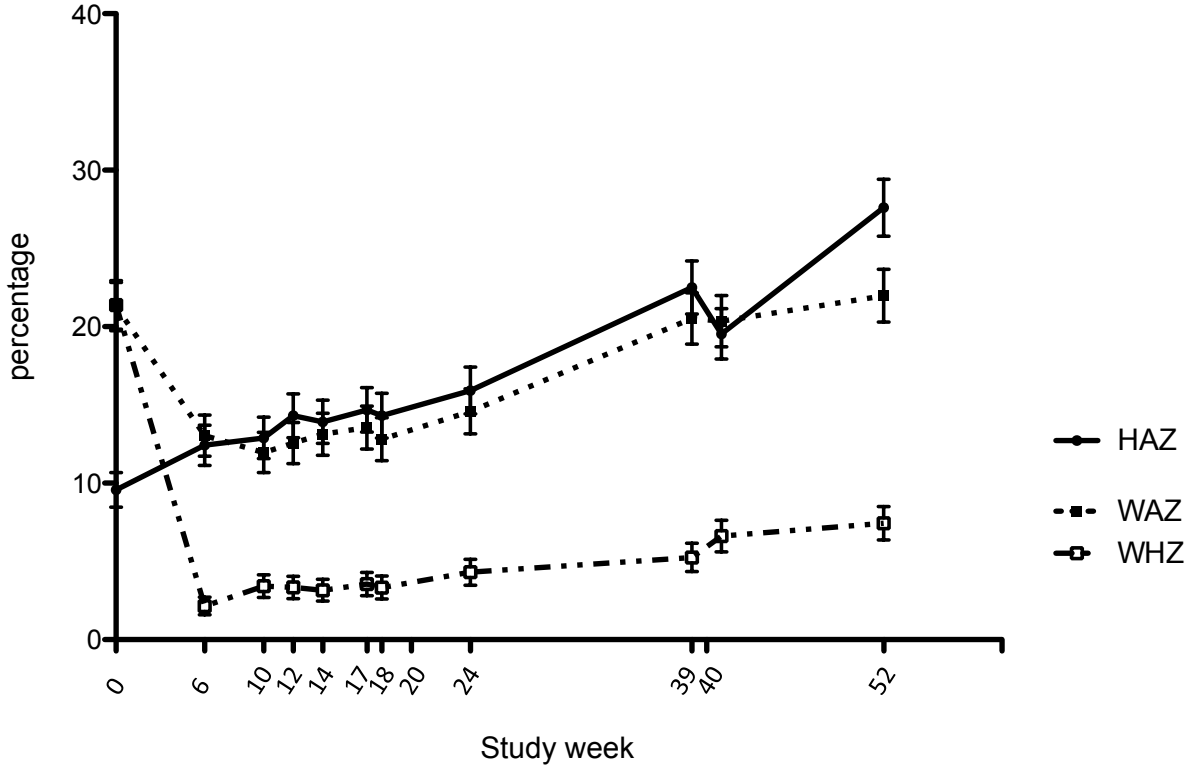
D

Marker	Amounts considered elevated*	% children with elevated amounts	n
Calprotectin	>200 ng/ mg	82.7%	596
MPO	>2000 ng/ mg	88.1%	591
Alpha1 anti-trypsin	>.27 mg/g	81.9%	592

Table 4.5. Rates of failure to OPV Sabin types. Failure is defined as an antibody titer of less than 1/8 ($\log_2 = 3$). N = 589.

Serum Neutralizing Antibody Type	Failure (N)	Failure (%)
Type 1	66	11.2%
Type 2	18	3.4%
Type 3	66	11.2%

Figure 4.3. Malnutrition z-scores over time. Height and weights were taken at scheduled clinic visits, and transformed to standardized Z scores (WHO ref). The X axis depicts the age of the child at which the measurement was taken, the Y axis depicts the percentage of children in the cohort who had an HAZ, WAZ or WHZ of -2 or less (stunted, underweight or wasted, respectively.


40
30
20
10
0
percentage
0
6
10
12
14
17
18
20
24
39
40
52
Study week
HAZ
WAZ
WHZ

Growth and OPV antibody response have similar patterns of correlation with biomarkers

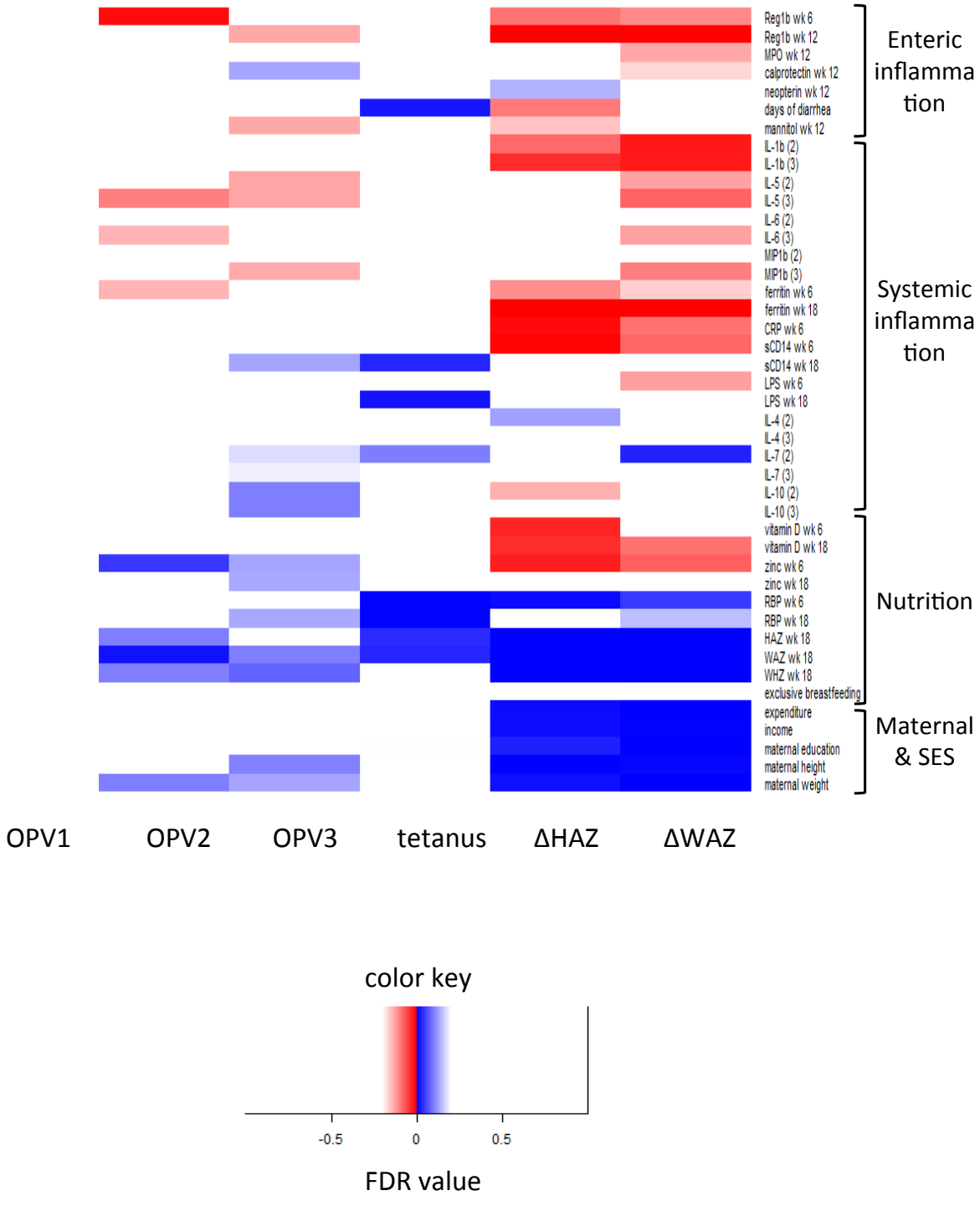
Linear regression analyses were performed for each outcome and biomarker. A false discovery rate of 20% was applied. Figure 4.4 depicts the biomarkers that had a FDR value of .2 or below for each outcome; red boxes indicate that the biomarker had a negative, or deleterious, effect on the outcome while blue boxes indicate that the biomarker had a positive, or improving, effect on the outcome. This figure shows the overall patterns of individual correlations with each outcome. The purpose of this univariate analysis was to get a sense of potential pathways that could be contributing to each outcome. By analyzing each marker individually, a pattern of significance was revealed.

There were similar patterns of correlations between all outcomes: enteric and systemic inflammation were negatively associated with growth and OPV antibody production, while nutrition and SES and maternal factors were positively associated with growth and OPV antibody production. ΔWAZ and ΔHAZ, our growth outcomes, exhibited strong negative correlations with Reg1b, IL-1b, ferritin, CRP and sCD14, biomarkers contained in both the enteric and systemic inflammatory groups. There were also additional negative correlations of ΔWAZ with markers in these groups, including MPO, IL-5, IL-6 and MIP-1b. All markers of maternal health & SES had strong positive correlations. Micronutrient correlations were surprising; zinc and vitamin D levels had negative correlations with both measures of growth, while exclusive breastfeeding up to 18 weeks had no correlation.

While OPV1 had no significant correlations with any markers, OPV2 and OPV3 had some negative correlations with the enteric and systemic inflammatory families of biomarkers, most notably Reg1b, IL-5 and MIP1b. Regulatory biomarkers IL-7 and IL-10 had a positive impact on OPV3 titers, a pattern not shared by the other OPV Sabin types or by the nutritional outcomes. Overall, growth as measured by ΔWAZ and ΔHAZ had stronger correlations with a larger number of individual markers, but both nutrition and OPV exhibited similar patterns of negative and positive correlations.

Tetanus IgG titer was used as a control outcome; as an intramuscular systemic vaccine it was not expected to be impacted by the same markers that affect growth or OPV titer. Only positive correlations were found with tetanus. These markers included nutritional markers: zinc, RBP and anthropometry, and some markers of systemic inflammation: LPS, sCD14 and IL-7. There was no overlap in the enteric and systemic inflammation groups with the primary outcomes. There was similarity in significant signals in the nutritional measures group: markers that positively correlated for tetanus IgG (RBP, WAZ, and HAZ) also correlated positively with the growth and OPV outcomes.

