Low dietary iron intake restrains the intestinal inflammatory response and pathology of enteric infection by food-borne bacterial pathogens

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Running head: Low dietary iron lowers gut inflammatory markers

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Abstract

Orally administrated iron is suspected to increase susceptibility to enteric infections among children in infection endemic regions. Here we investigated the effect of dietary iron on the pathology and local immune responses in intestinal infection models. Mice were held on iron-deficient, normal-iron, or high-iron diets and after two weeks they were orally challenged with the pathogen *Citrobacter rodentium*. Microbiome analysis by pyrosequencing revealed profound iron- and infection-induced shifts in microbiota composition. Fecal levels of the innate defensive molecules and markers of inflammation lipocalin-2 and calprotectin were not influenced by dietary iron intervention alone, but were markedly lower in mice on the iron-deficient diet after infection. Next, mice on the iron-deficient diet tended to gain more weight and to have a lower grade of colon pathology. Furthermore, survival of the nematode *Caenorhabditis elegans* infected with *Salmonella enterica* serovar Typhimurium was prolonged after iron-deprivation. Together, these data show that iron limitation restricts disease pathology upon bacterial infection. However, our data also showed decreased intestinal inflammatory responses of mice fed on high-iron diets. Thus additionally, our study indicates that the effects of iron on processes at the intestinal host-pathogen interface may highly depend on host iron status, immune status and gut microbiota composition.

Introduction

Iron deficiency is highly prevalent among the world and has major health consequences [1, 2]. Oral iron administration programmes can effectively correct iron deficiency [3], but concerns have been raised regarding the safety of iron supplementation. There is evidence suggesting that untargeted oral iron supplementation in regions with high prevalence of malaria transmission and infectious diseases can cause an increase in infections, hospital admission and mortality in young children [4-6]. This might be at least partly ascribed to iron also being an essential requirement for the growth of most bacterial species [7]. Importantly, iron uptake by the upper intestine is generally limited [8], which results in a large fraction of unabsorbed iron entering the colon, being potentially available for the gut microbiota. It is therefore not surprising that iron has been shown to influence the gut microbiota composition in a number of studies, among which were two studies among African children and infants which showed that iron fortification caused a potentially more pathogenic gut microbiota profile [9-11].

In the past few years it thus became apparent that supplementary iron can have a large impact on the gut microbiota composition, but the potential effects on host immune responses remained largely unexplored. Given the importance of the gut microbiota in shaping the host intestinal immune system [12] this issue however deserves further investigation as dietary iron could have an indirect effect on the responsiveness of the immune system via alteration of the gut microbiota [13]. Furthermore, host iron metabolism is largely intertwined with host immunity and it is known that host iron status affects the inflammatory response to pathogenic invaders [14]. We previously showed that the dysregulated iron metabolism in a mouse model of type I hemochromatosis resulted in an attenuated host immune response against *Salmonella enterica* serovar Typhimurium in the gastrointestinal tract [15]. Importantly, also iron deficiency is associated with an impaired immune response, but may remarkably increase the resistance against intracellular pathogens, probably due to increased nutritional immunity [6, 16, 17].

As iron status can affect the immune response it is likely that also the array of antimicrobial defenses that is secreted from the intestinal mucosa gets affected. Enterocytes and Paneth cells secrete

antimicrobial peptides (AMPs) such as defensins, cathelicidins and lipocalin-2 (a.k.a. neutrophil gelatinase-associated lipocalin (NGAL) or siderocalin). Lipocalin-2 is a molecule of our special interest as it is involved in host iron homeostasis and because it can prevent bacterial iron uptake via iron-scavenging siderophores, which they produce under iron-limiting conditions [18]. The importance of lipocalin-2 based defense is demonstrated by a study showing that lipocalin-2 knockout mice had an increased susceptibility to bacterial infection [19]. In the intestine, lipocalin-2 is only weakly expressed under normal conditions, but increases markedly during intestinal inflammation mainly due to the influx of neutrophils which secrete large amounts of lipocalin-2 [20-23].

