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Molecular Aspects of Medicine

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Abstract

Iron is an essential micronutrient for nearly all living organisms. In addition to facilitating redox reactions, iron is bound by metalloproteins that participate in a variety of biological processes. As the bioavailability of free iron in host environments is extremely low, iron lies at the center of a battle for nutrients between microbes and their host. Mucosal surfaces such as the respiratory and gastrointestinal tracts are constantly exposed to commensal and pathogenic microorganisms. Whereas a key strategy of mammalian antimicrobial defense is to deprive microbes of iron, pathogens and some commensals have evolved effective strategies to circumvent iron limitation. Here we provide an overview of mechanisms underpinning the tug-of-war for iron between microbes and their host, with a particular focus on mucosal surfaces.

1. Introduction

Iron (Fe) is a vital micronutrient for nearly all forms of life, and is involved in critical physiological processes including oxygen transportation, energy metabolism, and DNA replication (Lieu et al., 2001). By existing primarily in two oxidation states, ferrous (Fe²⁺) and ferric (Fe³⁺) iron, this element has the flexibility to serve as a key cofactor in many enzymes as well as play a crucial role in facilitating a variety of redox reactions. Nevertheless, despite iron's essential roles in the beneficial chemistry of life, its redox potential can negatively impact biological molecules and cells; for example, the Fenton reaction involving Fe²⁺ and hydrogen peroxide yields reactive oxygen species (ROS) that can damage biomolecules including lipids, proteins, and DNA. Oxidative stress, in turn, is linked to aging, cardiovascular disorders, neurodegenerative diseases, and cancer (Liu et al., 2018). To minimize iron's reactivity and toxicity, the vast majority of iron in mammals is found in intracellular compartments, complexed with hemoproteins (e.g., hemoglobin,

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myoglobin), or the iron-storage protein ferritin (Lieu et al., 2001). Thus, the bioavailability of free iron is low in the mammalian environment, even during homeostasis. Moreover, in a process known as nutritional immunity, extracellular and intracellular iron is further decreased during infection to prevent the outgrowth of pathogens (Hood and Skaar, 2012). Successful pathogens have therefore evolved strategies to compete for and obtain iron from the host in order to replicate and thrive despite limited nutrient availability.

Mucosal surfaces constitute a physical barrier between the external environment and the inner body. During homeostasis, many of these surfaces are colonized by an assortment of microbes that are collectively referred to as the commensal microbiota, and typically include non-pathogenic bacteria, fungi, archaea, and viruses (Lozupone et al., 2012). Among the body sites with a commensal microbiota, the gastrointestinal (GI) tract, and in particular the colon, is home to the body's largest and most diverse microbial community. The host and its commensal microbiota have evolved a mutualistic interdependency that significantly contributes to many aspects of the host's normal physiology, ranging from metabolism, to resisting pathogen colonization (Dethlefsen et al., 2007), to shaping and maintaining the host's immune system (Belkaid and Hand, 2014). The host, in turn, provides its microbiota with nutrients and environments suitable for growth. Even so, not all microbes are able to colonize and/or flourish in a given host, as the availability of specific nutrients controls the abundance of certain microbes (Pereira and Berry, 2017). The ability to acquire and utilize nutrients is thus essential for commensal microbes to stably colonize their host.

Despite this coevolved mutualism, the mucosal immune system must still prevent microorganisms, be they commensals or pathogens, from disseminating beyond mucosal surfaces. On this front, iron sequestration by the host represents a conserved innate immune mechanism for limiting microbial growth. Nevertheless, pathogens have evolved strategies to circumvent these defenses. Here we provide an overview of mechanisms underpinning the tug-of-war for iron between microbes and their host, which ultimately determines the health of the host and the survival of pathogenic microbes.

2. Iron regulation in humans

Regulation of iron metabolism is a balance of iron absorption, recycling, and loss. Iron levels must be tightly controlled to avoid iron deficiency (which can result in anemia) and to prevent toxicity due to excess iron. Trafficking and systemic metabolism of iron have been the subject of excellent reviews (e.g., (Hentze et al., 2010)). In this section, we provide a brief synopsis. Dietary iron is generally acquired as heme or non-heme iron, and is absorbed in the duodenum and proximal jejunum (Hentze et al., 2010). Whereas heme iron is efficiently absorbed, likely via receptor-mediated endocytosis, non-heme iron (typically ferric iron) has a much lower bioavailability (approximately 10%). In the duodenum, insoluble ferric iron (Fe^{3+}) is reduced to the soluble ferrous (Fe^{2+}) form by ferric reductases including duodenal cytochrome b (DCYTB). Ferrous iron is then taken up by various iron transporters, including its primary transporter DMT1 (also known as NRAMP2). Absorbed iron is either utilized by enterocytes, where it is stored in association with ferritin, or released into the plasma via the basolateral export protein ferroportin (FPN1). Once in the plasma, iron is complexed with transferrin (Tf), then distributed to target organs and cells

such as erythroid precursor cells (Hentze et al., 2010). Of important note, humans lack a discrete mechanism for excreting surplus iron from the body, so the body's total iron content is solely regulated at the level of intestinal absorption. Dietary iron absorption and distribution throughout the body are governed by the liver hormone hepcidin, the master regulator of iron homeostasis (Drakesmith and Prentice, 2012). In addition to maintaining a healthy level of iron in the body, hepcidin is also leveraged by the immune system to fight infection. One of the first acute phase responses following an infectious and/or inflammatory insult is the substantial and rapid decrease of plasma iron. This process, known as hypoferremia of inflammation, is cytokine-driven and results in the upregulation of hepcidin, which reduces intestinal iron absorption and promotes systemic iron sequestration (Soares and Weiss, 2015).