Figure 4.4 Heat map of FDR values of linear regression analysis. Biomarkers with a FDR value of .2 or below for at least one outcome are depicted on the heat map. Markers are grouped according to type. A positive correlation is indicated by a blue box, a negative correlation is indicated by a red box, and no correlation is indicated by a white box. An FDR value close to 0 indicates a strong correlation. The darker the color of a box, the closer to zero the FDR value is for that marker.


Reg1b wk 6
Reg1b wk 12
MPO wk 12
calprotectin wk 12
neopterin wk 12
days of diarrhea
mannitol wk 12
IL-1b (2)
IL-1b (3)
IL-5 (2)
IL-5 (3)
IL-6 (2)
IL-6 (3)
MIP1b (2)
MIP1b (3)
ferritin wk 6
ferritin wk 18
CRP wk 6
sCD14 wk 6
sCD14 wk 18
LPS wk 6
LPS wk 18
IL-4 (2)
IL-4 (3)
IL-7 (2)
IL-7 (3)
IL-10 (2)
IL-10 (3)
vitamin D wk 6
vitamin D wk 18
zinc wk 6
zinc wk 18
RBP wk 6
RBP wk 18
HAZ wk 18
WAZ wk 18
WHZ wk 18
exclusive breastfeeding
expenditure
income
maternal education
maternal height
maternal weight
Enteric inflammation
Systemic inflammation
Nutrition
Maternal & SES
OPV1
OPV2
OPV3
tetanus
ΔHAZ
ΔWAZ
color key
-0.5
0
0.5
FDR value

SCAD-selected independent biomarkers include enteric and systemic inflammatory markers negatively correlated with outcomes.

Univariate analysis of the individual biomarker linear regression analyses is based on the assumption that one marker at a time was the only factor influencing the outcome. Multivariable analysis in contrast is based on the anticipation that a given biomarker is not independent, but is itself influenced by multiple other biomarkers. This study measures 30+ markers, in general only a small number of biomarkers are independently informative with respect to the response of interest. Regression methods such as SCAD, used in this analysis, can identify the subset of markers that will independently correlate with an outcome. This method first produces effect sizes of each marker on an outcome, known as coefficient estimates; coefficient estimates indicate how much an increase in the marker will increase or decrease the outcome. By imposing some penalty, determined by the statistical algorithm, in the regression model, SCAD can shrink small coefficients of unimportant markers to zero, and thus select only important markers: those with coefficients greater than or less than zero after penalty shrinking (indicating a positive or negative correlation, respectively).

The biomarkers selected by SCAD (Table 4.6) also demonstrated linear correlation in the same directions (Figure 4.4). The fact that a different analysis yielded similar significant correlations increased confidence of the results. Table 4.6 contains the coefficient estimate sizes of the variables; the larger the estimate size, the larger the effect of a one unit change in the biomarker on the outcome. The markers have been standardized for comparison of estimate sizes. This multivariable analysis selects independent markers for each outcome. In contrast to the previous univariate analysis,

which is used to identify potential pathways, SCAD identifies the markers that are independently important for each outcome. These SCAD-selected markers thus show important points of overlap when the same marker is selected for more than one outcome; common markers indicate a common driver of poor or improved outcome, valuable in determining the origins of malnutrition and OPV failure.

Days of diarrhea, Reg1b, mannitol, and MPO at 12 weeks, zinc, vitamin D, CRP and sCD14 at 6 week, and ferritin at 18 weeks were negatively correlated with ΔHAZ, while RBP at 6 weeks, maternal education and height, and monthly expenditure were positively correlated. ΔWAZ was negatively correlated with Reg1b and MPO at 12 weeks, CRP and zinc at 6 weeks, ferritin at 18 weeks, IL-1b and exclusive breastfeeding; maternal education and weight, and monthly expenditure were positively correlated. Eight enteric or systemic biomarkers were chosen in total for both for ΔHAZ and ΔWAZ with four biomarkers (Reg1b and MPO at 12 weeks, CRP at 6 weeks, and ferritin at 18 weeks) selected for both nutritional outcomes. Maternal and SES factors had 100% overlap (maternal weight and height are closely correlated, so are considered as one marker here). Inflammation was always negatively correlated, and the biomarkers were equally distributed between both enteric and systemic inflammation. The enteric markers selected by SCAD focus on intestinal damage (Reg1b), neutrophil inflammation (MPO) and enteric infection (days of diarrhea). Systemic biomarkers CRP, ferritin and sCD14 are acute phase proteins that signal prolonged inflammation at an early age. Surprisingly, higher mannitol, indicative of increased intestinal absorption, and exclusive breastfeeding up to 18 days were also selected as negative markers.

Unsurprisingly, improved maternal health and socioeconomic status was positively correlated with increased growth.

The biomarkers selected for the three OPV antibody titers were more varied, though all showed a negative impact of any inflammatory biomarkers selected and a positive impact of nutritional and maternal health markers selected. OPV1 was negatively correlated with sCD14 at 6 weeks and positively correlated with sCD14 at 18 weeks and maternal education. OPV2 was negatively correlated with Reg1b and ferritin at 6 weeks, and positively correlated with RBP at 6 weeks and WAZ at 18 weeks. OPV3 was negatively correlated with Reg1b and mannitol at 12 weeks, and positively correlated with calprotectin at 12 weeks, sCD14 and zinc at 18 weeks, IL-10, WHZ at 18 weeks, and maternal height. OPV2 and OPV3 appear more related to each other than to OPV1. Reg1b, selected for OPV2 and OPV3, signals of epithelial cell damage, suggesting that it is specifically damage to the intestinal lining that reduces vaccine efficacy. Interestingly, each inflammatory biomarker selected for any of the OPV Sabin types is also selected for at least one of the nutritional outcomes, though occasionally at differing weeks.

Tetanus was positively correlated with days of diarrhea, sCD14 and LPS at 18 weeks, IL-6, RBP at 6 and 18 weeks, and WAZ at 18 weeks. All the inflammatory markers selected were systemic, reflecting perhaps the systemic vaccination strategy of the tetanus vaccine.

Table 4.6. SCAD-selected subset of important markers for all outcomes. SCAD multivariable analysis selects only markers with a non-zero effect after attaching a penalty on the regression model. Estimate sizes are listed for markers selected for an outcome. Results were standardized for direct comparison of variables. Standardization was achieved by subtracting the mean of each variable from the value of that variable, then dividing by the standard deviation. Each variable then has a mean of 0 and a variance of 1. Red numbers indicate a negative correlation (poorer outcome), while blue numbers indicate a positive correlation (improved outcome).

		ΔHAZ	ΔWAZ	OPV1	OPV2	OPV3	tetanus
Enteric Inflammation	Days of diarrhea	-0.02					+0.2
	Reg1b wk 6				-0.09		
	Reg1b wk 12	-0.03	-0.06			-0.03	
	Mannitol wk 12	-0.01				-0.06	
	MPO wk 12	-0.008	-0.005				
	Calprotectin wk 12					+0.07	
Systemic Inflammation	CRP wk 6	-0.04	-0.006				
	Ferritin wk 6				-0.02		
	Ferritin wk 18	-0.09	-0.06				
	sCD14 wk 6	-0.03		-0.04			
	sCD14 wk 18			+0.03		+0.02	+0.01
	LPS wk 18						+0.01
	IL-1b		-0.0007				
	IL-10					+0.02	
	IL-6						+0.0002
Nutrition	Zinc wk 6	-0.04	-0.13				
	Zinc wk 18					+0.01	
	Vitamin D wk 6	-0.02					
	RBP wk 6	+0.01			+0.02		+0.09
	RBP wk 18						+0.15
	Exclusive breastfeeding		-0.03				
Maternal & SES	WAZ at 18 weeks	+0.22			+0.09		+0.06
	HAZ at 18 weeks		+0.1				
	WHZ at 18 weeks					+0.18	
	Maternal education	+0.0002	+0.014	+0.04			
	Maternal height	+0.04				+0.05	
	Maternal weight		+0.01				
	expenditure	+0.02	+0.005				

Clustering of markers reveals relationships between outcomes and markers

Clustering analysis reveals linear relationships among the markers as a single group (Figure 4.5). The relationship between the clusters is expressed by "height" on the top X axis. Height refers to the decrease of the sum of the squared correlation between a marker and the center of the cluster, as a measure of distance. The distance between two markers is the common node on the height scale: the distance between days of diarrhea and mannitol at 12 weeks is .7. Distance between clusters is the delta height between a larger and smaller cluster. Ferritin at week 6 and 18 form cluster 1 with a height of 0.6, IL_10 (2) and activin form cluster 2 with a height of 0.8, the two together form cluster 3 with a height of 1.0; the distance between cluster 1 and cluster 3 is 1- 0.6 = 0.4. The smaller the distance between two markers or two clusters, the greater the positive correlation between them. In this manner the correlation of all clusters can be determined. The dendogram is split into 10 main clusters for analysis purposes, based on cluster patterns considered to be most interesting.