The effects of supplementary iron have not yet been investigated during gastroenteritis caused by a bacterial pathogen in an animal model. This is now warranted as the last few years it became apparent that intestinal inflammation can be exploited by specific enteric pathogens, such as *Salmonella* spp. and *Citrobacter* spp., a process leading to dysbiosis [24] and which may be influenced by supplementary iron [11]. To get more insight in the effects of iron during gastroenteritis we here for the first time examined the effects of dietary iron depletion and supplementation on the mouse gut microbiome and on intestinal immunity and pathology. We focused on the expression of intestinal lipocalin-2, which may be affected either through direct effects of iron on mammalian cells or indirectly through an altered gut microbiota. To investigate the effects of these iron-related modulations on the outcome during gastroenteritis we orally challenged mice with the mouse pathogen *Citrobacter rodentium*. This well-established model for infectious gastroenteritis closely mimics the pathology caused by human food-borne bacterial pathogens [25-27]. We show that mice on an iron-deficient diet as well as mice on a high iron-supplemented diet showed a decrease in fecal lipocalin-2 levels during intestinal inflammation, which may have result in impaired host defence against siderophilic pathogens in these mice. We also show that iron limitation restrains the pathology of enteric infection in a simple gut nematode model.

Results

General health and systemic responses to iron intervention and *Citrobacter rodentium* challenge

Previous studies have shown that the intestinal infection after ingestion of *C. rodentium* by mice closely mimics human colitis caused by food-borne bacterial pathogens, such as enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC respectively) [25-27]. Here we used this mouse model to investigate the effect of dietary iron levels on disease pathology by placing mice for 28 days on diets with three different iron levels (deficient, normal, high), with a *C. rodentium* challenge 14 days after start of the dietary intervention (**Figure 1A**). As expected, dietary intervention was effective in changing tissue iron stores at day 14 and 28 and fecal iron concentrations among the three groups at day 14 (**Figure 1B, C and Figure S1B**). Dietary iron intervention had no effect on strictly regulated serum iron levels at day 14 (**Figure S1A**).

Body weight as a measure for general health

During the period of iron intervention alone, body weight as a measure for general health was similar for all three groups, but body weights of mice tended to differ rapidly among groups after initiation of infection at day 14 ($p = 0.085$) (**Figure 1D**). Interestingly, mice on the iron-deficient diet seemed to recover most quickly as only this group had at day 28 an average body weight that was above that of day 14, while mice on a diet with normal iron content tended to suffer most from the colitis.

Lipocalin-2 levels in the circulation

Dietary iron intervention for two weeks in uninfected mice had no effect on serum lipocalin-2 levels, but systemic lipocalin-2 levels were markedly higher in the infected mice at day 28 compared to uninfected mice at day 14 (**Figure 2A**). Intestinal inflammation thus induces production of lipocalin-2 which can be detected systemically. In addition, systemic lipocalin-2 levels in infected mice were different among groups ($p = 0.002$) and highest in the mice on the normal-iron diet, which was significantly different from mice on the iron-deficient diet ($p < 0.05$) (**Figure 2A**).

Hepcidin as a marker for iron demand and inflammation

As mentioned above, the iron diets did not affect systemic iron levels. Systemic iron levels are mediated by the key iron-regulatory peptide hormone hepcidin (Hep-1 in mice), which blocks the ferroportin transporter in macrophages and the duodenum. Among others, hepcidin expression is regulated by host iron status and inflammation [28]. We here aimed to assess the effect of dietary iron on Hep-1 induction by colitis. As shown in **Figure 2B**, nearly all mice on the normal and high-iron diets had plasma Hep-1 levels that were clearly above those found in an independent group of uninfected reference control mice [29] on a standard diet ($p < 0.05$ for both comparisons), but were not markedly different from each other. In contrast, a subset of mice on the iron-deficient diet had very low Hep-1 levels that were just below the hepcidin levels found in uninfected control mice.

Intestinal responses to dietary iron intervention and *Citrobacter rodentium* challenge

Colon histopathology

Histological examination of colon sections at day 28 did not show statistical significant differences among groups, but did show a tendency for a linear trend towards a lower average histopathology score for mice on the iron-deficient diet compared to the normal and high-iron diets, with a large within-group variation ($p = 0.087$) (**Figure 3A**). Notably, this observation could fit with the observation that mice on the iron-deficient diet appeared to suffer less from the colitis as indicated by the outcome on body weights. However, based on the body weights, mice on a normal-iron diet seemed to suffer more from colitis than mice on the high-iron diet, something which is not evident from the histopathological examination.