Unabsorbed dietary iron (primarily heme and ferric iron) passes from the small intestine into the colon, where it can be scavenged by the microbiota and by pathogens. Indeed, several studies have shown that dietary iron and iron fortification can impact the composition of the microbiota as well as increase susceptibility to enteric pathogens (e.g., (Jaeggi et al., 2015)). This is particularly relevant in patients with inflammatory bowel disease (IBD) who develop anemia (Werner et al., 2011), and is discussed in a section further below. Beyond nourishing intestinal microbes, dietary iron can also have a profound impact on host physiology and on bacterial pathogenesis at extraintestinal sites (reviewed in (Lopez and Skaar, 2018)). Of note, as iron uptake primarily occurs in the proximal small intestine, patients that have their colon resected (colectomy) exhibit normal systemic iron levels, unless complications arise. For example, pouchitis is a complication resulting from the ileal pouch-anal anastomosis created during colectomy of UC patients (Ferrante et al., 2008). Recent studies have established a role for the microbiota in the pathophysiology of pouchitis (Machiels et al., 2017), and the administration of a probiotic cocktail has been shown to be effective in preventing onset and relapses of the disease (Gionchetti et al., 2012; Mimura et al., 2004). Whether and how iron impacts the development of pouchitis remains to be established.

3. Iron limitation at mucosal surfaces during health and disease

A number of mechanisms contribute to defense at mucosal surfaces, including colonization resistance mediated by the microbiota (Sassone-Corsi and Raffatellu, 2015) and microbial growth restriction mediated by nutritional immunity (Hood and Skaar, 2012). Iron represents an important battleground in both of these processes as it is a key nutrient that must be scavenged from the host environment by commensals and by pathogens. On the host side, intestinal homeostasis and restriction of pathogen growth at mucosal surfaces are, in part, accomplished by the secretion of potent metal-chelating proteins.

Lactoferrin (Lf), a widely conserved protein belonging to the transferrin family, contains two binding sites for ferric iron that enable tight but reversible iron sequestration (Ward and Conneely, 2004). A variety of functions are attributable to Lf, ranging from antimicrobial activities to iron-independent, immunomodulatory roles (Kruzel et al., 2017; Ward and Conneely, 2004). Lf's antimicrobial activity is thus far based on two characterized properties: (i) its strong iron-binding capacity; (ii) its high affinity for lipopolysaccharide (LPS). While predominantly found in milk, Lf is also present in mucosal secretions

including tears as well as bronchial and vaginal mucus (Levay and Viljoen, 1995). Lf is also an important component of neutrophil secondary granules and can bind to iron at a low pH, a trait that might be beneficial in inflammatory and infectious foci, which are often more acidic.

In addition to directly inhibiting pathogen growth *in vitro*, Lf can also promote colonization resistance and general health through shaping the microbiota. A newborn's immune system is immature and thus infants are at higher risk of infection. This susceptibility is partly compensated for by passive transfer of maternal antibodies through the placenta and by ingestion of human milk. In the latter context, Lf in human milk appears to beneficially modulate the neonatal microbiota composition. Although the antimicrobial effects of Lf are well supported by *in vitro* studies, the *in vivo* role of Lf against pathogens is less well defined (Sánchez et al., 1992). Nevertheless, evidence indicates that Lf promotes the growth of beneficial bacteria with low iron requirements, including intestinal and vaginal *Lactobacillus* spp. (Vega-Bautista et al., 2019). Moreover, Lf has been shown to promote the growth of probiotic strains belonging to the genus *Bifidobacterium*, which are among the first anaerobes to colonize and predominate the gut of breast-fed infants, although this effect might be attributable to roles associated with both the iron-bound and the iron-free forms of Lf (Vega-Bautista et al., 2019).

Lipocalin-2 (Lcn2; also known as neutrophil gelatinase-associated lipocalin [NGAL], siderocalin, or 24p3) is a small glycoprotein secreted by myeloid and epithelial cells in an interleukin-22-dependent manner upon infection and inflammation (Flo et al., 2004; Raffatellu et al., 2009). Lcn2's antimicrobial properties derive primarily from its ability to bind to a subset of bacterial siderophores, small molecules that are produced by bacteria to scavenge iron (described further in Section 4.1). One such siderophore is enterobactin, which is produced by commensal and pathogenic Gram-negative bacteria belonging to the family *Enterobacteriaceae*. By binding to enterobactin, Lcn2 limits bacterial iron acquisition and subsequently hampers the growth of enterobactin-dependent microbes (Figure 1) (Flo et al., 2004). In addition to siderophore binding, Lcn2 also exhibits activities that include inducing cytokines, mediating the recruitment of inflammatory cells, promoting apoptosis, and T-cell priming (Moschen et al., 2017).