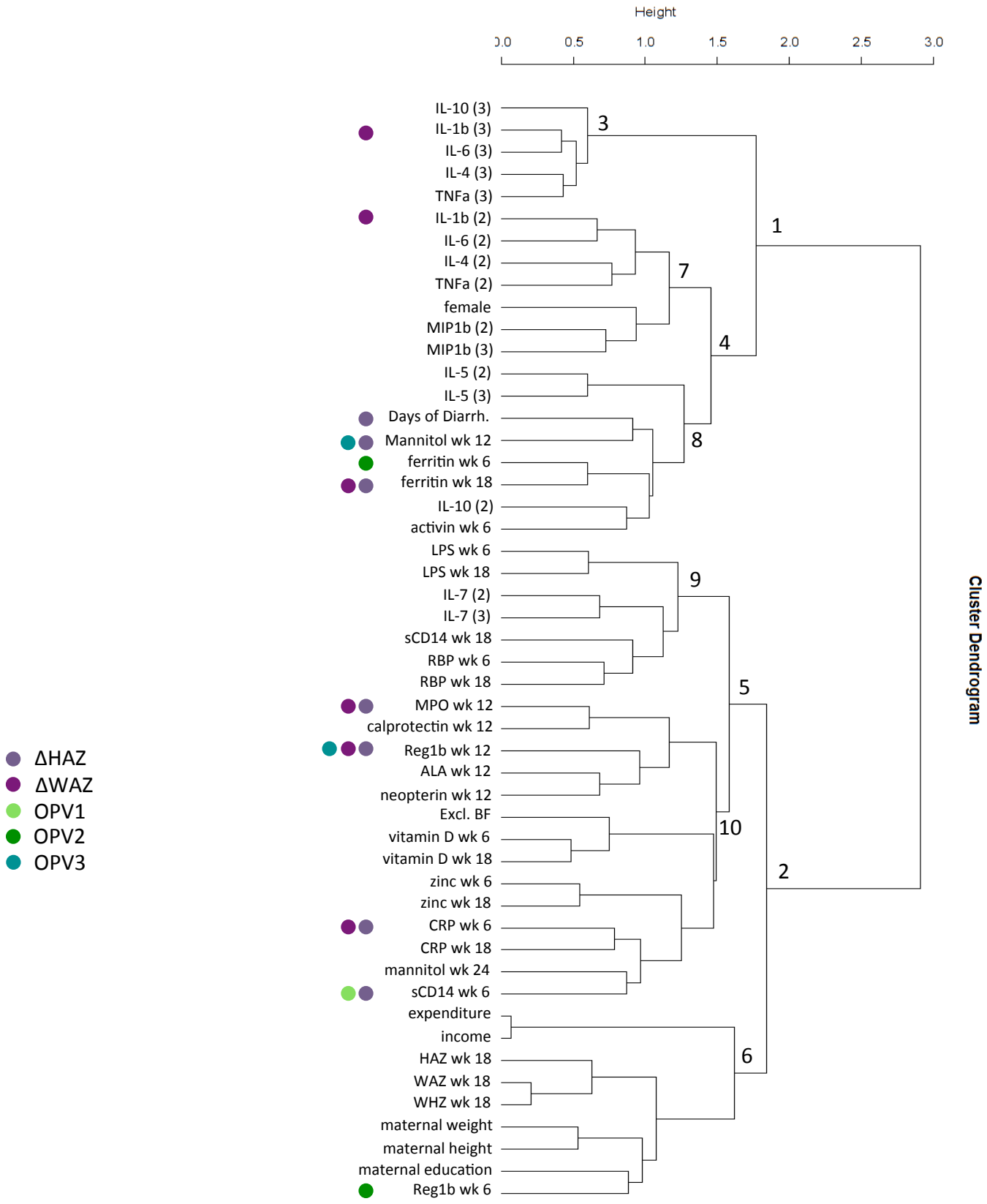
Cluster 1 was dominated by systemic inflammation biomarkers, particularly the cytokines. The cytokines clustered mainly in clusters 3 and 7; cluster 3 contained the 75^{th}-100^{th} percentiles while cluster 7 contained the 50^{th}-75^{th} percentile. These clusters show that the cytokines were usually all elevated at the same time. Days of diarrhea and mannitol levels were also on this arm.

Cluster two had three distinct sub-clusters: clusters 9 and 10 branch from a common cluster, 5, while cluster 6 branches directly out from cluster 2. Interestingly, Reg1b at 6 weeks was in this cluster, as opposed to cluster 10 where Reg1b at 12 weeks was located, indicating that Reg1b at 6 weeks was much more dependent on maternal

health than on enteric health or nutritional status, where Reg1b at 12 weeks clustered. The majority of the enteric inflammatory biomarkers grouped on cluster 10 are very close to one another, followed by a smaller cluster of micronutrients and systemic inflammatory biomarkers. It is interesting that the micronutrients are more closely correlated with both systemic and enteric inflammation rather than with SES factors, and could suggest a reason for the odd negative association of zinc with growth. Overall, cluster 2 is defined by the presence of enteric inflammatory markers and nutritional markers.

The colored circles on Figure 2 indicate inflammatory biomarkers that negatively associated with any of the outcomes as selected by SCAD analysis. Markers selected for ΔHAZ and ΔWAZ were on both main arms (arm 1 and arm 2), of the cluster tree. The two groups of markers were also closely correlated within the main arms. Days of diarrhea and ferritin had a distance of .1 within arm 8, while Reg1b and MPO had a distance of 1.2 within arm 10. IL-1b was slightly removed from the other markers on arm 1: IL-1b (2) and days of diarrhea had a distance of 1.45. The clustering patterns of the separate OPV antibody types were less apparent. The lack of overlap and clustering could indicate that different inflammatory processes had differing effects depending on OPV Sabin type.

Figure 4.5 Cluster analysis of biomarkers. Correlations of all markers are depicted in this dendogram. Height refers to the change in the sum of squared correlations between biomarkers, as a measure of distance. The larger the distance between clusters or markers, the smaller the correlation. Circles indicate the markers selected by SCAD analysis that have a negative effect on growth or OPV antibody titer.


Height
0.0
0.5
1.0
1.5
2.0
2.5
3.0
IL-10 (3)
IL-1b (3)
IL-6 (3)
IL-4 (3)
TNFa (3)
IL-1b (2)
IL-6 (2)
IL-4 (2)
TNFa (2)
female
MIP1b (2)
MIP1b (3)
IL-5 (2)
IL-5 (3)
Days of Diarrh.
Mannitol wk 12
ferritin wk 6
ferritin wk 18
IL-10 (2)
activin wk 6
LPS wk 6
LPS wk 18
IL-7 (2)
IL-7 (3)
sCD14 wk 18
RBP wk 6
RBP wk 18
MPO wk 12
calprotectin wk 12
Reg1b wk 12
ALA wk 12
neopterin wk 12
Excl. BF
vitamin D wk 6
vitamin D wk 18
zinc wk 6
zinc wk 18
CRP wk 6
CRP wk 18
mannitol wk 24
sCD14 wk 6
expenditure
income
HAZ wk 18
WAZ wk 18
WHZ wk 18
maternal weight
maternal height
maternal education
Reg1b wk 6
3
1
7
4
8
9
5
10
2
6
Cluster Dendrogram
ΔHAZ
ΔWAZ
OPV1
OPV2
OPV3

4.4 Discussion

This study investigated the link between malnutrition and OPV failure, and of the effect of inflammation on these outcomes, in a cohort of infants in Bangladesh. We have taken the approach of analyzing correlations between markers of inflammation, nutrition and socioeconomic status and the response to OPV and growth from birth to one year of age. The goal of this study was to understand what impacts growth and OPV response. We found that both enteric and systemic inflammation was correlated with reduced growth and OPV antibody response, while maternal health was positively correlated. We also found that diarrheal disease did not correlate with markers of enteric inflammation, an unexpected finding with implications for the pathogenesis of malnutrition, environmental enteropathy, and how we think about the interaction of diarrheal disease with nutrition.

Nutritional association with inflammation and maternal health

Our method of determining nutritional status was the change in HAZ and WAZ scores from birth to 1 year of age, thereby measuring the growth achieved in one year. In this way we sought to distinguish those children who are born with a healthy HAZ or WAZ (90% and 78% respectively) and become malnourished from those who maintain nutritional health, as well as those who are born malnourished and remain malnourished. Of note was the consistent positive correlation of maternal health and education with growth. A study by Mondal *et al* found the same association with a low HAZ and WAZ score at 12 months as well as at birth in a similar population in Dhaka[13]. Correlation of maternal stunting with newborn stunting has been found to occur across

the globe[8]. Maternal factors therefore play an extremely important role in the health of child, and highlight the importance of improving the education and health of women. These results also drive home the fact that malnutrition and the efforts to treat it do not just affect a particular child at one point in time, but will continue to affect further generations.

The biomarkers that negatively correlated with growth were of particular interest as they not only propose potential reasons for why some children become malnourished, but also provide potential markers of risk for use in stratifying children who are most in danger of becoming malnourished. Cluster analysis split the biomarkers into two main clusters (Figure 4.5), with SCAD-selected inflammatory biomarkers present in both clusters. Cluster group 1 included days of diarrhea, IL-1b and ferritin at 18 weeks. IL-1β is a pro-inflammatory cytokine produced primarily by blood monocytes, tissue macrophages and dendritic cells; roles include promoting cell infiltration, myeloid cell differentiation and further induction of inflammatory factors, including ferritin[101,102]. Ferritin is an acute phase protein as well as an iron store indicator that commonly rises during infections. The children in this study had 3-4 diarrheal episodes in the first year of life. Reaction to infection is likely responsible for the observed rise in IL-1b, which can in turn increase ferritin secretion[102]. Surprisingly, increased mannitol was also selected as a negative marker of growth, and clusters closely with days of diarrhea. This was unexpected as increased mannitol should be indicative of increased absorptive capacity of the gut[18], which is not usually associated with diarrheal disease or with poor nutrition.

Cluster 2 included the selected markers MPO and Reg1b at 12 weeks, and CRP and sCD14 at 6 weeks (Figure 4.5). MPO is a neutrophil enzyme indicative of inflammation, while Reg1b is indicative of epithelial damage as it is a regeneration and growth factor; their correlation and selection for negative growth is logical as inflammation and epithelial damage go hand-in-hand and have been observed in malnourished children[86,87,103,104]. CRP and sCD14 are acute phase proteins triggered by inflammation. It is interesting that MPO and Reg1b did not cluster with days of diarrhea; this could be because they are signaling the presence of environmental enteropathy or otherwise asymptomatic infections rather than overt diarrhea.

Enteric inflammation is independent of overt diarrheal disease: Two inflammatory pathways to malnutrition

The clustering of the biomarkers revealed a surprising outcome: that increased days of diarrhea were not correlated with measures of enteric inflammation. Diarrheal incidence was clustered instead with systemic cytokines. Enteric inflammatory markers including innate cell activation and cellular damage clustered together on a separate arm from days of diarrhea. This suggests that chronic enteric enteropathy is not only caused by diarrheal disease, but by subclinical inflammation.

We hypothesized that the PROVIDE children suffer from environmental enteropathy (EE), which would be reflected by high levels of enteric inflammatory markers. EE is most commonly characterized by blunted villi, an increased lactulose:mannitol ratio, and infiltrating immune cells in the lamina propria and intraepithelially. It has been found to predominantly affect children in settings of high

endemic infection and poor nutrition[18,87,103,104]. The elevated amounts of inflammation observed in the cohort (Figure 4.2) and the incidence of enteropathogen presence in stool samples from asymptomatic infection (Table 4.1) support the presence of subclinical infection inflammation. The majority of children had inflammation (Figure 4.2), which is not unusual for children in developing countries[103]. A study by Kosek *et al* found a similar result: utilizing stool samples from 8 different global sites, they measured alpha-1 anti-trypsin, neopterin, and MPO to develop an EE score to predict a loss in growth. While many children had elevated levels of these proteins, they found that increasing scores would predicted increasing loss of growth[105]. Our data supports these findings, as well as extending them by observing the selection of 2 of these markers as independently important for growth. The findings by Kosek *et al* combined with our own results emphasize the importance of enteric health for childhood nutrition.

