Old Herborn University Seminar Monograph 30:
The magnificent microbiome - future aspects.
Editors: Peter J. Heidt, Thomas C.G. Bosch, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 61-68 (2017).

REGULATION OF INTESTINAL BARRIER INTEGRITY BY IgA TARGETED GUT MICROBES IN UNDERNUTRITION

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SUMMARY

Undernutrition is a leading cause of childhood mortality worldwide. Environmental enteric dysfunction, characterized by recurrent exposure to environmental microbes and subsequent intestinal barrier dysfunction, is increasingly recognized as a major contributor to undernutrition. Analyses of faecal samples from children with undernutrition have documented compositional and functional alterations in their microbiota. IgA is the principal immunoglobulin produced by the gut mucosa. Using fluorescence-activated cell sorting to characterize bacterial taxa targeted by IgA in the gut communities of Malawian twins discordant for severe acute undernutrition (SAM) and then transferring purified IgA+ fractions to germ-free mice, we find that (i) the patterns of microbes targeted by the IgA response in undernourished children differ from that seen in healthy children with increased targeting of Enterobacteriaceae correlating with poorer growth outcomes; (ii) bacterial taxa with bound IgA recovered from SAM donor microbiota can transmit a diet-dependent enteropathy characterized by disruption of the small intestinal and colonic barrier to recipient germ-free mice, and (iii) IgA-targeted microbes present in healthy donor microbiota prevent development of this enteropathy. Together, these results illustrate the diagnostic and therapeutic opportunities provided by defining the gut mucosal IgA responses of children with undernutrition.

INTRODUCTION

The origins of undernutrition encompass both nutritional as well as biological causes. In particular, while there is clearly a role for food insecurity in the genesis of undernutrition, additional factors are likely contributory (*Richard* et al., 2014). Along with poor perinatal nutrition and recurrent acute infections, impairment of gut barrier function has been identified as a major factor contributing to childhood undernutrition (*Trehan* et al., 2016). Defective gut barrier function leads to reduced absorption of nutrients from foods, leakage of host proteins into the intestines and increased inflammation within the gut, leading to additional energy expenditure. The insults leading to impaired intestinal barrier function are not definitely known, however the prominent association of poor sanitation with undernutrition has led to the hypothesis that colonization with certain microbes leads to chronic impairment of the gut barrier (*Humphrey*, 2009; *Keusch* et al., 2014). This syndrome, called environmental enteric dysfunction (EED, formerly tropical

sprue, tropical enteropathy or environmental enteropathy) is thought to contribute greatly to the overall cycle of inflammation, gut functional loss, and undernutrition. Although the association of EED with poor hygienic conditions is well established (*Trehan* et al., 2016), considerably less is known about how perturbations of the gut microbiota may affect the gut barrier function in undernutrition.

GUT MICROBIOTA AND UNDERNUTRITION

Establishing an association between undernutrition and the gut microbiota poses many technical and scientific challenges since genetic, dietary and environmental exposures can all impact gut microbiota structure. In order to circumvent these obstacles, we performed a prospective clinical study on Malawian twins recruited shortly after birth. During study visits faecal microbiota specimens and anthropometric measurements were collected and the children were screened for severe undernutrition. Over the course of the study, 13 twin pairs were determined to be discordant for a form of severe acute undernutrition called kwashiorkor. Analyses of these samples revealed broad differences in the metabolic and functional capacities of these discordant microbiota. Transplantation of the faecal microbiota from the malnourished twin into gnotobiotic mice fed a macro- and micronutrient deficient diet resulted in significantly more weight loss compared to mice colonized with the microbiota originating from the healthy co-twin fed the same diet (*Smith* et al., 2013). These changes were associated with altered metabolic profiles in the recipients of the kwashiorkor microbiota, particularly a reduction in methionine and cysteine, indicating abnormal sulphur metabolism in these animals. Additional analysis of metagenomic data derived from the of the malnourished twins' faecal microbiota demonstrated delayed functional maturation compared to their healthy counterparts (*Smith* et al., 2013).