Calprotectin (CP; a heterodimer of the S100A8 and S100A9 proteins) is another antimicrobial host product with metal-chelating properties. Although CP has been widely characterized for its roles in scavenging zinc and manganese (Corbin et al., 2008), recent *in vitro* studies indicate that CP can also chelate Fe²⁺ (Nakashige et al., 2015) and induce Fe starvation in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Wang et al., 2019; Zygiel et al., 2019), however an *in vivo* context for these findings remains to be described. Neutrophils are the main source of CP, and release it at sites of infection (Corbin et al., 2008). Similar to Lf and Lcn2, fecal CP correlates with disease activity in patients with Crohn's disease and ulcerative colitis (Zollner et al., 2020), and is thus frequently utilized to aid in the diagnosis and monitoring of patients with IBD (Sipponen et al., 2008).

In addition to the iron-binding proteins produced by the host, members of the commensal microbiota can also play a key role in limiting iron availability to pathogens. For example,

the probiotic bacterium *E. coli* Nissle 1917 (EcN) deploys multiple iron acquisition systems to compete with pathogens in iron-limited environments such as the inflamed gut (Deriu et al., 2013).

4. Microbial iron metabolism, with a focus on Gram-negative bacteria

With the exception of some non-pathogenic lactic acid bacteria and the Lyme disease pathogen *Borrelia burgdorferi*, most microorganisms rely on micromolar iron concentrations for metabolism and replication (Archibald, 1983; Posey and Gherardini, 2000; Weinberg, 1978). However, iron in the mammalian host is largely bound to host proteins (e.g., ferritin, Tf, Lf, hemoproteins), thereby reducing iron's toxicity and accessibility. As such, microbes have evolved a variety of iron acquisition strategies to colonize iron-limited niches within the host, with some bacteria even being able to reside within intracellular compartments. Microbial iron acquisition mechanisms include the secretion and uptake of siderophores, and the import of host-derived Tf and heme. Many mechanisms of bacterial iron homeostasis are regulated at the transcriptional level. Under iron-rich conditions, iron-acquisition mechanisms are repressed by Fur, the ferric uptake regulator. Fur is a global iron regulator in many characterized Gram-negative (e.g., enterobacteria) and some Gram-positive (e.g., *Bacillus* spp.) bacteria (Miethke and Marahiel, 2007). The diphtheria toxin regulator DtxR plays a comparable role in many characterized Gram-positive bacteria with a high genomic GC content (e.g., corynebacteria, mycobacteria) (Miethke and Marahiel, 2007). In Gram-negative bacteria, TonB-dependent receptor complexes play a key role for energizing the transport of iron across the bacterial outer membrane via various uptake systems for siderophores, heme, or Tf/Lf, among others (Krewulak and Vogel, 2008; Noinaj et al., 2010). Nevertheless, much like mammals, iron overload can be toxic to bacteria, which is why iron assimilation is tightly regulated. Therefore, the mechanisms described below are generally expressed in iron-limiting conditions.

4.1. Siderophore-based iron acquisition systems

Many bacterial species, including members of the microbiota and various human pathogens, secrete structurally diverse, high-affinity, iron-chelating molecules known as siderophores (Figure 2a) (Crosa, 1989). For many Gram-negative bacteria, siderophores are essential virulence factors. Enterobactin, a catecholate-type siderophore, and the related siderophore salmochelin, a C-glucosylated derivative of enterobactin, are the most common siderophores utilized by enterobacteria such as *E. coli*, *Klebsiella* spp., and *Salmonella enterica* (Figure 1) (Raymond et al., 2003). Other siderophores include yersiniabactin (produced by *Yersinia pestis* and *Y. enterocolitica*, but also by other *Enterobacteriaceae* including *E. coli* and *Klebsiella*), aerobactin (*E. coli*), and vibriobactin (*Vibrio cholerae*) (Miethke and Marahiel, 2007). Of note, in a process termed 'iron piracy', some bacterial species are capable of acquiring siderophores that they do not synthesize, generally termed xenosiderophores (e.g., *Salmonella* and *Yersinia* acquire, but do not make, ferrioxamine). Moreover, a subset of these bacteria do not produce siderophores at all (e.g., *Bacteroides thetaiotaomicron* does not produce siderophores, but can acquire enterobactin and salmochelin) (Figure 3b) (Zhu et al., 2020). As the biosynthesis of siderophores is an energy-intensive process, iron piracy via xenosiderophores is a lucrative iron-acquisition mechanism for the non-producing strains. In

addition to bacteria, fungi also produce and uptake siderophores. For reference, in 1952, the fungal siderophores ferrichrome and coprogen were among the first siderophores to be described (Hesseltine et al., 1952; Neilands, 1952). Furthermore, several fungal species including *Candida* spp. and *Saccharomyces* spp. engage in iron piracy by importing xenosiderophores (Almeida et al., 2009; Winkelmann, 2007).