The presence of biomarkers associated with malnutrition on both main clusters points to dual pathways leading to malnutrition that are independent of one another. This is a striking finding, as it was hypothesized that diarrheal incidence would be intimately connected with increasing enteric inflammation. Instead, diarrheal incidence is correlated with systemic inflammation, while enteric inflammation occupies a cluster by itself. Both diarrhea and EE have been theorized to lead to malnutrition[26,87,106]. Our data supports both hypotheses, but suggests that rather than directly informing one another, EE and diarrheal disease contribute to independent pathogeneses that both contribute to malnutrition.

OPV antibody response is affected to a lesser extent by inflammation

There were fewer biomarkers that correlated with OPV response compared to growth, and surprisingly there was little overlap between the Sabin strains in regards to specific biomarkers. There was however a common theme between all three Sabin strains in that intestinal disruption and acute phase protein response had a negative effect on antibody production, whereas maternal education and health had a positive effect. While the markers correlating with OPV antibody response did not overlap individually by strain a great deal, it was noteworthy that every negatively associated marker selected for the strains was also selected for ΔHAZ or ΔWAZ, although occasionally at different weeks. This suggests that there is a common pathology leading to both impaired growth and OPV antibody response. In addition, the nutritional status of the child at 18 weeks was itself important, possibly due to the immunosuppression attendant to malnutrition[16,107]. The markers that were not mirrored in growth tended to overlap with those selected for tetanus, and included indicators of controlled immune response and T cell maturation. Tetanus, as an intramuscular vaccine, is unlikely to be affected by enteric inflammation; markers that correlated for both OPV and tetanus likely indicated the importance of a functioning immune response in order for antibody to be produced. The markers of enteric inflammation that were specific to reduced OPV antibody response implied that inflammation at the time of vaccination was detrimental.

Chapter 5:

Summary and Future Work

4.1 Summary

This dissertation has sought to further the understanding of the interaction between nutritional status and the immune system. By looking at both the causes of malnutrition, and the resulting effects, we have gained knowledge about how the immune system is shaped by disease and malnutrition. Leptin, a nutritional hormone, is a significant contributor to the regulation of the immune system. In a malnourished child that regulation is compromised. Enteric disease is a burden exacerbated by malnutrition; this research has found that altered leptin signaling due to a leptin receptor mutation results in impaired neutrophil chemotaxis towards leptin. This in turn likely results in fewer neutrophils trafficking to the site of an *E. histolytica* infection, resulting in reduced parasite clearance. Immune inflammation is hypothesized to be a potential aggravator of malnutrition as well . A panel of markers for systemic inflammation, enteric inflammation, micronutrient status and socioeconomic status were analyzed against growth and OPV response over a year in a cohort of infants in Bangladesh. We found that all inflammation correlated with malnutrition, and that poor OPV response was also correlated with inflammation. Of importance was the finding that malnutrition was independently correlated with markers of enteric inflammation and systemic inflammation, suggesting dual pathways of inflammation triggered by subclinical or diarrheal infection, respectively. The research performed not only extends current knowledge, but also opens up new avenues of research for the future.

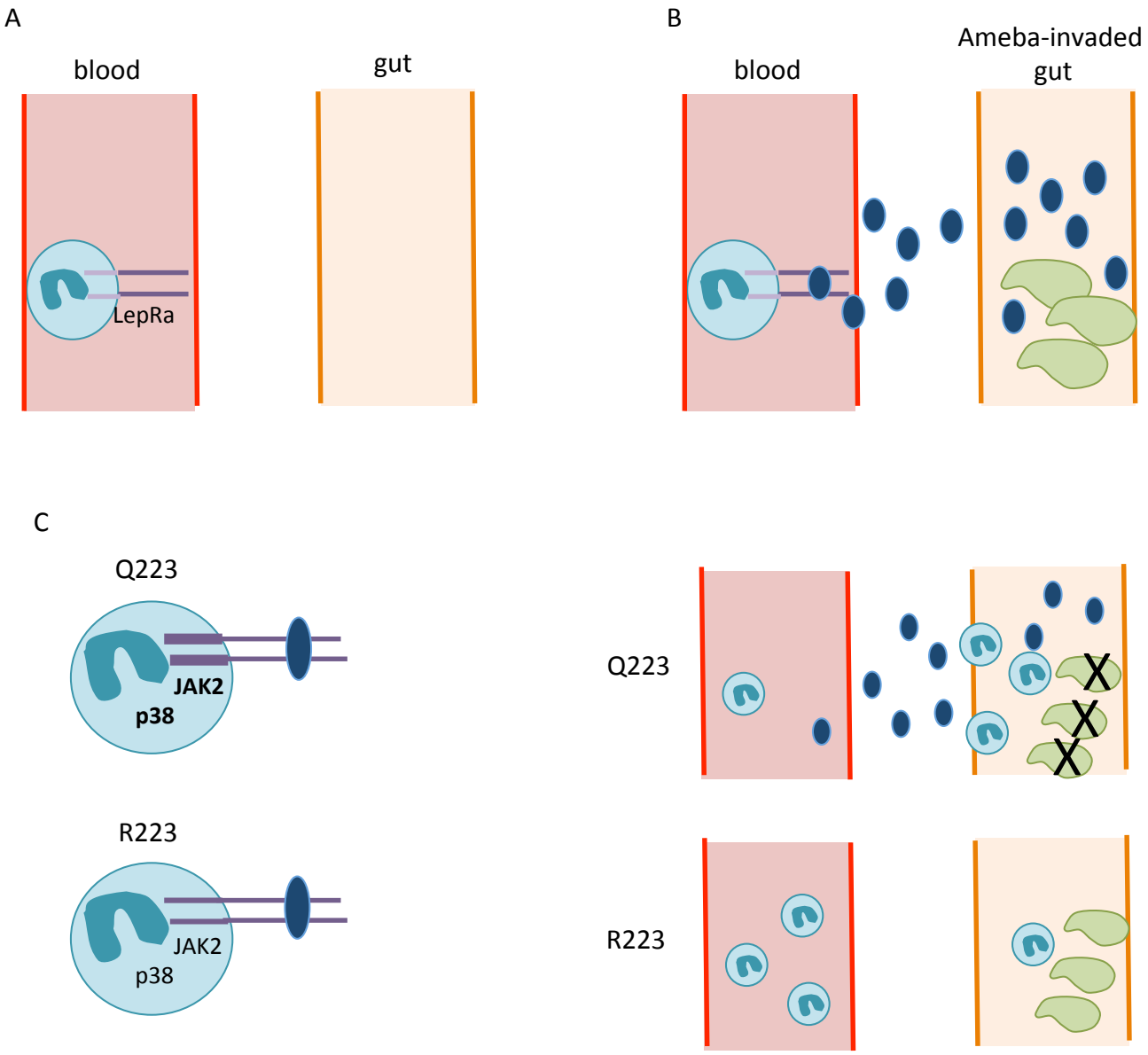
4.1.1 The R223 mutation results in reduced neutrophil chemotaxis to the site of an *E. histolytica* infection

E. histolytica infection is worsened by malnutrition[64], which is itself associated with reduced circulating leptin. Leptin signaling has significant effects on proliferation, apoptosis and cellular function within the immune system, suggesting that susceptibility observed could be due to reduced leptin. A candidate gene analysis found that likelihood of infection was increased fourfold by a glutamine to arginine single nucleotide polymorphism in the leptin receptor[63]. This effect was recapitulated in the mouse model: 100% of Q223 mice were able to clear *E. histolytica* infection by 72 hours, while 40% of R223 mice remained infected. Divergent infection rates were observed by 12 hours after infection[70]. We analyzed the immune response to an *E. histolytica* challenge 12 hours after infection to test if there any differences that could explain the increased susceptibility associated with the R223 genotype.

Neutrophil influx to the site of infection is a characteristic of the host immune response an *E. histolytica* infection[67,69]; R223 mice have a reduced neutrophil population in the cecal lamina propria after 12 hours of infection compared to Q223 mice. We found that the presence of neutrophils in the cecum after challenge is important for rapid clearance, thus this reduction provides a testable rationale for increased duration of infection in the R223 mice. Production of common neutrophil chemotactic factors was equivalent between the two genotypes, with high levels of most at both 6 and 12 hours after infection. Leptin was also produced in the cecum, with a significant increase at 12 hours after infection in both genotypes. R223 neutrophils were found to have reduced chemotaxis towards leptin compared to Q223 neutrophils.

These results suggest that the susceptibility to *E. histolytica* infection conferred by the mutation is at least in part due to impaired neutrophil chemotaxis to the site of infection. Figure 5.1 posits a possible model for the effect of the R223 mutation on neutrophil response to infection. Determining why this mutation would result in reduced chemotaxis of neutrophils is the next step. JAK2 signaling leading to p38 activation has been shown to lead to chemotaxis towards leptin in neutrophils[60]; examining the activation of these proteins after leptin signaling in an R223 neutrophil would indicate if this pathway is being altered.

Figure 5.1. Postulated model of R223 neutrophil-mediated susceptibility to *E. histolytica*. Neutrophils expressing the short-form leptin receptor circulate in the bloodstream (A). When ameba invade the gut, leptin (blue ovals) is produced by epithelial cells to act as a chemoattractant for neutrophils (B). Leptin binding activates JAK2 and $p38^{60}$ downstream to initiate chemotaxis towards the leptin gradient. R223 neutrophils could have reduced signal transduction after leptin binding, posited here as reduced JAK2 and p38 activation (C). Neutrophils chemotax to the site of infection, and kill the invading ameba. In an R223 mouse, fewer neutrophils chemotax due to reduced leptin signaling, and ameba are not cleared (D).