Alterations in the maturation of the gut microbiota were also observed in a Bangladeshi birth cohort of healthy and malnourished children (*Subramanian* et al., 2014). In this study, a cohort of healthy Bangladeshi children was used to develop a model of microbiota maturation over the first two years of life. Comparison of this model to children with SAM demonstrated a chronological delay in their microbiota maturation (*Subramanian,* et al., 2014). Alarmingly, intervention with a therapeutic diet did not entirely reverse the measured microbiota immaturity, emphasizing the need for new approaches to effect long-term changes in microbiota immaturity (*Subramanian,* et al., 2014).

INTESTINAL BARRIER FUNCTION AND THE MICROBIOTA

Alterations in gut microbiota membership during undernutrition have both short and long term consequences on a child's metabolic, immune and absorptive capacities. It is reasonable to infer that these changes are probably most dramatic at the gut barrier, the site of closest contact between the host and the gut microbiota. The gut intestinal barrier is the series of host defences that allow the peaceful coexistence of our intestinal microbiota and immune

system. These defences are necessarily both complex and overlapping because they must simultaneously maintain a selective partition between some components of the intestinal lumen while allowing the absorption of nutrients and the surveillance of the microbiota by the immune system. To achieve this, an overlapping and multi-tiered "defence in depth" has co-evolved with the microbiota.

First, physical barriers prevent penetration of bacteria through the intestinal epithelium. For example, mucins secreted by goblet cells create a glycoprotein mesh that prevents incursion of bacteria (*McGuckin* et al., 2011). Disruption of the microbiota by antibiotic treatment can trigger thinning of the mucus layer, resulting in increased susceptibility to enteric infection (*Wlodarska* et al., 2011). Additionally, the intestinal epithelium itself regulates the penetration of the intestinal contents into the lamina propria through the modulation of tight junctions between cells (*Groschwitz* et al., 2009). These tight junctions are necessarily malleable because of their critical role in allowing uptake of nutrients from digested foods (*Marchiando* et al., 2010). Second, chemical barriers help to dissuade bacteria from venturing too close to the intestinal epithelium. Antimicrobial peptides released by intestinal epithelial cells as well as Paneth cells segregate the epithelium and gut microbiota (*Vaishnava* et al., 2011). Third, immunologic barriers functioning to prevent, repel and repair insults to the epithelium constitute yet another layer of the intestinal barrier.

Among these immunologic components of the intestinal barrier, immunoglobulin A (IgA) is uniquely suited for studying the interplay between the gut microbiota and intestinal barrier function. IgA is abundant on all mucosal surfaces particularly the gut, where it functions by binding bacteria, toxins and other antigens to prevent them from interacting with the host, a process known as "immune exclusion" (*Stokes* et al., 1975). The synthesis of IgA occurs in response to both innate and adaptive immune signals (*Bunker* et al., 2015). Also, IgA production changes rapidly in response to alterations in the microbiota (*Hapfelmeier* et al., 2010). Thus, identifying which members of the microbiota are targeted by the IgA response will help better elucidate the dynamic changes that occur at the gut barrier in response to diet, infection or breakdown of other components of the gut barrier.

BARRIER INTEGRITY AND THE ROLE OF IgA

We and others have developed a flow cytometry based method for identifying IgA-coated bacteria from faecal samples (*Kau* et al., 2015; *Palm* et al., 2014). Using this approach, which we have termed "BugFACS", we characterized the patterns of IgA targeting in mice colonized with the faecal microbiota of either a Malawian co-twin diagnosed with kwashiorkor, or the co-twin that remained healthy.

