Title: The potential of the microbiota to influence vaccine responses

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Abbreviations Page

Bacille de Calmette et Guérin (BCG)
Cholera toxin (CT)
Dendritic cells (DC)
Dietary fibre (DF)
Diphtheria, tetanus and acellular pertussis (DTPa)
Hepatitis B virus surface antigen (HBsAg)
Human serum albumin (HSA)
Immunoglobulin (Ig)
Inflammatory bowel disease (IBD)
Interferon gamma (IFNγ)
Interleukin (IL)
Lipopolysaccharide (LPS)
Mesenteric lymph nodes (MLN)
Muramyl dipeptide (MDP)
Nucleotide-binding oligomerization domain containing 2 (Nod2)
Ovalbumin (ova)
Pattern recognition receptors (PRRs)
Pneumococcal conjugate vaccine 13 (PCV13)
Receptor interacting serine/threonine kinase 2 (Ripk2)
Short chain fatty acids (SCFAs)
Toll-like receptor 5 (TLR5)
ABSTRACT

After clean water, vaccines are our primary public health intervention providing protection against serious infectious diseases. Antigen-specific antibody-mediated responses play a critical role in the protection conferred by vaccination, however these responses are highly variable between individuals. Additionally, vaccine immunogenicity is frequently impaired in developing world populations, for reasons that are poorly understood. Although the factors that are associated with inter-individual variation in vaccine responses are likely manifold, emerging evidence from mouse models and studies in human populations now suggests that the gut microbiome may play a key role in shaping systemic immune responses to both orally and parenterally administered vaccines. Here, we review the evidence to date that the microbiota can influence vaccine responses and discuss the potential mechanisms through which these effects may be mediated. Additionally, we highlight the gaps in this evidence and suggest future directions for further research.
Introduction
Vaccination is second only to clean water in reducing infectious disease burden, preventing an estimated 6 million deaths per year globally [1]. Vaccines primarily mediate protection by inducing B cells to produce antigen-specific antibodies, although T cells also contribute to protection mediated by several vaccines and are critical to ensuring the induction of high-affinity antibodies and immune memory [2]. Antigen-specific antibodies are produced following the initial exposure to a vaccine antigen (the primary antibody response) and for many vaccines, these initial responses are boosted via re-exposure to the same antigen, inducing a secondary, usually stronger and longer-lasting, memory response. The concentration of antigen-specific antibodies in serum (the antibody titer) is commonly used as a correlate of vaccine-mediated protection. Specific antibody titer thresholds, above which an individual is considered to be protected, have been defined for many vaccines. Although the success of vaccination programs are indisputable [1], the magnitude of antibody and T cell responses, induced by vaccination, can vary substantially among individuals. For example, the magnitude of neutralizing antibody titers and CD8+ effector T cell responses induced by vaccination of humans with the live attenuated yellow fever vaccine 17D, one of the most successful vaccines ever developed [3], can vary by more than 10-fold between individuals [4]. Furthermore, the magnitude of hemagglutinin inhibition titers induced in individuals vaccinated with the inactivated seasonal influenza vaccine can vary by more than 100-fold [5]. In a randomized trial of 1,709 pneumococcal conjugate vaccine 13 (PCV13)-vaccinated infants in the U.S.A., the geometric mean concentration of antigen-specific IgG responses was 1.91 µg/mL with a standard deviation of 1.47, indicating considerable variation in the response between infants [6]. Cytokine responses to mycobacterial antigens have also been shown to vary up to 10 log-fold in *Bacille de Calmette et Guérin* (BCG)-vaccinated infants [7]. It is possible that such differences in vaccine induced immune responses could translate into
differences in vaccine efficacy, both in terms of the proportion protected and the duration of protection. Vaccine efficacy of the diphtheria, tetanus and acellular pertussis (DTPa) vaccine, for example, has been reported as 71-78% for preventing milder symptoms of pertussis and 84% for preventing typical disease [8], while estimates of BCG efficacy against pulmonary TB in children are highly variable (0-80%) [9]. Additionally, clinical trials consistently show lower immunogenicity for vaccines in developing world populations, including those against poliomyelitis and rotavirus, and for the BCG vaccine [10, 11]. While many factors, such as genetics [12], prior antigen exposure, and maternal vaccination [13], can potentially influence vaccine immunogenicity, several lines of evidence now suggest that the microbiota may also be a significant factor [14, 15]. In this review, we summarize the evidence that the gut microbiota can influence vaccine responses and discuss potential mechanisms through which this can occur (Figure 1).