A
blood
gut
LepRa
B
blood
Ameba-invaded
gut
C
Q223
JAK2
p38
R223
JAK2
p38
Q223
R223

These results have revealed a novel effect of the mutation on neutrophil function and disease susceptibility, and of the role of leptin during an *E. histolytica* infection. Our results agree with a previous study in 2014 that found that leptin was important for airway neutrophil infiltration after bacterial pneumonia[54]. This study found that leptin was up-regulated in the lungs after LPS challenge, and that treatment with leptin in a murine model would result in an increased airway neutrophil population, ultimately aiding in bacterial clearance. Neutrophil chemotaxis appears to be an important effect of leptin for a range of infections.

Previous data from our lab found that R223 epithelial cells exhibited increased caspase-3 activation 3 days after infection, and that leptin receptor expression on hematopoietic cells could not rescue the mortality observed in db/db mice after *E. histolytica* infection[38]. The results seen here do not contradict previous findings. The epithelial layer undoubtedly plays an important role for host resistance to infection; *E. histolytica* causes tissue destruction, and invasion of the lamina propria results in the more severe disease forms[65]. The R223 mutation is likely to have effects on susceptibility to *E. histolytica* infection beyond simply increasing apoptosis of intestinal epithelial cells. The neutrophil data presented here is another link in the chain of host reactions necessary for resistance to infection. The failure of leptin receptor-expressing hematopoietic cells in a Db/Db mouse to rescue resistance to infection is likely due to importance of leptin in many cell types. As discussed in the introduction, leptin has a wide variety of roles in many cell types. Presence on the intestinal epithelium appears to be particularly important: knockout of leptin receptor in these cells resulted in increased susceptibility to infection[38]. The intestinal lining of Db/Db

cells is likely to be very fragile due to the lack of leptin receptor, an important anti-apoptotic factor, so a robust immune response would cause a great deal of damage.

Our results indicate that leptin production and signaling are important immune indicators, and work to recruit neutrophils to the site of infection. Reduction in this recruitment due to the R223 mutation is associated with reduced pathogen clearance, reinforcing that neutrophil influx is an integral part of host resistance.

4.1.2 Reduced signaling in the R223 leptin receptor is not due to impaired leptin binding or surface expression

Leptin receptor signaling occurs when leptin binds to the dimeric receptor, causing a conformational change and a signaling cascade through several signaling pathways. The Q223R leptin receptor mutation is located in one of the extracellular domains; the functional impact was unclear. Previous work in a transfected HEK model has shown that a STAT3 reporter exhibits reduced activation in cells transfected with the R223 leptin receptor. After stimulation with leptin at varying concentrations, the R223 leptin receptor appears to have reduced signaling capabilities compared to the wild-type Q223 leptin receptor. We tested two of three possible explanations for reduced signaling: impaired ligand binding, reduced surface expression, and inhibited conformational change.

Leptin binding to the receptor was analyzed utilizing BiaCore surface plasmon resonance. This analysis revealed that there was no difference in affinity between the two receptor types.

Surface expression was analyzed using a monoclonal antibody to murine leptin receptor. Flow cytometric analysis of murine splenocytes found that B220+ cells had the highest expression, and that there was no difference in leptin receptor MFI between R223 and Q223 cells.

These results indicate that the R223 mutation does not impair ligand binding or surface expression. This suggests that the most likely reason for the reduced STAT3 activation previously seen lies in the conformational change that occurs after leptin binding. The CHR1 domain is not required for successful leptin receptor signaling[108,109], yet we have demonstrated that a mutation in this region has clear functional effects. It is possible the arginine substitution causes some mild steric hindrance of the conformational change that reduces but does not obliterate the signaling cascades emanating from leptin binding to the receptor. It is also possible that the mutation affects dimerization of the receptor. Previous BiaCore studies report that the receptor exists as a dimer[82]. Recent analysis has also shown that a further quaternary structure results from leptin binding secondarily to the Ig domain of a second leptin receptor dimer [84]. The presence of the mutation in the CHR1 domain makes it unlikely that it would have an effect on dimerization or tetramerization. Fluorescent microscopic analysis of oligomerization or conformational change after leptin binding could be employed to further investigate the functional consequences of the mutation.

4.1.3 Malnutrition is correlated with two pathways of inflammation

Malnutrition affects 20% of children, and results in increased infectious disease mortality and morbidity, reduced IQ and reduced vaccine efficacy. Environmental

enteropathy (EE), subclinical chronic inflammation of the intestinal tract, is hypothesized to be a driver of malnutrition. The pathogenesis of EE is unclear; it could be the result of repeated diarrheal episodes or subclinical infections resulting in elevated immune inflammation. We hypothesized that malnutrition as measured by change in HAZ and WAZ over 1 year would negatively correlate with increased diarrhea and enteric infection, as would reduced oral polio vaccine (OPV) antibody production.

Univariate linear correlation analysis revealed that malnutrition was positively associated with increased maternal education and health and greater socioeconomic status. OPV antibody production was also positively correlated with these markers. SCAD selected markers of maternal health for malnutrition included maternal education, height and weight. These data reveal the cyclical nature of malnutrition; women who were malnourished were more likely to have malnourished children, as were women who raised their children in poverty with a lack of education. This effect has been discussed by Black *et al*; children born to short mothers are significantly more likely to have a low birthweight, die before five years of age, and be stunted compared to children born to tall mothers. This result was after adjustment for wealth, education and urban or rural residence[3]. Efforts to improve the health and education of women will not just benefit one generation, but will have lasting contributions to the health of future generations as well.

Inflammatory markers correlated with reduced growth. Univariate analysis illustrated the negative effect both enteric and systemic inflammation had on growth. The SCAD-selected subset of markers included markers from both inflammatory groups as well, indicating that these were independent drivers of malnutrition. OPV was

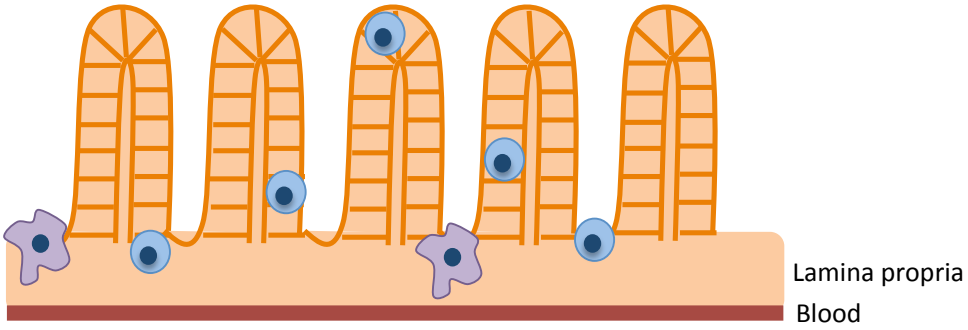
interesting in that inflammation had a reduced role in antibody production, especially when compared to the striking correlations with malnutrition. Few inflammatory biomarkers were identified as significant with univariate analysis, a trend carried on with SCAD analysis. However, of the inflammatory markers selected by SCAD for all OPV Sabin types, 4 out of 7 were also selected for malnutrition, implying that at least part of OPV response could be tied to the same pathogenesis that drives malnutrition.

One of the most fascinating outcomes of this study was the finding that enteric inflammation and diarrheal incidence did not correlate. Cluster analysis was used to depict correlational distance between markers. The biomarkers were split between 2 main clusters. Systemic cytokines were grouped closely together on the first arm, as expected, while enteric inflammation markers MPO, alpha-1 anti-trypsin, Reg1b, calprotectin and neopterin were grouped on the second arm. Interestingly, diarrheal incidence was located on the first arm with the systemic inflammatory markers. A high rate of enteropathogen presence in surveillance stools taken at 6 and 10 weeks could suggest a mechanism for this observation: otherwise asymptomatic infections, recurrent and possibly chronic, result in elevated enteric inflammation, ultimately driving malnutrition. Diarrheal disease, while likely to also induce enteric inflammation as well, appeared to primarily have the effect of elevating systemic inflammation. Our growth outcomes had SCAD-selected independent markers included in both arms. Considering these data along with the univariate and multivariable analyses, this could imply that there are two separate pathways of inflammation that result in malnutrition. The biomarkers selected by univariate analyses represent possible pathway members, and SCAD-selected biomarkers indicate which markers are independent of each other.

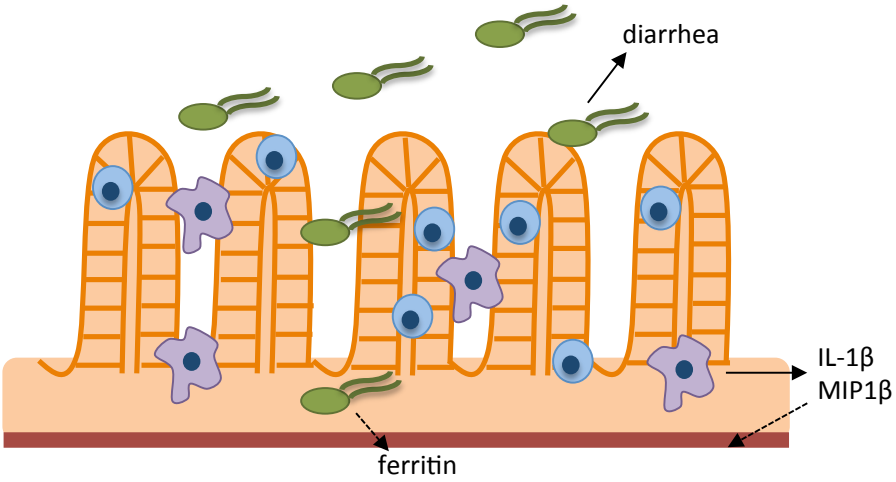
Cluster analysis reveals the relationships between the markers, revealing that days of diarrhea and MPO, two markers selected by SCAD, are not on the same arms, suggesting that they each represent a different pathway. The correlations revealed by univariate linear analysis, meanwhile, fill in pieces of the proposed pathways. Figure 5.2 depicts the two hypothesized inflammatory pathways based on the three analyses.

Figure 5.2. Potential pathways of inflammation leading to malnutrition. A) Represents a healthy intestinal tract: defined villi with healthy epithelial cells, small number of intraepithelial immune cells. B) Theoretical pathway 1: diarrheal disease leads to systemic inflammation. Infection with diarrhea-causing pathogen results in acute immune response in gut. Macrophage production of IL-1b and MIP1b leads to systemic inflammation; inflammation induces ferritin release for iron sequestration. In addition to iron sequestration, increased diarrheal episodes result in frequently activated immune system, potentially usurping energy that could be used for growth. C) Theoretical pathway 2: subclinical gut infection results in chronic enteric inflammation, resulting in damaged epithelial cells, villous blunting, and systemic CRP production. Epithelial death is potentially a result of both pathogen and neutrophil enzymatic cytotoxicity.