Similar to our previous findings (*Smith* et al., 2013), mice colonized with the malnourished microbiota lost more weight than mice colonized with the healthy microbiota fed the same macro- and micronutrient deficient diet. In addition, BugFACS analysis showed that in mice colonized with the malnourished microbiota, there was preferential targeting of members of Enterobacteriaceae. In mice colonized with the healthy co-twin's microbiota, *Akkermansia mucinaphila*, a member

of the phylum Verrucomicrobia, was preferentially targeted. The capacity of the IgA targeted microbes from the malnourished microbiota to induce barrier dysfunction was demonstrated by their ability to induce severe weight loss and mortality when transplanted to another generation of gnotobiotic mice. This barrier dysfunction was associated with mislocalization of the cell adhesion molecule, EpCAM, within the small intestine and epithelial shedding within the large intestine. Interestingly, IgA targeted Enterobacteriaceae alone were insufficient to produce this pathology and required other IgA targeted bacteria, including members of Bacteroidales, to produce overt disease (*Kau* et al., 2015).

The importance of both Bacteroidales and Enterobacteriaceae in undernutrition has also been described in a model of Environmental Enteric Dysfunction (EED) (*Brown* et al., 2015). In this study, oral exposure to the combination of Bacteroidales and *E. coli* resulted in growth stunting accompanied by histopathologic changes consistent with EED including small intestinal villous blunting and disruption in the expression of tight junction genes CLDN2 and CLDN4. Similar to our findings, neither the Bacteroidales nor *E. coli* were capable of inducing barrier dysfunction when administered singly (*Brown* et al., 2015).

Interaction between *E. coli* and Bacteroidales has been noted before in several contexts. In a mixed infection model of abdominal abscess, the presence of *Bacteroides* sp. potentiated abscess formation (*Rotstein* et al., 1989a), possibly mediated through inhibition of neutrophil function by a soluble Bacteroides molecule (*Rotstein* et al., 1989b). Additionally, transcriptional analysis of *E. coli* and *B. thetaiotaomicron* in mono- and bicolonization experiments have shown that these species may crossfeed, potentially enhancing their *in vivo* fitness (*Li* et al., 2015). These data emphasize the need for consideration of bacterial interactions in future experiments directed at understanding the microbiota in modulation of barrier function.

IGA TARGETED BACTERIA IN HUMANS

The ability to determine the targets of the gut mucosal IgA response offers the opportunity to address many important questions regarding the developmental maturation of the gut mucosal barrier in early childhood. In order to tackle some of these questions, we examined a cohort of 40 healthy American twin pairs with faecal samples collected monthly from birth to 2 years of age and performed BugFACS analysis as well as whole community V4-16S rRNA sequencing (*Planer* et al., 2016). Analyses of these data show that IgA responses to the gut microbiota amongst twins were very similar in the first two years of life compared unrelated twins. However, by 2 years the similarity in IgA targeting profiles between unrelated individuals began to converge such that family membership was no longer distinguishable based on IgA profiles. Also, at two years, the children's' IgA profile largely resembled the mothers' implying that IgA mucosal responses have adapted an adult-like configuration by this time. Remarkably, there was a surprising consistency in the most prominent members of the microbiota targeted in both the children and mothers, especially *A. muciniphila* and *Ruminococcus torques*. Both of these taxa were nearly always targeted by the host IgA re-

sponse when present in an individual's faecal sample (*Planer* et al., 2016).

In malnourished children, different taxa predominate amongst the IgA targeted taxa. Consistent with our findings from humanized gnotobiotic mice, members of Enterobacteriaceae were significantly targeted by the IgA response in two cohorts of Malawian children with varying degrees of undernutrition (*Kau* et al., 2015). The degree of IgA targeting appeared to be negatively associated with anthropometric measurements, with individuals having the highest degree of IgA targeting of Enterobacteriaceae also showing the poorest (lowest) weight-for-age and weight-for-height Z-scores. Additionally, the IgA response to Enterobacteriaceae was significantly associated with the presence of virulence factors for enteropathogenic *E. coli* (EPEC) and enteroaggragative *E. coli* (EAEC), likely reflecting mucosal immune activation in response to colonization with these pathogens (*Kau* et al., 2015). Compared to healthy American cohorts (*Planer* et al., 2016), which demonstrated limited targeting of Enterobacteriaceae, the strong IgA responses to Enterobacteriaceae may reflect a compensatory response to the recurrent exposure to these bacteria and could be indicative of a degraded mucosal barrier.