During iron limitation, microbes release siderophores into the environment to capture iron. Importantly, siderophores' high affinity for ferric iron enables them to outcompete host proteins (e.g., Tf) for this metal (Caza and Kronstad, 2013). To recover the captured iron, Gram-negative bacteria express outer membrane receptors that import iron-laden siderophores in a TonB-dependent fashion. The evolutionary importance of siderophores is reflected by the co-evolution of siderophore neutralization strategies by mammals. As discussed in Section 3, the host antimicrobial protein Lcn2 efficiently neutralizes bacterial enterobactin; accordingly, *Lcn2*-deficient mice are highly susceptible to infection with microbes that utilize enterobactin for iron acquisition (Flo et al., 2004; Moschen et al., 2016). However, many bacterial pathogens circumvent Lcn2-mediated enterobactin sequestration by producing salmochelin, a chemically modified form of enterobactin that cannot be bound by Lcn2 (Fischbach et al., 2006; Raffatellu et al., 2009). Thus, salmochelin and other “stealth siderophores” enable pathogens to evade host nutritional immunity (Figure 1 and Figure 2b).

4.2. Heme acquisition systems

Heme is comprised of an iron ion bound to a porphyrin ring. Most of the heme in humans is associated with intracellular metalloproteins (hemoproteins) including hemoglobin, myoglobin, and cytochromes. Many bacterial pathogens have evolved specific mechanisms to liberate heme from vertebrate hemoproteins (Runyen-Janecky, 2013). Moreover, although free heme is limited and usually scavenged by host hemopexin or serum albumin, small amounts can become available to microbes, for example from lysed erythrocytes or from host cells damaged during infection (Runyen-Janecky, 2013). In the gut, dietary heme represents another source of iron that can be utilized by intestinal bacteria (Figure 2). In Gram-negative bacteria, general mechanisms of heme acquisition involve receptors that directly bind heme or hemoproteins (e.g., hemoglobin, hemopexin, haptoglobin) or involve scavenging and uptake of heme via bacterial hemophores (Runyen-Janecky, 2013). Heme is then internalized across the outer membrane via TonB-dependent transport, after which it is brought across the cytoplasmic membrane by ABC transporters. Once heme arrives in the cytoplasm, specific heme oxygenases cleave the porphyrin ring to release the iron (Runyen-Janecky, 2013).

One example of a Gram-negative pathogen that relies on heme for its growth is the “heme-loving” opportunistic human pathogen *Haemophilus influenzae*, which colonizes the upper respiratory tract and is a frequent cause of pneumonia, otitis, and meningitis. The iron-limited host environment triggers the pathogen to express several heme acquisition systems (Hup, HgpA, HgpB, HgpC, HxuA, HxuC), which in turn mediate acquisition of heme directly and from various hemoproteins (Runyen-Janecky, 2013).

Alternative bacterial strategies to obtain iron from heme include secreting hemolysins to lyse red blood cells or producing proteases that degrade hemoglobin. Such mechanisms are employed by several Gram-negative pathogens including pathogenic *E. coli*, *V. cholerae*, and *Bordetella pertussis*, as well as by the Gram-positive pathogen *Staphylococcus aureus* (Caza and Kronstad, 2013).

Heme uptake is also employed by certain fungal pathogens (Bairwa et al., 2017). The polymorphic fungus and opportunistic pathogen *Candida albicans* can be part of the commensal microbiota and inhabit the oral cavity, the GI tract, and/or the female reproductive tract. In most individuals, *C. albicans* is harmless because it is kept under control by the microbiota through nutrient competition. However, under circumstances such as immunodeficiency, antibiotic treatment, or immunosuppressive therapy, *C. albicans* can cause diseases ranging from cutaneous infections to life-threatening systemic candidiasis (Mayer et al., 2013). In addition to xenosiderophore uptake, *C. albicans* also relies on iron acquisition via heme/hemoglobin (Bairwa et al., 2017).

4.3. Transferrin/lactoferrin receptor-based systems

The iron-carrying host glycoproteins Tf and Lf exhibit structural similarities, and this is reflected in the similarities of bacterial Tf and Lf receptors (Gray-Owen and Schyvers, 1996). Tf in the serum is primarily exploited as a source of iron by pathogens during bloodstream or systemic infections, whereas Lf is generally leveraged by pathogens at mucosal surfaces (Morgenthau et al., 2013). Tf and Lf can be exploited by pathogens expressing bacterial outer membrane receptors including transferrin-binding-protein A and B (TbpAB) and lactoferrin-binding-protein A and B (LbpAB), respectively (Morgenthau et al., 2013). Both uptake systems function in a similar fashion in that the receptors remove ferric iron from Tf or Lf, and then transport the iron across the outer membrane in a TonB-dependent manner (Caza and Kronstad, 2013; Morgenthau et al., 2013). The specific mechanisms and the order of the steps, however, remain to be elucidated. The utilization of Tf or Lf as iron sources has been described for important human pathogens including *Neisseria* spp. and *Helicobacter pylori* (Caza and Kronstad, 2013; Morgenthau et al., 2013).

5. Regulation of mucosal iron metabolism during infection and inflammation

Mucosal surfaces including the eyes and oropharynx, as well as the GI, respiratory, and urogenital tracts, use various antimicrobial defense strategies that interfere with microbial adhesion and survival. Among these defenses is the upregulation of Lf and Lcn2. Although both host proteins play additional roles in other cells and body sites (Kruzel et al., 2017; Moschen et al., 2017), here we focus on their roles at mucosal surfaces.