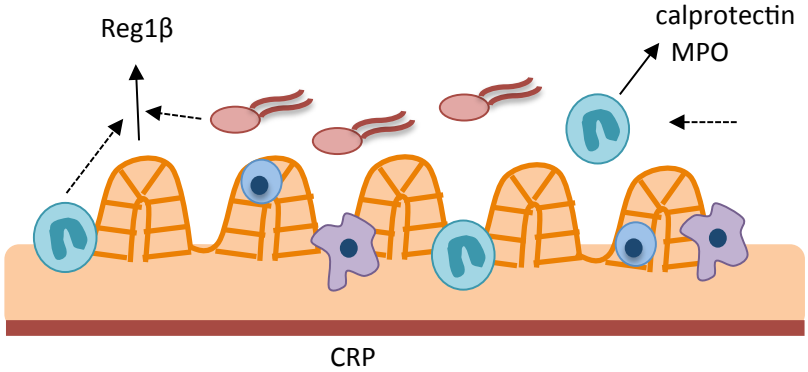
A Healthy Gut



B Systemic Inflammatory pathway



C Enteric Inflammatory pathway



4.2 Future work

Leptin signaling in human populations

Malnutrition-mediated immune dysfunction is an area of active interest, and is shifting from an idea of random failure of function towards one of hormonal-regulation for adaptation to a stressful environmental situation. Leptin is known to be an important regulator of immune function and could be a significant contributor to this regulation. While some strides have been made investigating leptin signaling in human populations, further research in this area is needed, particularly for neutrophils and the innate system.

We have shown that a single mutation within the receptor can alter the neutrophil response enough such that susceptibility to disease is enhanced. While this mutation is also affecting other aspects of the host response[38,70], the impact on neutrophils is significant. Leptin clearly has a role to play in a broad array of immune responses, and neutrophils are included in this. However, the extent to which this is true under malnutrition conditions is unknown. Future work could include *in vitro* rescue of neutrophil function. Working in a cohort of malnourished children, a hypoleptinemic environment, neutrophil function would be evaluated before and after incubation with leptin to test if leptin can improve the function of these cells. Quantitative flow cytometry methods for the measurement of oxidative burst, phagocytosis and cell surface activation markers would greatly add to the store of knowledge about neutrophil impairment during malnutrition as well as inform about the impact of leptin signaling.

Further pursuing the Q223R leptin receptor mutation is an additional method to dissect the function of leptin signaling in neutrophils. A strength of this mutation is that it is common, present in 50-90% of populations[63]. The mutation appears to reduce leptin binding signals, creating a mild mimic of what happens during malnutrition-induced circulating leptin decrease. Our research into Q223R did not investigate the full range of neutrophil function; we found that while chemotaxis towards leptin was diminished in R223 neutrophils, chemotaxis towards two unrelated chemokines was undiminished. It would be important to test if the mutation results in any impairment of oxidative burst or phagocytosis. The effect of the mutation on other diseases is a promising avenue of research. Investigating the effect of the mutation on severity or susceptibility to a variety of enteropathogens could assess the specificity of the effect of the mutation. Viral and bacterial infections require different immune responses; the outcome of the infection in a Q223 or R223 population is influenced by the nature of the pathogen. The ability of the mutation to protect against some infections is also a fascinating avenue of study. We are currently pursing this avenue of study via collaboration with Dr. Tom Braciale and Amber Cardani investigating influenza infections in Q223 and R223 mice, and have observed that R223 mice are protected from influenza mortality while Q223 mice are more susceptible. We also hope to enter into collaboration with the Sanger Center in Cambridge, the UK, to investigate severity of influenza infections in a cohort of genotyped patients.

Enteric enteropathy in malnourished populations

The most fascinating finding from our work in the PROVIDE population was the observation that markers of enteric inflammation did not correlate with days of diarrhea. This finding is extremely exciting as it suggests that our assumption that environmental enteropathy occurs as a result of diarrheal infections is incorrect, and that instead asymptomatic subclinical infections could be driving it. Additionally, because days of diarrhea and systemic cytokine levels were associated with malnutrition as well as enteric inflammation, there could be two independent pathways to malnutrition.

Replication of these results in an independent cohort is critical. The PROVIDE study has a sister cohort in Calcutta, India. Stool samples have been taken from these children at the same time points as those in Bangladesh. A subset of the biomarkers would be selected with the intent to test 3 hypotheses: 1) days of diarrhea correlates with systemic inflammation and not enteric inflammation, 2) asymptomatic infections correlate with enteric inflammation, 3) reduced growth over one year of age is correlated with, and predicted by, increased enteric inflammation at 12 weeks of age. The subset should include 2-3 enteric inflammatory markers, such as MPO and Reg1b at 12 weeks, systemic inflammatory markers such as IL-1b and ferritin, and days of diarrhea. Enteropathogen detection of surveillance stool at 6 and 10 weeks of age should also be included. Further replication in cohorts on other continents such as Africa and South America would provide valuable data as well as it would be informative to test if our observations in the Bangladesh cohort are part of global phenomena.

Future work could also include investigation into causal organisms. It is possible that a particular enteropathogen or set of enteropathogens is responsible for the bulk of inflammation leading to malnutrition. Work of this nature would provide a possible target for antibiotic or antiviral treatment, as well as open up avenues of research in murine models. Evaluation of nutritional rehabilitation strategies in the context of these markers would also be a clinically relevant avenue of research. It has been observed that nutritional supplementation does not always result in nutritional recovery or recovery of the mucosal architecture associated with EE[85,86]. Enteric inflammatory markers could be used to determine the effectiveness of treatment, likelihood of failure and likelihood of remission (return to poor nutrition).

4.3 Impact

The effect of leptin on the innate immune system is of only recent interest. Work previous to this dissertation had demonstrated that signaling by the short form leptin receptor expressed by neutrophils had effects on oxidative burst and surface expression of activation markers. Leptin was also shown to be a chemoattractant for neutrophils. *In vivo* work had found that leptin receptor knockout mice had reduced neutrophil response to respiratory infection, and that nutritionally obese mice had increased numbers of neutrophils after peritonitis compared to lean mice. Leptin therefore appeared to have an impact on neutrophil response, but how was not clear. The Q223R leptin receptor mutation was found to increase susceptibility to *E. histolytica*, but again the importance of leptin signaling during this infection was imperfectly understood. This dissertation has extended knowledge concerning leptin and enteric infections. For the first time leptin was shown to be elevated in the cecum

after an *E. histolytica* infection, which coincided with a reduced cecal neutrophil population in R223 mice compared to Q223 mice. It was also shown that neutrophils expressing the R223 leptin receptor did not chemotax towards leptin to the same extent as Q223 neutrophils, a previously unknown effect of the mutation. This work provides a link between observed reductions in neutrophil populations after infections and the chemotactic properties of leptin.

Malnutrition is a condition requiring action, but that action can only be successful if the causes and effects of malnutrition are thoroughly understood. The cause of malnutrition has been traditionally ascribed to an inadequate supply of calories, protein and nutrients. This undoubtedly is a root cause, but it is not the only one; nutritional supplementation can result in nutritional gain but it often fails, and children can relapse. As a result of malnutrition children experience an increase in disease susceptibility, and, as was observed recently by our lab, a decrease in OPV antibody response. This dissertation has added to our understanding of both the cause and effects of malnutrition. The broad panel of biomarkers, socioeconomic factors, and rigorous anthropometry measurements has allowed a study of unprecedented depth to take place. We found that enteric and systemic inflammation negatively correlate with a child's growth over one year; markers such Reg1b and ferritin were selected for both ΔHAZ and ΔWAZ, making them potential risk-stratification markers. Of particular note was the finding that there appeared to be two independent pathways of inflammation, a systemic pathway that included diarrheal burden, and an enteric pathway that focused on gut inflammation and epithelial damage. This had not been observed or hypothesized before, and has powerful implications for future research. In addition, we

found that OPV antibody response shared a limited similarity to malnutrition: systemic and enteric inflammation was negatively correlated with antibody response, but not to the same extent as malnutrition. This unexpected outcome is informative as it suggests that while there is a common pathogenesis contributing to both malnutrition and OPV antibody production, vaccine response is complex and is impacted by other untested factors.

Overall this dissertation has contributed to the store of knowledge concerning nutrition and the immune system. Cause and effect has been explored. Leptin signaling is inhibited in malnourished children due to reduced circulating levels. Using the genetic leptin receptor mutation Q223R, which is hypothesized to diminish signaling of the receptor and results in increased susceptibility to *E. histolytica*, this work has identified an important for leptin in neutrophil chemotaxis during an enteric infection. This dissertation has also found that increased inflammation, systemic and enteric, both correlate with malnutrition. Reduced OPV antibody production, an effect of malnutrition, is also impacted by this inflammation. Understanding both why malnutrition occurs and endures, and how it subsequently shapes a child's immune system, will provide avenues of treatment and identification of at-risk children, bringing us closer to providing a healthy life for all.

References

1. The World Health Organisation & the United Nations Children's Fund. *WHO child growth standards and the identification of severe acute malnutrition in infants and children*. 11 (2009).

2. UNICEF, World Health Organisation & World Bank. Joint Child Malnutrition Estimates. *World Bank* at <http://data.worldbank.org/child-malnutrition>

3. Black, R. E. *et al.* Maternal and child undernutrition and overweight in low-income and middle-income countries. *Lancet* **382,** 427–51 (2013).

4. Schaible, U. E. & Kaufmann, S. H. E. Malnutrition and infection: complex mechanisms and global impacts. *PLoS Med.* **4,** e115 (2007).

5. The World Bank. *Repositioning Nutrition as Central to Development*. (The World Bank, 2006). doi:10.1596/978-0-8213-6399-7

6. Jr, W. A. P. *et al.* Enteric infections, diarrhea, and their impact on function and development. *J. Clin. Invest.* **118,** 1277 – 1290 (2008).