The potential disease-causing role of IgA targeted bacteria is also supported by data from the inflammatory bowel disease literature. Inducing colitis using dextran sulphate sodium, gnotobiotic mice colonized with bacteria found to be targets of the IgA response in patients with inflammatory bowel disease results in severe colonic inflammation. In contrast, colonization with bacteria untargeted by the IgA response from the same microbiota donor displayed only minimal inflammation (*Palm* et al., 2014). When weighed together with our own observations in undernutrition, these studies indicate that IgA targeted intestinal microbes have at least some potential to exacerbate, if not precipitate, intestinal barrier dysfunction.

Perhaps equally interesting, IgA targeted bacteria from healthy individuals may have a role in protecting barrier integrity. Both *A. muciniphila* and *Bifidobacterium bifidum*, identified as targets of the IgA response in children (*Planer* et al., 2016), have both been proposed as probiotics. *B. bifidium* has long been associated with the healthy infant gut microbiota and has more recently been shown to prevent TNF-α mediated gut epithelial barrier disruption *in vitro* (*Hsieh* et al., 2015). Likewise, *A. muciniphila* reduces diet induced metabolic endotoxaemia and its presence correlates improved metabolic parameters of a variety of obesity induced abnormalities including insulin levels, glucose and triglycerides (*Everard* et al., 2013; *Schneeberger* et al., 2015). In our gnotobiotic model undernutrition, we found that *A. muciniphila*, administered with another IgA targeted organism, *Clostridium scindens*, was capable of preventing the barrier dysfunction caused by the administration of IgA targeted microorganisms from an undernourished individuals (*Kau* et al., 2015). These results hint that IgA targeted microbes from healthy individuals may occupy a privileged niche that simultaneously allows prolonged immunological contact with the host while avoiding overt inflammation that would result in degradation of the intestinal barrier. In return, the presence of these "friendly" IgA targets may prevent invasion of harmful taxa, synthesize useful metabolites or provide a basal amount of signalling that promote a healthy gut barrier such as epithelial turn over or mucus secretion.

The factors that result in a bacterial

taxa becoming a target of IgA in the intestine are not known, but will undoubtedly encompass multiple variables. However, one unifying factor for many of the bacteria that we have identified as being targeted by the IgA response is that the lifestyle of these bacteria may bring them in close association with the host epithelium. Both *A. muciniphila* and *R. torques* are known to degrade host mucins (*Derrien* et al., 2004; *Png* et al., 2010) while both EPEC and EAEC are known to adhere to the intestinal epithelium as part of their pathogenic lifecycles (*Kaper* et al., 2004). Alternatively, IgA responses may be driven primarily by bacterial encoded factors, such as capsule (*Peterson* et al., 2015), that modulate antigenicity in response to the environment. Regardless, understanding the features that promote IgA responses in the mucosa is of great practical importance as efforts to rationally select next-generation probiotics for undernutrition will need to take into account their effects on host barrier function.

CONCLUSIONS

Future therapies directed at improving barrier function in undernutrition will rely on a deeper understanding of the microbial and host dynamics at the gastrointestinal barrier. In the absence of biopsy specimens, proxy measurements of barrier function such as identifying IgA targeted bacteria, will greatly aid in our understanding of EED and may constitute a new diagnostic modality for barrier function. Further, efforts to recover IgA-targeted microbes from healthy individuals may lead to next-generation probiotics that favourably modulate barrier function.

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