5.1 Iron metabolism during intestinal inflammation

The lower GI tract is a complex ecosystem and harbors the most abundant and diverse microbial community of the human body (Huttenhower et al., 2012). The commensal gut microbiota is dominated by anaerobic microbes that mainly belong to the phyla *Firmicutes* and *Bacteroidetes*. *E. coli* and other *Enterobacteriaceae* family members (belonging to the

phylum *Proteobacteria*) typically comprise a minor fraction of the microbiota in the non-inflamed gut. One of the main mechanisms by which beneficial commensal microbes prevent the outgrowth of potentially harmful microbes is through the competition for iron. In the healthy gut, commensal microbes consume available micronutrients and heme, which provides an ideal environment for the microbiota to reside in. In turn, the gut microbiota provides a variety of beneficial metabolites to the host, such as B vitamins (Celis and Relman, 2020). This mutual relationship between the host and commensal microbes makes it difficult for pathogens to become established in these densely colonized host compartments (Vonaesch et al., 2018) (Figure. 3a).

Numerous intrinsic and extrinsic factors modulate the microbiota's composition. For example, intestinal inflammation (regardless of etiology) and antibiotic administration have a major impact on the microbiota which is typified by a decrease in microbial diversity (Bäumler and Sperandio, 2016). The inflamed gut exhibits an altered nutritional environment, generated in part by host-mediated iron limitation (Deriu et al., 2013; Raffatellu et al., 2009) (Figure 2b). Gut inflammation is also frequently associated with the loss of *Bacteroidetes* and *Firmicutes* in favor of a bloom of *Gammaproteobacteria* (usually members of the *Enterobacteriaceae*, Figure 3c) (Baumler and Sperandio, 2016). Overall, the observed decreases in microbial diversity generally lead to, and sometimes overtly favor, pathogen outgrowth. Lcn2 is highly upregulated in the gut as part of the inflammatory response to restrict blooms of harmful microbes (Moschen et al., 2016; Raffatellu et al., 2009), for instance during chronic intestinal inflammation. In this context, Lcn2 greatly prevented the exacerbation of colitis and spontaneous colonic tumor formation by impeding the growth of enterobactin-utilizing, pro-colitogenic bacterial species in mice (Figure 3c) (Moschen et al., 2016). Nevertheless, some successful enterobacterial pathogens, including *Salmonella*, *Klebsiella* and pathogenic *E. coli*, circumvent Lcn2-mediated iron restriction by secreting stealth siderophores including salmochelin, which cannot be sequestered by Lcn2 (Figure 2b) (Bachman et al., 2012; Fischbach et al., 2006; Flo et al., 2004; Raffatellu et al., 2009).

It is worth noting that Lcn2 can also restrict the growth of enterobactin-secreting commensal bacteria (Figure 2b). However, certain members of the commensal microbiota have, similar to pathogens, evolved resistance to some host antimicrobial responses. For example, beneficial species including EcN, *Bifidobacterium* spp., and *Lactobacillus* spp. exhibit some resistance to host-mediated iron limitation (Deriu et al., 2013; Vega-Bautista et al., 2019). Intriguingly, the aforementioned microbes have been administered as probiotics for a long time, despite the mechanistic basis of their beneficial properties being unknown. Recent studies, however, have shed light on some of these mechanisms. The probiotic EcN employs multiple systems to acquire iron from the intestinal environment, including multiple siderophores (enterobactin, yersiniabactin, aerobactin, salmochelin) (Valdebenito et al., 2006). These mechanisms of iron acquisition collectively increase EcN's ability to compete with other microbes, including *Salmonella*, during colitis (Deriu et al., 2013) (Figure 3b). Siderophore cross-feeding between different bacterial taxa offers another mechanism for commensals to obtain iron. For example, *B. thetaiotaomicron*, a member of the phylum *Bacteroidetes*, pirates xenosiderophores from commensal and pathogenic *Enterobacteriaceae*

(phylum *Proteobacteria*) in the inflamed gut, thereby contributing to the resilience of the commensal gut microbiota (Zhu et al., 2020) (Figure 3b).

Some studies in model organisms have proposed that the host might benefit from microbe-associated iron metabolism and sensing. A study by *Qi et al.* demonstrated that enterobactin produced by commensal *E. coli* increased mitochondrial iron levels and promoted growth of the worm *Caenorhabditis elegans* (Qi and Han, 2018). Another study reported that intestinal commensals such as *Lactobacillus* spp. can sense levels of iron in the gut and subsequently modulate the host's systemic iron metabolism. Here, microbe-derived metabolites suppressed the host transcription factor HIF-2 α and increased the expression of ferritin, thereby reducing systemic iron overload in an experimental model of hemochromatosis (Das et al., 2020). The microbiota has also been shown to modulate host expression of hepcidin in the gut (Bessman et al., 2020). In this study, commensal microbes were shown to induce hepcidin expression in dendritic cells, which in turn promoted iron sequestration by macrophages. In addition to modulating gut microbiota composition, this process also contributed to mucosal healing in an experimental IBD model (Bessman et al., 2020).

5.2. Iron in the context of inflammatory bowel disease

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), comprises a heterogeneous spectrum of chronic and/or relapsing episodes of intestinal inflammation. Alterations of the gut microbiota and microbial interactions with the perturbed mucosal barrier are prominent features of IBD (Caruso et al., 2020). The inflamed gut mucosa is also responsible for chronic GI blood loss and reduced intestinal iron absorption, which is frequently accompanied by iron deficiency and anemia of chronic disease (Nielsen et al., 2018). Iron supplementation in anemic IBD patients, however, differs from standard approaches, and several considerations need to be taken into account (outlined in (Nielsen et al., 2018)).