7. The World Health Organisation. Children: Reducing Mortality. *World Heal. Organ.* at <http://www.who.int/mediacentre/factsheets/fs178/en/>

8. Black, R. E. *et al.* Maternal and child undernutrition: global and regional exposures and health consequences. *Lancet* **371,** 243–60 (2008).

9. Olofin, I. *et al.* Associations of suboptimal growth with all-cause and cause-specific mortality in children under five years: a pooled analysis of ten prospective studies. *PLoS One* **8,** e64636 (2013).

10. Caulfield, L. E., de Onis, M., Blössner, M. & Black, R. E. Undernutrition as an underlying cause of child deaths associated with diarrhea, pneumonia, malaria, and measles. *Am. J. Clin. Nutr.* **80,** 193–8 (2004).

11. Man, W. D.-C. *et al.* Nutritional status of children admitted to hospital with different diseases and its relationship to outcome in The Gambia, West Africa. *Trop. Med. Int. Heal.* **3,** 678–686 (1998).

12. Mondal, D. *et al.* Contribution of enteric infection, altered intestinal barrier function, and maternal malnutrition to infant malnutrition in Bangladesh. *Clin. Infect. Dis.* **54,** 185–92 (2012).

13. Mondal, D., Haque, R., Sack, R. B., Kirkpatrick, B. D. & Jr, W. A. P. Short Report : Attribution of Malnutrition to Cause-Specific Diarrheal Illness : Evidence from a Prospective Study of Preschool Children in Mirpur , Dhaka , Bangladesh. *Am. J. Trop. Hyg. Med.* **80,** 824–826 (2009).

14. Mcmurray, N., Ph, D. & Reyes, A. Effect of renutrition mediated immunity on humoral in severely It malnourished. *Am. J. Clin. Nutr.* **34,** 2117–2126 (1981).

15. Vasquez-Garibay, E. *et al.* Effect of Renutrition on Natural and Cell-Mediated Immune Response in Infants With Severe Malnutrition. *J. Pediatr. Gastroenterol. Nutr.* **34,** 296–301 (2002).

16. Palacio, A., Lopez, M., Perez-Bravo, F., Monkeberg, F. & Schlesinger, L. Leptin levels are associated with immune response in malnourished infants. *J. Clin. Endocrinol. Metab.* **87,** 3040–6 (2002).

17. Bartz, S. *et al.* Severe acute malnutrition in childhood: hormonal and metabolic status at presentation, response to treatment, and predictors of mortality. *J. Clin. Endocrinol. Metab.* jc20134018 (2014). doi:10.1210/jc.2013-4018

18. Korpe, P. S. & Petri, W. a. Environmental enteropathy: critical implications of a poorly understood condition. *Trends Mol. Med.* **18,** 328–36 (2012).

19. Faggioni, R., Feingold, K. R. & Grunfeld, C. Leptin regulation of the immune response and the immunodeficiency of malnutrition. *FASEB J.* **15,** 2565–71 (2001).

20. Fantuzzi, G. & Faggioni, R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. *J. Leukoc. Biol.* **68,** 437–46 (2000).

21. La Cava, A. & Matarese, G. The weight of leptin in immunity. *Nat. Rev. Immunol.* **4,** 371–9 (2004).

22. Margetic, S., Gazzola, C., Pegg, G. G. & Hill, R. a. Leptin: a review of its peripheral actions and interactions. *Int. J. Obes. Relat. Metab. Disord.* **26,** 1407–33 (2002).

23. Loffreda, S. *et al.* Leptin regulates proinflammatory immune responses. *FASEB J.* **12,** 57–65 (1998).

24. Matarese, G., Moschos, S. & Christos, S. Leptin in Immunology. *J. Immunol.* **174,** 3137–3142 (2005).

25. Procaccini, C., Jirillo, E. & Matarese, G. Leptin as an immunomodulator. *Mol. Aspects Med.* **33,** 35–45 (2012).

26. Guerrant, R. L., Oriá, R. B., Moore, S. R., Oriá, M. O. B. & Lima, A. a M. Malnutrition as an enteric infectious disease with long-term effects on child development. *Nutr. Rev.* **66,** 487–505 (2008).

27. Sitaraman, S. *et al.* Colonic leptin: source of a novel pro-inflammatory cytokine involved in inflammatory bowel disease. *FASEB J.* (2004).

28. Gil-Campos, M., Aguilera, C. M., Ramirez-Tortosa, M. C., Cañete, R. & Gil, a. Fasting and postprandial relationships among plasma leptin, ghrelin, and insulin in prepubertal obese children. *Clin. Nutr.* **29,** 54–9 (2010).

29. Klok, M. D., Jakobsdottir, S. & Drent, M. L. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obes. Rev.* **8,** 21–34 (2007).

30. Korbonits, M. *et al.* Leptin levels do not change acutely with food administration in normal or obese subjects, but are negatively correlated with pituitary-adrenal activity. *Clin. Endocrinol. (Oxf).* **46,** 751–7 (1997).

31. Mackintosh, R. & Hirsch, J. The effects of leptin administration in non-obese human subjects. *Obes. Res.* **9,** 462–469 (2001).

32. Soliman, A. T., Elzalabany, M. M., Salama, M. & Ansari, B. M. Serum Leptin Concentrations During Severe Protein-Energy Malnutrition: Correlation With Growth Parameters and Endocrine Function. *Metabolism.* **49,** 819–825 (2000).

33. Barrenetxe, J. *et al.* Distribution of the long leptin receptor isoform in brush border, basolateral membrane, and cytoplasm of enterocytes. *Gut* **50,** 797–802 (2002).

34. Yang, R. & Barouch, L. a. Leptin signaling and obesity: cardiovascular consequences. *Circ. Res.* **101,** 545–59 (2007).

35. Zarkesh-Esfahani, H. *et al.* Leptin Indirectly Activates Human Neutrophils via Induction of TNF- . *J. Immunol.* **172,** 1809–1814 (2004).

36. Lammert, a, Kiess, W., Bottner, a, Glasow, a & Kratzsch, J. Soluble leptin receptor represents the main leptin binding activity in human blood. *Biochem. Biophys. Res. Commun.* **283,** 982–8 (2001).

37. Ptak, A., Kolaczkowska, E. & Gregoraszczuk, E. L. Leptin stimulation of cell cycle and inhibition of apoptosis gene and protein expression in OVCAR-3 ovarian cancer cells. *Endocrine* **43,** 394–403 (2013).

38. Guo, X. *et al.* Leptin signaling in intestinal epithelium mediates resistance to enteric infection by Entamoeba histolytica. *Mucosal Immunol.* **4,** 294–303 (2010).

39. Marie, C. S., Verkerke, H. P., Paul, S. N., Mackey, A. J. & Petri, W. a. Leptin protects host cells from Entamoeba histolytica cytotoxicity by a STAT3-dependent mechanism. *Infect. Immun.* **80,** 1934–43 (2012).

40. Wang, D. *et al.* Leptin regulates proliferation and apoptosis of colorectal carcinoma through PI3K/Akt/mTOR signalling pathway. *J. Biosci.* **37,** 91–101 (2011).

41. Lai, Q. *et al.* Leptin signaling maintains B-cell homeostasis via induction of Bcl-2 and Cyclin D1. 1–6 (2010). doi:10.1073/pnas.1004185107/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1004185107

42. Bruno, A., Conus, S., Schmid, I. & Simon, H. Apoptotic Pathwyas are Inhibited by Leptin Receptor Activation in Neutrophils. *J. Immunol.* **174,** 8090–8096 (2005).

43. Fukuda, J. *et al.* Effects of leptin on the production of cytokines by cultured human endometrial stromal and epithelial cells. *Fertil. Steril.* **80,** 783–787 (2003).

44. Ogunwobi, O. O. & Beales, I. L. P. The anti-apoptotic and growth stimulatory actions of leptin in human colon cancer cells involves activation of JNK mitogen activated protein kinase, JAK2 and PI3 kinase/Akt. *Int. J. Colorectal Dis.* **22,** 401–9 (2007).

45. Rodríguez, L., Graniel, J. & Ortiz, R. Effect of leptin on activation and cytokine synthesis in peripheral blood lymphocytes of malnourished infected children. *Clin. Exp. Immunol.* **148,** 478–85 (2007).

46. Huttunen, R. & Syrjänen, J. Obesity and the risk and outcome of infection. *Int. J. Obes. (Lond).* **37,** 333–40 (2013).

47. Faggioni, R. *et al.* Leptin deficiency enhances sensitivity to endotoxin-induced lethality. *Am. J. Physiol.* **276,** R136–42 (1999).

48. Madan, R. *et al.* Role of leptin-mediated colonic inflammation in defense against Clostridium difficile colitis. *Infect. Immun.* **82,** 341–9 (2014).

49. Savino, W. The thymus gland is a target in malnutrition. *Eur. J. Clin. Nutr.* **56,** 46–49 (2002).

50. Moore, S. E. *et al.* Early-life nutritional and environmental determinants of thymic size in infants born in rural Bangladesh. *Acta Paediatr.* **98,** 1168–75 (2009).

51. Farooqi, I. S. *et al.* Beneficial effects of leptin on obesity , T cell hyporesponsiveness , and neuroendocrine / metabolic dysfunction of human congenital leptin deficiency. *J. Clin. Invest.* **110,** 1093–1103 (2002).

52. Lord, G. M. *et al.* Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* **394,** 897–901 (1998).

53. Saucillo, D. C., Gerriets, V. a, Sheng, J., Rathmell, J. C. & Maciver, N. J. Leptin metabolically licenses T cells for activation to link nutrition and immunity. *J. Immunol.* **192,** 136–44 (2014).

54. Ubags, N. D. *et al.* The role of leptin in the development of pulmonary neutrophilia in infection and acute lung injury. *Crit. Care Med.* **42,** e143–51 (2014).

55. Moshyedi, A. K. *et al.* Increased Leptin Expression in Mice with Bacterial Peritonitis is Partially Regulated by Tumor Necrosis Factor Alpha Increased Leptin Expression in Mice with Bacterial Peritonitis is Partially Regulated by Tumor Necrosis Factor Alpha. *Infect. Immun.* **66,** 1800–1802 (1998).

