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HOST-ASSOCIATED VIROMES IN HEALTH AND DISEASE

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SUMMARY

The microbiome (defined here as all microorganisms, commensal, symbiotic or pathogenic that share our body space) plays a significant role in human health and disease (*Virgin*, 2014; *Norman* et al., 2015). Many studies have focused on the bacterial component of the microbiome. For example, Crohn's disease (CD) and ulcerative colitis (UC), two forms of inflammatory bowel disease (IBD), are characterized by changes in the gut bacterial microbiome in adults and children (*Gevers* et al., 2014; *Kostic* et al., 2014). The virome is composed of both eukaryote infecting viruses and bacteriophages (phages, hereafter). Recently, associations of the virome with disease have been made in HIV/AIDS (*Handley* et al., 2012, 2016), malnutrition (*Reyes* et al., 2015), and IBD (*Norman* et al., 2015). AIDS in both humans and non-human primate models is characterized by an expansion of enteric eukaryotic viruses, in particular picornaviruses and adenoviruses (*Handley* et al., 2012, 2016). In IBD, we showed that in a cross-sectional comparison of adult IBD cases and household controls there was a significant expansion of the *Caudovirales*, an order of phages, and anelloviruses, a family of eukaryotic DNA viruses of unknown pathogenicity (*Norman* et al., 2015). Importantly, we also observed statistically significant associations between phages and bacteria that might play a protective or causative role in IBD.

INTRODUCTION

Recent associations of the virome with disease have been observed in a diverse set of diseases including in HIV/AIDS (*Handley* et al., 2012, 2016), malnutrition (*Reyes* et al., 2015), and IBD (*Norman* et al., 2015). AIDS in both humans and non-human primate models is characterized by an expansion of enteric eukaryotic viruses, in particular picornaviruses and adenoviruses (*Handley* et al., 2012, 2016). In IBD, there is a significant expansion of the *Caudovirales*, an order of phages and anelloviruses, a family of eukaryotic DNA viruses of unknown pathogenicity (*Norman* et al., 2015). The pathological consequences of viral community changes in the enteric microbiome are unclear. As eukaryotic viruses infect host cells, it is safe to speculate that expansion of these viruses may impact host cell physiology. Our understanding of the impact for many of these viruses is relatively well-established as these viruses regularly cause infectious disease. The role of bacteriophage expansion is less clear due to the fact that they can infect, modulate and lyse bacterial cells, as well as influence host-cell physiology via their free-virion state. This later concept has an anaemic literature and

would benefit from further scientific investigation. This review focuses on summarizing what is known about the largely ignored associations between phage and human disease, and highlights an even greater challenge for the virome research community, that of the currently unclassifiable components of the virome frequently referred to as 'dark matter'. The impact of viral dark matter is that we are blind to a significant component of host-associated viromes and may be missing important disease associated viruses. A concerted research effort is required to define the mechanisms behind virome changes in causing disease in concert with defining the full virome by shedding light onto the void of our own viromes.

REVIEW

The human intestinal microbiome consists of an estimated 10^{14} bacterial cells that play a vital role in gut metabolism, nutrient uptake, immune system development, intestinal physiology, and competition with pathogenic bacteria (*Van Praet* et al., 2015). Bacterial communities are parasitized by complex array of bacteriophages. Next generation sequencing (NGS)-based studies have revealed that enteric bacteriophage communities are highly variable among individuals, incredibly diverse, and affected by environmental stimuli (*Reyes* et al., 2010; *Minot* et al., 2013). Since bacteriophage influence bacterial fitness by killing bacteria with specific receptors on their surfaces and by transferring mobile genetic elements and antibiotic resistance genes among bacteria, an altered intestinal virome might well trigger shifts in bacterial flora relevant to diseases such as IBD (*Duerkop* et al., 2012; *Sougakoff* et al., 1988; *Doucet-Populaire* et al., 1991).

Bacteriophages have been proposed to be associated with inflammatory disease (*Riley*, 2004), however their role in disease remains largely undefined. Increased tailed bacteriophage (order *Caudovirales*) have been observed by electron microscopy in intestinal biopsy washes from adults with CD compared to healthy controls (*Colombet* et al., 2008). These findings were further supported by a sequence-based study of paediatric CD, where the analysis of mucosal washes and intestinal biopsies revealed an increase in bacteriophage reads when compared to non-IBD tissue biopsies (*Wagner* et al., 2013). Increased bacteriophages may be explained by the activation of bacterial-integrated prophage by environmental stimuli, such as oxidative stress or antibiotic use (*Fortier* and *Sekulovic*, 2013), which are commonly associated with IBD, but this specific association has yet to be proven.

Free bacteriophage particles in the intestine could come into contact with epithelial cells as well as the lamina propria and antigen presenting cells, via breaks in the intestinal mucosa, the lamina propria and host myeloid cells resulting in host immune surveillance and systemic spread (*Górski* et al., 2006). It has long been established that antibody responses are made against bacteriophage particles, indicating that they are immunogenic (*Uhr* et al., 1962). Additionally, even though a known eukaryotic cell receptor is unknown, endotoxin-free bacteriophage particles stimulate inflammatory cytokine production (including IL-1β and TNF-α) by macrophages in a MyD88-dependent manner (*Eriksson* et al., 2009). This may be due in part to CpG motifs present in bacteriophage

genomes, which stimulate interferon production and protect against vaccinia virus challenge *in vivo* (*Mori* et al., 1996). Alternately, the cytokine production may be related to the immunogenicity of bacteriophage coat proteins that have been reported to enhance DNA vaccine potency by stimulating adaptive immune responses (*Cuesta* et al., 2006). This adjuvant effect of bacteriophages has not been explored in mechanistic detail. Therefore, changes in bacteriophage populations may have effects on intestinal inflammation indirectly by pruning the structure of the enteric bacterial microbiome or directly by stimulating inflammation.