In animal models of IBD, it has been shown that high concentrations of luminal iron or heme iron alter gut microbial communities, further exacerbating the bloom of *Gammaproteobacteria* and worsening colitis (Constante et al., 2017; Mahalhal et al.; Werner et al., 2011) (Figure 3c). Importantly, these effects were only observed when iron was administered orally, whereas intravenous iron did not induce such changes. These findings are in line with a study in IBD patients, showing that oral iron therapy is associated with distinct and unfavorable changes in gut microbial ecology (Lee et al., 2017).

In stark contrast to these findings, a recently published study demonstrated that a short course of dietary iron supplementation attenuated the virulence of an enteric pathogen (Sanchez et al., 2018). Following infection of mice with the murine pathogen *Citrobacter rodentium*, a common model for human enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) infections, dietary iron supplementation promoted host survival by increasing intestinal glucose content and insulin resistance. Elevated glucose resulted in suppression of virulence factors in the pathogen, which resulted in the pathogen colonizing, but not killing, its host. This phenomenon was termed a 'cooperative response' by the authors (Sanchez et al., 2018). Although iron supplementation during infection has previously been shown to be detrimental to humans and model organisms by promoting the growth of pathogens, it is possible that oral iron supplementation may promote such a

cooperative response in some contexts of localized infection or microbiota composition. Future studies are needed to show whether this result applies to other microbes and in the context of other hosts.

In patients with ileal CD, a distinct pathovar of *E. coli* is abnormally prevalent. Termed adherent-invasive *E. coli* (AIEC), these mucosa-associated bacteria are able to adhere to and invade intestinal epithelial cells, a process that likely contributes to the progression of CD (Shaler et al., 2019). In addition to other virulence factors, iron metabolism likely plays a key role in AIEC's pathogenicity (Figure 3c). Iron acquisition systems that are most frequently found in pathogenic *Enterobacteriaceae*, such as the synthesis and uptake of yersiniabactin and salmochelin, are present in many AIEC strains. Moreover, the presence of a heme acquisition system correlates with AIEC's intracellular persistence in macrophages (Dogan et al., 2014). Notably, there are currently no treatments available that specifically eradicate CD-associated AIEC from the gut of these patients. Although the gut microbiota plays a key role in the pathogenesis of IBD, current standard treatments focus on targeting the immune system rather than the intestinal microbiota. Routine administration of antibiotics in IBD patients remains controversial because it further disrupts the gut microbiota, promoting bacterial dysbiosis. Moreover, antibiotics have been linked to potentiating AIEC infection and outgrowth (Shaler et al., 2019). Thus, antimicrobial strategies that selectively target pathogenic *Enterobacteriaceae* in IBD are highly desirable. Because iron metabolism is an important virulence factor for IBD-associated bacteria, developing strategies to impede pathogenic iron metabolism could be useful.

6. Iron metabolism in the respiratory and genito-urinary tract

6.1. The respiratory tract

Bacterial communities of the respiratory tract are more dynamic, albeit less well characterized, than those of the gut (Huffnagle et al., 2017). Similar to the gut, however, iron acquisition is also important for bacterial pathogens that infect the airways. Correspondingly, Lcn2 is also highly upregulated in human pulmonary tissue during bacterial pneumonia (Warszawska et al., 2013). However, whether this increased expression of Lcn2 results in beneficial or detrimental outcomes strongly depends on the pathogen. The Gram-negative pathogen *Klebsiella pneumoniae* is a common cause of bacterial pneumonia and sepsis. Clinical isolates of *K. pneumoniae* have been shown to either produce enterobactin alone, or a combination of enterobactin, salmochelin, and yersiniabactin (Bachman et al., 2011). Whereas salmochelin and yersiniabactin enabled *K. pneumoniae* isolates to evade Lcn2-mediated iron sequestration and to disseminate systemically, Lcn2 conferred substantial protection against isolates that only produce enterobactin (Bachman et al., 2012). Of note, siderophores in this context also exhibited a noncanonical role by acting directly on the host to induce inflammatory cytokines (Holden et al., 2016). In contrast to *K. pneumoniae*, the Gram-positive pathogen *Streptococcus pneumoniae*, the most prevalent bacterial cause of respiratory tract infections, acquires iron via hemoproteins instead of siderophores. Furthermore, instead of helping the host, increased Lcn2 expression worsened the outcome of *S. pneumoniae* infection by skewing macrophage polarization towards an anti-inflammatory phenotype (Warszawska et al., 2013). The differing impact of Lcn2 in

these examples highlights the protein's context-dependent roles during infection, which stem from its siderophore-neutralizing and immunomodulatory properties.

Increased iron levels have been observed in the sputum of patients with the chronic pulmonary disease cystic fibrosis (CF), which has implications for a range of CF-related bacterial and fungal pathogens (Tyrrell and Callaghan, 2016). For example, *Pseudomonas aeruginosa* is a frequent cause of CF exacerbations, and studies have shown that this pathogen has a plethora of iron acquisition systems, including heme uptake and the production of siderophores such as pyoverdine and pyochelin (Minandri et al., 2016; Vasil and Ochsner, 1999).