56. Siegl, D. *et al.* Obesity-induced Hyperleptinemia Improves Survival and Immune Response in a Murine Model of Sepsis. *Anesthesiology* 1–17 (2014). doi:10.1097/ALN.0000000000000192

57. Azuma, T. *et al.* Gastric leptin and Helicobacter pylori infection. *Gut* **49,** 324–329 (2001).

58. Caldefie-Chezet, F., Poulin, a, Tridon, a, Sion, B. & Vasson, M. P. Leptin: a potential regulator of polymorphonuclear neutrophil bactericidal action? *J. Leukoc. Biol.* **69,** 414–8 (2001).

59. Caldefie-Chezet, F., Poulin, A. & Vasson, M. Leptin Regulates Functional Capacities of Polymorphonuclear Neutrophils. *Free Radic. Res.* **37,** 809–14 (2003).

60. Montecucco, F. *et al.* Induction of neutrophil chemotaxis by leptin: crucial role for p38 and Src kinases. *Ann. N. Y. Acad. Sci.* **1069,** 463–71 (2006).

61. Kordonowy, L. L. *et al.* Obesity is associated with neutrophil dysfunction and attenuation of murine acute lung injury. *Am. J. Respir. Cell Mol. Biol.* **47,** 120–7 (2012).

62. Peelman, F., Zabeau, L., Moharana, K., Savvides, S. N. & Tavernier, J. 20 Years of Leptin: Insights Into Signaling Assemblies of the Leptin Receptor. *J. Endocrinol.* **223,** T9–23 (2014).

63. Duggal, P. *et al.* A mutation in the leptin receptor is associated with Entamoeba histolytica infection in children. *J. Clin. Invest.* **121,** 1191–1198 (2011).

64. Petri, W. a, Mondal, D., Peterson, K. M., Duggal, P. & Haque, R. Association of malnutrition with amebiasis. *Nutr. Rev.* **67 Suppl 2,** S207–15 (2009).

65. Haque, R., Huston, C. D., Hughes, M., Houpt, E. & Petri, W. A. Amebiasis. *N. Engl. J. Med.* **348,** 1565–1573 (2003).

66. Ralston, K. S., Solga, M. D., Mackey-lawrence, N. M., Bhattacharya, A. & Petri, W. A. Trogocytosis by Entamoeba histolytica contributes to cell killing and tissue invasion. *Nature* **508,** 526–530 (2014).

67. Mortimer, L. & Chadee, K. The immunopathogenesis of Entamoeba histolytica. *Exp. Parasitol.* **126,** 366–80 (2010).

68. Asgharpour, A., Gilchrist, C., Baba, D., Houpt, E. & Hamano, S. Resistance to Intestinal Entamoeba histolytica Infection Is Conferred by Innate Immunity and Gr-1+ Cells. *Infect. Immun.* **73,** 4522–4529 (2005).

69. Guo, X., Houpt, E. & Petri, W. A. Crosstalk at the initial encounter: Interplay between host defense and ameba survival strategies. **19,** 376–384 (2009).

70. Mackey-Lawrence, N. M. *et al.* Effect of the leptin receptor Q223R polymorphism on the host transcriptome following infection with Entamoeba histolytica. *Infect. Immun.* **81,** 1460–70 (2013).

71. Houpt, E. R. *et al.* The mouse model of amebic colitis reveals mouse strain susceptibility to infection and exacerbation of disease by CD4+ T cells. *J. Immunol.* **169,** 4496–503 (2002).

72. Bracho-Riquelme, R. L., Loera-Castañeda, V., Torres-Valenzuela, A., Loera-Castañeda, G. a & Sánchez-Ramírez, J. P. Leptin and leptin receptor polymorphisms are associated with poor outcome (death) in patients with non-appendicular secondary peritonitis. *Crit. Care* **15,** R227 (2011).

73. Wang, B. *et al.* Effect of leptin receptor mutation on the development of chronic bronchitis. *Asia. Pac. J. Public Health* **25,** 80S–7S (2013).

74. Chadee, K. Human Neutrophils Activated by lnterferon-y and Tumour Necrosis Factor-a Kill Entamoeba histolytica Trophozoites In Vitro. **274,** 270–274 (1989).

75. Yang, S. K., Eckmann, L., Panja, a & Kagnoff, M. F. Differential and regulated expression of C-X-C, C-C, and C-chemokines by human colon epithelial cells. *Gastroenterology* **113,** 1214–23 (1997).

76. Song, F. *et al.* Expression of the neutrophil chemokine KC in the colon of mice with enterocolitis and by intestinal epithelial cell lines: effects of flora and proinflammatory cytokines. *J. Immunol.* **162,** 2275–80 (1999).

77. Ohtsuka, Y., Lee, J., Stamm, D. S. & Sanderson, I. R. MIP-2 secreted by epithelial cells increases neutrophil and lymphocyte recruitment in the mouse intestine. *Gut* **49,** 526–33 (2001).

78. Papathanassoglou, E. *et al.* Leptin Receptor Expression and Signaling in Lymphocytes: Kinetics During Lymphocyte Activation, Role in Lymphocyte Survival, and Response to High Fat Diet in Mice. *J. Immunol.* **176,** 7745–7752 (2006).

79. Summers, C. *et al.* Neutrophil kinetics in health and disease. *Trends Immunol.* **31,** 318–24 (2010).

80. Fernández-Riejos, P. *et al.* Role of leptin in the activation of immune cells. *Mediators Inflamm.* **2010,** 568343 (2010).

81. Biener, E. *et al.* Quantitative FRET imaging of leptin receptor oligomerization kinetics in single cells. *Biol. Cell* **97,** 905–19 (2005).

82. Mistrík, P., Moreau, F. & Allen, J. M. BiaCore analysis of leptin-leptin receptor interaction: evidence for 1:1 stoichiometry. *Anal. Biochem.* **327,** 271–7 (2004).

83. Couturier, C. & Jockers, R. Activation of the leptin receptor by a ligand-induced conformational change of constitutive receptor dimers. *J. Biol. Chem.* **278,** 26604–11 (2003).

84. Mancour, L. V *et al.* Ligand-Induced Architecture of the Leptin Receptor Signaling Complex. *Mol. Cell* **48,** 655–661 (2013).

85. Bhutta, Z. a *et al.* What works? Interventions for maternal and child undernutrition and survival. *Lancet* **371,** 417–40 (2008).

86. Sullivan, P. B. *et al.* Persistent Diarrheae and Malnutrition - The Impact of Treatment on Small Bowel Structure and Permeability. *J. Pediatr. Gastroenterol. Nutr.* **14,** 208–215 (1992).

87. Prendergast, A. & Kelly, P. Enteropathies in the developing world: neglected effects on global health. *Am. J. Trop. Med. Hyg.* **86,** 756–63 (2012).

88. Pasetti, M. F., Simon, J. K., Sztein, M. B. & Levine, M. M. Immunology of Gut Mucosal Vaccines. *Immunol. Rev.* **239,** 125–148 (2011).

89. Levine, M. M. developing countries : lessons from a live cholera vaccine. *BMC Biol.* **8,** 2–11 (2010).

90. Haque, R. *et al.* Oral polio vaccine response in breast fed infants with malnutrition and diarrhea. *Vaccine* **32,** 478–82 (2014).

91. Liu, J. *et al.* A laboratory-developed TaqMan Array Card for simultaneous detection of 19 enteropathogens. *J. Clin. Microbiol.* **51,** 472–80 (2013).

92. Liu, J. *et al.* Development and assessment of molecular diagnostic tests for 15 enteropathogens causing childhood diarrhoea: a multicentre study. *Lancet. Infect. Dis.* **14,** 716–24 (2014).

93. Fan, J. & Li, R. Variable selection via nonconcave penalized likelihood and its oracle properties. *J. Am. Stat.* **96,** 1348–1360 (2001).

94. Fan, J. & Ly, J. A selective overview of variable selection in high dimensional feature space. *Stat. Sin.* **20,** 101–148 (2010).

95. Huang, J., Breheny, P. & Ma, S. A Selective Review of Group Selection in High-Dimensional Models. *Stat. Sci.* **4,** 481–499 (2012).

96. Tibshirani, R. Regression shrinkage and selection via the LASSO. *J. Roy. Stat. Soc* **58,** 267–288 (1996).

97. Wang, H., Li, R. & Tsai, C.-L. Tuning parameter selectors for the smoothly clipped absolute deviation method. *Biometrika* **94,** 533–568 (2007).

98. Zeng, L. & Xie, J. Group variable selection via SCAD-L2. *Statistics (Ber).* 1–18 (2012).

99. Sharpstone, D., Rowbottom, A., Nelson, M. & Gazzard, B. Faecal ac antitrypsin as a marker of gastrointestinal disease in HIV antibody positive individuals. *Gut* **38,** 206–210 (1996).

100. Sutter, R. W. & Cochi, S. L. in 631–686 (2007).

101. Dinarello, C. a. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* **27,** 519–50 (2009).

102. Wang, W., Knovich, M. A., Coffman, L. G., Torti, F. M. & Torti, S. V. Serum ferritin: Past, present and future. *Biochim. Biophys. Acta* **1800,** 760–9 (2010).

103. Campbell, D. I. *et al.* Chronic T cell-mediated enteropathy in rural west African children: relationship with nutritional status and small bowel function. *Pediatr. Res.* **54,** 306–11 (2003).

104. Keusch, G. T. *et al.* Environmental enteric dysfunction: pathogenesis, diagnosis, and clinical consequences. *Clin. Infect. Dis.* **59 Suppl 4,** S207–12 (2014).

105. Kosek, M. *et al.* Fecal markers of intestinal inflammation and permeability associated with the subsequent acquisition of linear growth deficits in infants. *Am. J. Trop. Med. Hyg.* **88,** 390–6 (2013).

106. Keusch, G. T. Symposium : Nutrition and Infection , Prologue and Progress Since 1968 The History of Nutrition : Malnutrition , Infection and Immunity 1 , 2. 336–340 (2003).

107. Rodríguez, L. *et al.* Assessment by Flow Cytometry of Cytokine Production in Malnourished Children Assessment by Flow Cytometry of Cytokine Production in Malnourished Children. *Clin. Vaccine Immunol.* **12,** 502–507 (2005).

108. Fong, T. M. *et al.* Localization of Leptin Binding Domain in the Leptin Receptor. *Mol. Pharmacol.* **240,** 234–240 (1998).

109. Zabeau, L. *et al.* Functional analysis of leptin receptor activation using a Janus kinase/signal transducer and activator of transcription complementation assay. *Mol. Endocrinol.* **18,** 150–61 (2004).