A recent study defined the faecal virome in IBD patients and controls (*Norman* et al., 2015). This study found that the enteric virome differs between CD and UC patients and controls with a significant, and disease-specific, expansion of bacteriophages that was not secondary to changes in bacterial populations. These data support a 'virus-predator-bacterial prey' model in which the virome may contribute to IBD associated intestinal inflammation through altering bacterial community structure or through direct interactions with the host.

In animal models, viruses that infected eukaryotic cells (murine norovirus) have been shown to interact with IBD risk genes to alter intestinal disease (*Cadwell* et al., 2010; *Basic* et al., 2014). In the same study that identified enteric bacteriophage expansion associations with IBD, an increased abundance of anellovirus was observed in IBD patients when compared to controls. Taken together with the bacteriophage data, these data suggest that both bacterial and eukaryotic viruses may contribute to the IBD pathologies.

The virome expansion observed in IBD was extraordinarily patient specific. Bacteriophage populations were different between individuals, but remained relatively constant within a patient over time. This parallels what is observed for bacterial community structure in IBD patients, suggesting that a full understanding of a patient's personal microbiome may be required to properly realize safe and effective personal treatment strategies. It is well recognized, for example, that the course of disease (or prognosis) varies substantially between patients with IBD. Recent studies have begun to investigate the biology that determines prognosis, and have demonstrated that pathways associated with CD8+ T-cell activation are up-regulated in a subset of IBD patients who subsequently experience a significantly more aggressive disease course (*Lee* et al., 2011). Indeed, the balance between T-cell activation and exhaustion seems to determine prognosis in a range of autoimmune diseases, including IBD (*McKinney* et al., 2015). The causes of T-cell exhaustion in IBD, however, remain unknown, but it is striking that this phenomenon is typically associated with chronic viral infection. Nevertheless, to date, no studies have investigated the associations between an individual's personal enteric viral content virome and their disease status.

THE DARK MATTER CHALLENGE

Typically a majority of the sequences present in purified virus preparations cannot be classified due to the lack of statistically significant sequence similarity to reference virus sequence (*Reyes* et al., 2010, 2015; *Minot* et al.,

2013; *Norman* et al., 2015). Unlike bacteria, viruses lack a universally conserved marker sequence (*Rohwer* and *Edwards*, 2002). Thus, viromes require analysis via metagenomic (sequencing random fragments) methods. These fragments are classified using sequence alignment to known viral sequences. In all previous virome studies, fewer than 50% of viral sequences are classifiable with the rest remaining as viral "dark matter" with unknown taxonomic and functional assignment, compromising our ability to detect important associations. Recent efforts have attempted to address the dark matter in human enteric viromes. One striking example was the discovery of crAssphage, a previously unrecognized phage that is present in ~50% of the population and is the most abundant known phage in the human gut (*Dutilh* et al., 2014). The majority of crAssphage proteins have no sequence similarity to known viral proteins and its extraction from the viral dark matter relied on novel computational approaches. Thus, there is a fundamental need to apply novel computational tools to analyse viral dark matter.

In addition to more robust computational analysis of virome sequence data, concerted wet laboratory techniques need to be employed for functional characterization of unclassifiable sequence data. The advent of NGS methods has dramatically increased our ability to detect nucleic acid sequences derived from novel eukaryotic viruses and phages, forming the basis of most virome studies (*Minot* et al., 2013; *Ogilvie* et al., 2013; *Lim* et al., 2015; *Krishnamurthy* et al., 2016; *Manrique* et al., 2016), but the potential to bring this knowledge to clinical application has not yet been realized. Over the last decade, a tremendous number of previously unrecognized eukaryotic viruses from human stool samples have been discovered (*Finkbeiner* et al., 2008a, 2008b, 2009; *Holtz et al.,* 2008; *Kapoor* et al., 2008, 2009; *Phan* et al., 2016). However, of these a cell culture system has only been described for human Theiler's-like cardiovirus (*Chiu* et al., 2010) and astrovirus VA1 (*Janowski* et al., 2017). Likewise, many novel phage sequences in the human enteric tract have been discovered, but culture has not been attempted (*Reyes* et al., 2010; *Minot* et al., 2011, 2013; *Reyes* et al., 2015). This paucity of culturable eukaryotic viruses and phages precludes any functional assessment of the role of these agents in disease. Therefore, it is essential that culture systems be developed for eukaryotic viruses and phages identified in virome studies in order to study their impact on disease. These efforts will aid in functional characterization of dark matter as classifiable viruses, making these efforts extraordinarily valuable for characterizing the viromes influence of health and disease.

The increased literature emerging on enteric virome demonstrate that both eukaryotic viruses and phage populations take on different forms in disease states. In particular, the emergence of both eukaryotic and bacterial viral populations in people with IBD is a prominent display of the extent and complexity of the virome in context of a dysbiotic bacterial microbiome and diseased intestine. How these viruses are contributing to disease, or if they are just innocuous passengers or markers of the diseased state has yet to be answered. As highlighted here, the path to establishing causal connections between viromes and disease require advancements in total understanding of the virome through advances in both computational and laboratory biology. Only then will we fully appreciate the impact our smallest enteric passengers on our own health.

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