Colon pro-inflammatory cytokine secretion ex vivo

To evaluate the effects of intestinal infection during dietary iron intervention on the intestinal cytokine response, colon sections were sampled at day 28 (14 days after *C. rodentium* challenge) and *ex vivo* secreted levels of IL-6, TNF-α and IL-17 were measured by ELISA. **Figure 3B** shows that secreted levels from animals on the iron-deficient diet were lowest for all three pro-inflammatory

cytokines. For IL-17 this was significantly different from the normal-iron diet ($p < 0.01$), but secreted IL-17 was also significantly lower in the high-iron group ($p < 0.05$). Secreted IL-6 levels showed a similar tendency. TNF-α tended to increase with increasing amounts of iron in the diet (not significantly). Together, these findings suggest that the intestinal cytokine response is about maximum at normal dietary iron levels, and which generally appears diminished at a low dietary iron level.

Fecal levels of innate defense molecules lipocalin-2 and calprotectin

Analogous to findings in serum/plasma, ELISA measurements in feces showed that lipocalin-2 levels were low and not significantly different among groups after iron intervention alone at day 13. Lipocalin-2 levels were however markedly increased after infection at day 27, most prominently in the feces of mice on the normal-iron diet. After infection, fecal lipocalin-2 levels were significantly higher in the normal-iron group compared to both the iron-deficient and high-iron group ($p < 0.01$ and $p < 0.05$ respectively) (**Figure 4A**).

Fecal calprotectin is a common and validated marker for intestinal inflammation [30]. We therefore measured fecal calprotectin next to fecal lipocalin-2, to examine whether the effect of dietary iron on lipocalin-2 levels were also found on calprotectin levels and to examine the grade of inflammation. Interestingly, like lipocalin-2 levels, fecal calprotectin levels were increased at day 27 compared to day 13 and most prominently in the normal-iron diet group, which was significantly different from the high-iron group ($p < 0.05$) (**Figure 4B**). Notably, although levels of lipocalin-2 were generally still higher in inflamed mice compared to non-inflamed mice, fecal calprotectin levels in inflamed mice on the high-iron diet were mostly not elevated compared to levels in non-inflamed mice. The similar response of lipocalin-2 and calprotectin is underscored by the finding that their fecal levels had a strong correlation (Pearson $r = 0.50$; $p = 0.007$) (**Figure 4C**). Together, these findings suggest that the intestinal inflammatory response was blunted in both the iron-deficient and high-iron diets.

The combined effect of iron intervention and intestinal inflammation on the gut microbiome

Effects of dietary iron intervention and colitis on the gut microbiome profile

On average, 4,875 bacterial 16S rDNA sequences per sample were analyzed by pyrosequencing. At baseline, the mouse gut microbiome consisted of the phyla Bacteroidetes (71.2% of the 16S rDNA reads), Firmicutes (27.3%), Tenericutes (0.8%) and Actinobacteria (0.6%). After 2 weeks of dietary intervention the phylogenetic diversity index (a measure of α-diversity) was decreased in the iron-deficient group compared to baseline ($p < 0.05$), in the normal and high iron groups a significant decrease was only observable at day 27. Interestingly, the diversity was lower in the high-iron group compared to the normal-iron group at day 13 ($p < 0.05$) and at day 27 the diversity in the iron-deficient group was significantly higher compared to the high-iron group ($p < 0.05$) (**Figure 5**). This analysis shows that during infection mice that were held on the iron-deficient diet were able to maintain part of their gut microbial diversity.

Multivariate Redundancy Analysis (RDA) shows that the gut microbiome of mice at baseline was similar for all dietary iron groups ($p = 0.422$), as expected. Clearly, after two weeks of dietary iron intervention, the mice had a distinct non-overlapping gut microbiome composition ($p = 0.002$)(**Figure 6A**). Correspondingly, hierarchical clustering analysis clearly clustered the baseline microbiomes together and separated them from the later time points (**Figure 6C**). The most prominent change at day 13 appeared to be the shift from a *Barnesiella* dominated profile to an *Allobaculum* dominated profile in mice on the high-iron diet.

After infection at day 27 the dietary iron intervention also elicited a distinct non-overlapping gut microbiome profile ($p = 0.002$). Additionally, the gut microbiome profile of each group at day 27 did not overlap with the groups at day 13 (**Figure 6A**). Next, hierarchical clustering analysis indicates that the microbiomes of the mice on the normal-iron and high-iron diets had most extensively changed (**Figure 6C**). Again, the most prominent change was a shift from a *Barnesiella* dominated profile to an *Allobaculum* dominated profile. The microbiome of the mice on the iron-deficient diet also changed and was dominated by *Parabacteroides* at day 27. Together, these analyses show that dietary iron had a large impact on the mouse gut microbiome, especially during intestinal inflammation. In the subsequent section the differences among groups in both the dominant and subdominant taxa are shown in more detail.