**The human microbiome**

Humans are colonised by a large and diverse group of microorganisms, collectively known as the microbiota. The intestinal microbiome, in particular, hosts an enormous abundance and diversity of microbes, which perform a range of essential and beneficial functions including the metabolism of nutrients, the maintenance of gut homeostasis, and the regulation of gut mucosal immunity [16]. In neonates, the intestinal microbiome is rapidly established and, in vaginally-born infants, its composition is strongly determined by the maternal microbiota [17]. A range of factors, including gestational age, route of birth, infant diet, the use of pre- or probiotics, and maternal diet and weight, can potentially influence the infant microbiome [17-19], resulting in considerable variation in the composition of the microbiota between human infants. High inter-individual variation in the composition of the microbiota is also evident in adults and is correlated with a large number of exogenous and intrinsic factors including dietary
factors, exposure to drugs, disease, and smoking [20]. Moreover, antibiotic exposure can profoundly change the composition of the human microbiota and can lead to a long-lasting loss of diversity and dysregulation of the microbiome (termed dysbiosis) [21, 22]. The effects of a single course of intrapartum antibiotics on the infant microbiota, for example, has been shown to persist to at least 3 months of age [23]. Dysbiosis during this critical developmental window may have particularly profound and long-lasting consequences on metabolism and immune responses systemically.

**Dysbiosis, disease and dysregulation of systemic immunity**

It is becoming well-established that the consequences of intestinal dysbiosis extend far beyond the gut. Early-life intestinal dysbiosis is associated with a wide variety of diseases including metabolic syndrome [24], obesity [25], and allergic asthma [26]. The developing microbiome also plays a key role in programming the neonatal immune system, and dysregulation of the microbiome can significantly impact systemic immunity. The microbiota, for example, has been shown to play a key role in driving early postnatal innate immune development in mice [27]. Neonatal antibiotic exposure has also been associated with increased susceptibility to late-onset sepsis in premature infants [28]. The microbiota also play a key role in shaping adaptive immune responses, including the regulation of T helper 17 and regulatory T cell responses [29]. Inter-individual variation in the composition and function of the gut microbiome of healthy adult humans has also been shown to be associated with the capacity for inflammatory cytokine production in blood stimulated with microbial ligands [30]. Given the growing list of ways that the microbiome can influence the immune system, it would be surprising if the microbiome did not also influence vaccine responses, however, evidence that this is the case is to date relatively limited.
The influence of antibiotics on vaccine responses in animal models

Several lines of evidence now suggest that antibiotics can modulate vaccine responses. Early work, published in 1999, investigated the effects of the antibiotics, clarithromycin and doxycycline, on antibody responses in mice immunized with tetanus toxoid, pneumococcal polysaccharide vaccine, or the hepatitis B virus surface antigen (HBsAg) vaccine [31]. Although there were minor differences in antibody responses between control and antibiotic treated mice, the results of the study were highly variable and clear conclusions could not be drawn. At the time, the gut microbiota was also not considered as a potential mediator of these effects and altered vaccine responses were thought to be due to possible immunomodulatory properties of the antibiotics [32]. More recently, mice born to dams treated with a cocktail of antibiotics (ampicillin, streptomycin and clindamycin) were shown to have impaired IgG responses following immunization with the model antigen ovalbumin (ova), although the differences were very modest (e.g. 540ng/ml versus 400 ng/ml, in control versus antibiotic treated mice, respectively) [33]. Interestingly, this modest impairment was only observed when the pups that were born to antibiotic treated mothers were immunized at 7 days of age. Adult mice and mice vaccinated at 14 days old did not have impaired responses. Germ-free mice were also observed to have impaired responses to ova-immunization, which could be restored 4 weeks after the colonization of the germ-free mice with a mixed flora. These data suggested that the microbiota had a role to play in supporting optimal antibody response to systemic immunization, though the effect seemed to be limited to very early in life and the impairment was modest. In contrast, antibiotic treatment has been shown to enhance serum and mucosal rotavirus-specific antibody responses in mice orally inoculated with the virus [34]. Germ-free mice were also observed to have enhanced rotavirus antibody responses in this study. These data suggest that the gut microbiota may enhance systemic vaccine responses but suppress oral
vaccine responses, however, further work is needed to determine if this is the case in all circumstances.