6.2. The genitourinary tract

Similar to other mucosal sites, the genitourinary tract is colonized by microbes, although distinct differences exist between males and females (Gottschick et al., 2017). The healthy female vaginal microbiota is largely dominated by members of the lactic acid-producing bacteria *Lactobacillus* spp. (Huttenhower et al., 2012). These iron-independent commensals together with host-derived Lf represent essential factors to maintain a healthy vaginal environment. Bacterial vaginosis (BV) is a common disorder in women of reproductive age and is predominantly caused by *Gardnerella vaginalis*. Besides BV, *G. vaginalis* can also cause urinary tract infections (UTIs), amniotic infections, and pelvic inflammatory disease. Numerous iron-acquisition mechanisms have been shown for this pathogen, including siderophore and heme utilization as well as Lf uptake (Jarosik et al., 1998).

UTIs are another common infectious disease in otherwise healthy women, which is complicated by the rise of antimicrobial resistance. Uropathogenic *E. coli* (UPEC) is the primary cause of such infections, and iron acquisition gene clusters have been identified as important virulence factors to successfully colonize the urinary tract (Garcia et al., 2011; Mike et al., 2016). Lcn2 is secreted by the urinary tract mucosa of women with UTI and represents a colonization barrier that pathogens must overcome (Steigedal et al., 2014). UPEC is able to successfully colonize this environment by importing heme and by deploying the stealth siderophores salmochelin, yersiniabactin, and aerobactin (Garcia et al., 2011; Mike et al., 2016). In light of recent findings indicating the presence of a urinary tract microbiome (the so-called urobiome) (Wolfe and Brubaker, 2019), future studies may elucidate mechanisms of competition for iron between pathogens and the urobiome.

7. Exploiting microbial iron acquisition for therapy and diagnosis

As many pathogens require iron during infection, their means of iron acquisition constitute attractive therapeutic targets (Figure 4). For example, as described above, many pathogens require siderophores for iron uptake. As such, siderophore immunization might be a promising approach to limit microbial iron acquisition during infection. To this end, immunization against yersiniabactin and aerobactin in the context of UTIs protected from systemic infection with UPEC (Figure 4a) (Mike et al., 2016). Similarly, immunization with the siderophore enterobactin, which resulted in the production of antibodies against both enterobactin and salmochelin, protected against intestinal *Salmonella* infection (Figure 4a) (Sassone-Corsi et al., 2016a). Intriguingly, the reduced *Salmonella* burden in enterobactin-

immunized mice was paralleled by increased levels of *Lactobacillus spp.* This could be due to a growth advantage of *Lactobacillus* over other commensals in the inflamed, iron-deprived intestine, although the overall impact of enterobactin immunization on the gut microbiota warrants further investigation.

Another way to exploit siderophore-mediated iron acquisition is by employing “Trojan horse” antimicrobials, wherein an antimicrobial molecule is conjugated to a natural product that is taken up by the targeted microbe. Although such compounds can be generated in the lab, it is worth noting that siderophore-antibiotic conjugates exist in nature; these so-called sideromycins constitute important fitness factors as they mediate microbial competition in nutrient-limited environments (Braun et al., 2009). One class of sideromycins are the microcins, which are comprised of an antibacterial peptide that is covalently attached to a siderophore (Braun et al., 2009). By secreting microcins, *EcN* is able to inhibit the growth of enteric pathogens during intestinal inflammation without broadly affecting the gut microbiota (Figure 4a, panel II) (Sassone-Corsi et al., 2016b). Inspired by sideromycins that exhibit narrow-spectrum activity, researchers have generated synthetic siderophore-antibiotic conjugates with the goal of more specifically targeting pathogens. For example, conjugating enterobactin to the broad-spectrum antibiotic ciprofloxacin yielded a narrow-spectrum antibacterial compound. This molecule only targeted *E. coli* that express the salmochelin system’s IroD esterase, an enzyme that hydrolyzes enterobactin and salmochelin, and is thus predicted to preferentially target pathogens instead of commensals (Neumann et al., 2018) (Figure 4b, panel I).

In addition to being a target for antimicrobial therapy, siderophores can be used directly as therapeutic and diagnostic agents. Desferrioxamine B (desferal), a siderophore naturally produced by *Streptomyces spp.*, is an iron chelator approved for use in humans to treat iron overload syndromes. Siderophores are also being investigated for use in the molecular imaging of bacterial and fungal infections (Petrik et al., 2017) (Figure 4c). As a proof of principle, the *Aspergillus* siderophore triacetylfusarinine C was labeled with a positron-emitting gallium radioisotope (^{68}Ga), then injected into mice and imaged by positron emission tomography (PET) to detect localization of the fungal pathogen *Aspergillus fumigatus* (Haas et al., 2015). This approach could be helpful to better diagnose and monitor infectious agents in complex disease scenarios.

8. Conclusions

With antimicrobial resistance posing an increasing threat to humans, alternative strategies to combat infections are in urgent need. As iron is a key micronutrient for most microbes, its abundance is tightly controlled by the host in order to restrict microbial growth. In addition to host-encoded mechanisms of iron sequestration, the microbiota also contributes to reduce iron availability to pathogens. Thus, to colonize the host, pathogens often deploy a variety of virulence factors that evade or subvert these iron-withholding mechanisms. Targeting microbial iron metabolism and virulence factors associated with iron acquisition hold promise as therapeutic strategies to reduce pathogen colonization and limit the severity of infection. Furthermore, such strategies could be advantageous for shifting the composition of the microbiota at mucosal surfaces by promoting the growth of beneficial microbes (e.g.,

Bifidobacterium and *Lactobacillus spp.*). These properties are particularly attractive in the context of diseases with underlying dysbiosis, including IBD. Collectively, iron metabolism at the host-microbe interface constitutes an important area for future investigations and for the potential development of novel therapeutics.

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Figure 1. Evasion of Lcn2-mediated antimicrobial activity.

Members of the family *Enterobacteriaceae* can secrete siderophores such as enterobactin and salmochelin into the intestinal lumen to scavenge iron from the host environment. Some immune cells (e.g., neutrophils) and epithelial cells (in response to cytokines including interleukin-22) produce Lcn2, which sequesters enterobactin and thus limits the growth of enterobactin-dependent bacteria by depriving them of iron. In contrast, salmochelin and other 'stealth' siderophores enable certain bacteria (mostly pathogens) to evade Lcn2-dependent iron limitation.

Figure 2. Model of host-microbe competition for iron in the gut during homeostasis and disease. (a) Under homeostatic conditions, members of the commensal microbiota acquire iron in the form of heme or by scavenging ferric iron with siderophores such as enterobactin. Low levels of antimicrobial proteins including lactoferrin (Lf) and lipocalin-2 (Lcn2) are present in the gut lumen. Lf sequesters luminal ferric iron, whereas Lcn2 sequesters iron-laden enterobactin. (b) Antibiotic treatment, infection and inflammation can induce IL-22-mediated secretion of Lcn2 from intestinal epithelial cells, and of Lcn2 and Lf from neutrophils that are recruited to the gut. The consequent iron limitation, in turn, induces commensals and pathogens to upregulate various iron uptake mechanisms, including increasing production and secretion of siderophores. Many enteric pathogens secrete stealth siderophores that promote evasion of lipocalin-2-mediated iron sequestration, thus enabling these pathogens to outcompete the gut microbiota and thrive.

Figure 3. Iron acquisition by pathogens and commensals.

(a) In the healthy gut, the microbiota is primarily comprised of obligate anaerobes (the phyla *Firmicutes* and *Bacteroidetes*). These commensals occupy local niches, consume luminal nutrients and uptake iron (Fe), thereby providing colonization resistance to pathogenic microbes. (b) Intestinal inflammation causes a dramatic reduction in luminal Fe content. The enteric pathogen *Salmonella* is able to scavenge Fe^{3+} in this environment, due to the production of salmochelin. However, secretion of multiple siderophores enables the probiotic bacterium *E. coli* Nissle 1917 to outcompete *Salmonella*. Moreover, the commensal bacterium *Bacteroides thetaiotaomicron* sustains colonization during inflammation by utilizing xenosiderophores, a critical mechanism to restore its original colonization state after inflammation is resolved. (c) IBD is characterized by chronic relapsing or remittent intestinal inflammation. During colitis, the increased secretion of host antimicrobial proteins lactoferrin (Lf) and lipocalin-2 (Lcn2) limits the availability of iron to gut microbes. However, the low Fe content induces the upregulation of bacterial Fe uptake genes, which contributes to the blooms of *Proteobacteria* frequently observed. Even high luminal Fe content (e.g., due to Fe supplementation) promotes the growth of *Proteobacteria* in the inflamed gut. In patients with Crohn's disease, adherent-invasive *E. coli* (AIEC) strains express a variety of Fe-uptake genes, which contribute to the pathogen's growth during colitis.

Figure 4. Siderophores as therapeutic targets and diagnostic tools.

(a) During infection, various siderophores are secreted by pathogens to thrive in the host despite host-mediated iron limitation. In experimental models, siderophores have been targeted for vaccination by linkage to a carrier protein in order to generate anti-siderophore antibodies. Whereas pathogens (e.g., *Salmonella*, UPEC) were able to successfully replicate in naïve hosts, siderophore-based immunization reduced their colonization levels. **(b)** Conjugation of antimicrobial peptides or antibiotics to siderophores. **(b.I)** An enterobactin-ciprofloxacin conjugate is transported through a common siderophore receptor found in the outer membrane of all *E. coli* strains. Once in the cytoplasm, the IroD esterase, which is mainly found in pathogenic *E. coli* strains, processes the enterobactin-ciprofloxacin prodrug and thereby enables ciprofloxacin-mediated inhibition of DNA synthesis. **(b.II)** Certain strains of *Enterobacteriaceae*, such as *E. coli* Nissle or *K. pneumoniae*, secrete microcins in response to nutrient (e.g., Fe) limitation. It is generally thought that the siderophore moiety enables these small antimicrobial peptides to better target susceptible strains of *Enterobacteriaceae* by utilizing their siderophore receptors for import. **(c)** Microbial siderophore uptake during infection can be leveraged for imaging applications. During lung infection, *Aspergillus* scavenges iron (Fe) via siderophores. Injection of siderophores radiolabeled with ^{68}Ga in experimental *Aspergillus* infections enabled the visualization of the lung infection site by PET/CT.