**The microbiota as a vaccine adjuvant**

The most convincing evidence in mice to date that the microbiota can influence vaccine responses is a study which investigated mice immunized with the seasonal influenza vaccine and showed that germ-free or antibiotic treated mice had significantly impaired IgG and IgM antibody responses to this vaccine [35]. Interestingly, the seasonal influenza vaccine is relatively unusual in that it is an unadjuvanted vaccine (adjuvants are pharmacological or immunological agents that act to accelerate, prolong, or enhance antigen-specific immune responses). Previous work by the same group has shown that the expression of toll-like receptor 5 (TLR5) (a pattern recognition receptor for flagellin [36]) in human peripheral blood mononuclear cells was induced 3 to 7 days post-vaccination, and the level of TLR5 expression correlated with the magnitude of hemagglutination inhibition titers (a measure of response to the influenza vaccine) 4 weeks later [5]. The study in mice [35] subsequently showed that TLR5-mediated sensing of flagellin that was produced by the gut microbiota was necessary for antibody responses to the influenza vaccine, as mice deficient for TLR5 had substantially impaired responses to the vaccine at day 7 and 14 post-vaccination. Furthermore, antibody responses could be restored in antibiotic treated or germ-free mice by oral reconstitution of the gut microbiota with a flagellated, but not an aflagellated strain, of *E. coli*. This indicated that the microbiota could in effect act as a natural adjuvant for the influenza vaccine [37]. A similar effect was also observed in this study in mice vaccinated with the inactivated polio vaccine, however, antibody responses to several adjuvanted and live vaccines, including yellow fever 17D, were not found to be impaired in these mice. This study focused on adult mice, and further
work is now needed to determine the impact of the microbiota in early life on infant vaccine responses.

Aside from flagellin, the microbiota also produce a range of molecules that signal through other pattern recognition receptors (PRRs) of the innate immune system and could also potentially adjuvant vaccine responses [38]. Recently, for example, recognition of the microbiota by another PRR, nucleotide-binding oligomerization domain containing 2 (Nod2), has been found to be essential for the mucosal adjuvant activity of cholera toxin (CT) in mice intranasally immunized with the model antigen human serum albumin (HSA) and CT [39]. CT was essential for the antigen-specific responses to HSA. Antibiotic treated, germ-free, *Nod2*−/−, and receptor interacting serine/threonine kinase 2 (Ripk2) deficient mice (Ripk2 is an adaptor downstream of Nod2, required for Nod1 and Nod2 signalling [40]), all had substantially impaired IgG responses to HSA. Nod2 recognises peptidoglycan molecules that contain muramyl dipeptide (MDP) [41] and reconstitution of germ-free mice with MDP or colonization with a bacterium with high Nod2 stimulatory activity was sufficient to restore antibody responses. The importance of Nod2 activity for antibody responses to routinely administered commercial vaccines, however, remains to be investigated.

Another potent immunomodulatory molecule produced by the microbiota is lipopolysaccharide (LPS). LPS is recognized by the PRR, TLR4 [42]. Different species in the microbiota produce different types of LPS and variation in the immunogenicity of these different types of LPS has been shown to contribute to autoimmunity in humans [43], and to differences in the class of T helper cell responses [44]. It is not currently known whether LPS produced by the microbiota can influence vaccine antibody responses but it seems possible given that immunization of mice with synthetic nanoparticles containing antigens and ligands which signal through TLR4
lead to significantly enhanced and more persistent antigen-specific antibody responses [38]. The immunomodulatory activity of LPS produced by the microbiota may be particularly relevant if vaccines are administered following antibiotics. Antibiotic exposure is commonly associated with a subsequent overgrowth of specific species of bacteria such as members of the *Enterobacteriaceae* [45], which produce high levels of endotoxin (LPS). For example, germ-free mice monocolonized with *Enterobacter* and fed a high-fat diet, developed obesity, insulin resistance, and had elevated serum endotoxin levels and increased inflammation [46]. Increased serum endotoxin was likely due to increased intestinal permeability, allowing microbial products to translocate out of the gut [47, 48]. Microbial translocation has been shown to be a cause of systemic immune activation in chronic HIV infection [49], to worsen graft-versus-host disease [50], and is a common feature of inflammatory bowel disease (IBD) [51]. Interestingly, TLR4 signalling in B cells has also been shown to be critical for the production of circulating microbiota-specific IgG, which can provide protection against systemic infection by certain bacteria [52]. Further work is now required to determine whether immune responses to vaccines are altered in mice or people who have been recently been exposed to antibiotics and have high levels of gut dysbiosis.

**Cell-types mediating the effects of the microbiota on vaccine responses**

There are currently conflicting data on which cell-types may mediate the effects of the microbiota on systemic vaccine responses. B cells express a range of PRRs that could potentially sense microbial products produced by the microbiota [53] and regulatory B cells have been shown to be induced in the spleen and mesenteric lymph nodes (MLN) by gut microbiota-driven interleukin (IL)1B and IL6 [54]. However, the effects of flagellin on B cells, which has been shown to be critical for TLR5-mediating sensing of the microbiota on influenza vaccine responses, was shown to be relatively modest [35]. Instead, macrophages were
identified as playing a critical role in mediating the influence of the microbiota on antibody responses to the influenza vaccine. Interestingly, dendritic cells (DC) did not seem to be required, as vaccination of $Cd11c^{\text{Cre}}\text{Myd88}^{\text{loxP}}$ mice (which have defective TLR signalling in CD11c+ DCs) with the influenza vaccine resulted in only a small reduction in antibody responses. In contrast, Nod2-mediated recognition of the microbiota by cells expressing CD11c has been shown to be critical for the adjuvant activity of cholera toxin in mice intranasally immunized with HSA [39]. Interestingly, however, intraperitoneal immunization with HSA and CT resulted in comparable antibody responses in antibiotic treated and untreated mice. This may suggest that the critical cell-types that mediate sensing of the microbiota to adjuvant vaccine responses depend on the vaccine itself, the adjuvant used, or on the site of vaccination. Further work is necessary to clarify whether this is the case.

**Metabolites produced by the microbiota**

Another potential mechanism through which the microbiome can influence vaccine immune responses is through changes in the levels of microbially produced metabolites which have been shown to have immunomodulatory properties on a number of cell-types including macrophages, DCs, T cells and B cells [55]. Undigested dietary fibre is an abundant substrate for bacterial fermentation in the colon and the main metabolic end products of this process are short chain fatty acids (SCFAs) such as acetic, butanoic and propionic acid [56]. Recently, SCFAs have been shown to regulate B cell gene expression and energy metabolism to support optimal antibody responses [57]. Mice fed diets rich in dietary fibre (DF) or administered propionate in their drinking water had significantly increased frequencies of IgA+ B cells in the small and large intestine, the MLN, and in the spleen. DF also led to increased levels of secreted IgA and serum levels of IgG. Antibiotic treatment abolished the positive effects of DF on antibody responses. Whether SCFAs alter antigen-specific responses to vaccines was not
assessed in this study, however, SCFAs promoted enhanced antibody responses to infection with *Citrobacter rodentium*, suggesting that this is a strong possibility. There are also a number of indirect mechanisms through which the microbiota may regulate B cell responses (reviewed recently in [58]), though none of the studies to date have assessed antigen-specific vaccine responses.

**T cell responses to vaccination**

Vaccine-mediated protection against disease is highly dependent on the induction of antibody responses against vaccine antigens. However, the generation of long-term T cell memory responses has also been shown to regulate the protective effects of some vaccines, including those against influenza, measles, pertussis and tuberculosis [2]. The composition of the gut microbiota and the metabolites they produce have been shown to alter T cell differentiation and function within the intestine and in peripheral lymphoid tissues [29]. For example, butyrate produced by *Clostridia* species in the gut microbiome has been shown to enhance regulatory T cell differentiation and function [59], while segmented filamentous bacteria have been shown to induce differentiation of Th17 cells [60]. However, whether the microbiome can influence the formation of long-term antigen-specific T cell memory post-vaccination is currently unknown. Interestingly, a recent study reported that maternal antibiotic treatment in mice led to significantly reduced antigen-specific IFNγ production by CD8+ T cells in the mice born to these mothers following vaccinia virus infection [61]. This effect could be partially rescued by the oral administration of LPS [62]. This suggests that the microbiota can indeed influence antigen-specific T cell responses, though whether these effects were long-lasting was not assessed. Similarly, the recall production of interferon gamma (IFNγ) or IL5 from antigen-stimulated splenocytes isolated from mice immunized with HSA and CT, was completely
Evidences from human clinical studies

Most of the evidence to date that the microbiota can influence vaccine responses comes from studies in mice. A few recent clinical studies also suggest a potential role of the microbiota in determining optimal vaccine responses in humans. The relative abundance of several bacterial species in the stool microbiota of 48 Bangladeshi infants, for example, have been associated with vaccine-specific IgG and T cell proliferation responses to the oral polio, BCG, tetanus toxoid and hepatitis B virus vaccines [63]. Increased abundance of Actinobacteria was positively associated with vaccine responses, while higher levels of several other species such as Enterobacter, were negatively associated. The composition of the infant gut microbiome in infants from rural Ghana was also found to be correlated with rotavirus vaccine responses, where a higher abundance of Bacteroidetes was associated with impaired responses [64]. Interestingly, it is not only the gut microbiota that may influence vaccine responses as the composition of the nasal microbiome has also been found to be associated with IgA responses to the live attenuated influenza vaccine [65]. In addition to these studies, a number of studies have also shown significant effects of probiotic or prebiotic administration on vaccine responses (recently reviewed in [66]), however, equally as many studies have also shown no effect of these treatments. It is difficult to compare between studies due to differences in the strains of probiotics used, when they were administered, and the vaccines investigated. All of the human clinical studies to date have also been limited by relatively small sample sizes and it must be emphasized that all of the evidence to date in humans is correlative. There is, as yet, no human study that demonstrates a causal impact of the microbiota on immune responses to
vaccination and properly powered clinical studies are urgently needed to determine whether the microbiota is a significant determinant of vaccine responses in humans.

CONCLUDING REMARKS

Recent data in adult mice demonstrate a key role for the gut microbiota in inducing antibody responses to some vaccines. This is consistent with numerous previous studies in mice which have shown that the microbiota can modulate many aspects of innate and adaptive immune responses [29, 67]. Further work is now needed to investigate whether B and T cell responses to infant vaccinations are influenced by the microbiota in early life. A major challenge for the field is to determine the potential impact of the microbiome on vaccine immune responses in humans, particularly in infants. Numerous elegant studies in humans have established a correlative link between the composition of the gut microbiota and various disease conditions such as diabetes [68] and autoimmunity [43]. Additionally, the composition of the microbiota has also been linked to an individual’s capacity for inflammatory cytokine production [30] and many of the species in the human microbiota have been shown to be immunomodulatory in colonized mouse models [69]. Currently, however, studies which demonstrate a causal link between the gut microbiota and vaccine immune responses in humans are lacking. Therefore, we await future studies that investigate the potential role of the gut microbiome on modulating vaccine responses in diverse human populations.
AUTHORSHIP

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DISCLOSURES

The authors declare no conflict of interest
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**Figure Legends**

**Figure 1.** Mechanisms through which the gut microbiota has been shown to influence antibody responses to vaccination/infection in mice. **A)** TLR5-mediating sensing of flagellin from the gut microbiota by macrophages is necessary for optimal antibody responses in mice to the seasonal influenza vaccine. **B)** Nod2-mediated recognition of muramyl dipeptide (MDP) from the gut microbiota by CD11c⁺ DCs is necessary for optimal antibody responses to intranasal immunization of mice with human serum antigen (HSA) and cholera toxin (CT). **C)** Dietary fibre supports the production of short-chain fatty acids (SCFAs) by the gut microbiota which promote optimal antibody responses in mice to *Citrobacter* infection by boosting B cell metabolism and gene expression. **D)** These positive effects of the microbiota on vaccine responses are abrogated in antibiotic treated or germ-free mice.