Effects of dietary iron intervention and colitis on the relative abundance of gut microbial taxa

The relative abundances of all detected bacterial taxa among groups at day 13 and day 27 were compared in an explorative manner. At day 13 the overall main differences between diets with a higher iron level compared to diets with a lower iron level were an increased abundance of *Clostridium* and a lower abundance of *Dorea* (**Figure S2**). Although the normal-iron group did not show a lower abundance of *Lactobacillus* and *Bifidobacterium* compared to the iron-deficient group, in the high-iron group there was a consistent relative lower abundance of these taxa compared to the iron-deficient and normal-iron groups. Furthermore, *Peptococcus*, *Bacteroides* and *Allobaculum* were consistently more abundant in the high-iron group (**Figure S2**), which is also indicated by multivariate RDA (**Figure 6B**).

After infection at day 27, main differences were a higher abundance of *Allobaculum* and *Enterorhabdus* in the normal/high-iron groups compared to the iron-deficient group ($p = 0.005$ and $p = 0.004$ respectively for both the normal-iron and high-iron groups vs the iron-deficient group). In addition, in the high-iron group a higher abundance of *Bacteroides* was found compared to both the iron-deficient and the normal-iron groups ($p = 0.004$ for both). For the taxa *Bifidobacterium*, *Lactobacillales*/*Lactobacillus* and *Parabacteroides* we consistently found a lower abundance in all comparisons, meaning that that their abundance was lower in the normal/high-iron groups compared to the iron-deficient group, as well as in the high-iron group compared to the normal-iron group (**Figure 7**). Multivariate RDA also generally associates the taxa mentioned in this paragraph with the respective dietary iron groups (**Figure 6B**).

Citrobacter was lowly abundant relative to all 16S rDNA reads at day 27, but was not detected at all in samples from baseline and day 13 by pyrosequencing. We therefore performed a qPCR specific for *C. rodentium* to be able to quantify this low-abundant pathogen which initiated the intestinal inflammation. This analysis showed that there were no differences in the abundance of *C. rodentium* among the dietary groups at day 27 (data not shown). This suggests that *in vivo C. rodentium* colonization was not influenced by the iron diets at this point. However, differences may have existed at an earlier time point after infection, which was not assessed in this study.

Iron limitation prolongs survival of *Salmonella*-infected nematodes

To confirm the potential protective effect of iron limitation on the pathology of enteric infection as observed in our mouse model, we investigated the effect of iron on the pathogenicity of the human gut pathogen *S*. Typhimurium in a live nematode gut model. This has previously been shown to be a suitable model host for *S*. Typhimurium infection [31]. Survival of *C*. *elegans* that forages on *S*. Typhimurium decreased in the presence of increasing concentrations of iron. This was reflected in the nematode survival time (LT50), which was on average decreased by 1.3 and 2 days in the 10 and 100 μmol/L ferric ammonium citrate conditions respectively, compared to the no-iron condition ($p < 0.01$ and $p < 0.001$, respectively) (**Figure 8**). Furthermore, the AUCs of the survival curves of the conditions with 10 and 100 μmol/L ferric ammonium citrate were significantly lower compared to the no-iron condition over the course of the experiment ($p < 0.05$ and $p < 0.001$ respectively) (**Figure S3**). At the same time this iron-dependency was absent when *C*. *elegans* foraged on a *E*. *coli* control strain. These data confirm a subtle, but reproducible, protective effect of low dietary iron intake on the pathology of enteric infection. Importantly, these data also confirm that iron can increase the virulence of an enteric pathogen as we previously showed *in vitro* [32].

Discussion

It is well known that both oral iron administration and intestinal inflammation can alter the gut microbiota composition and that host iron status influences the inflammatory response [11]. Furthermore, oral iron administration has been associated with increased levels of fecal calprotectin (indicating increased gut inflammation) and with an increased incidence of diarrhea [9, 10, 33]. Nonetheless, little is known about the effects of nutritional iron on the gut microbiota composition and the host immunological response during periods of intestinal inflammation. We therefore investigated the effects of iron on pathology, gut microbiota composition and host intestinal immune responses in the non-inflamed and inflamed colon and we here for the first time show that dietary iron has profound effects on the gut microbiome composition and on the host immune response during colonic infection by common food-borne bacterial pathogens in a mouse model.

Although iron diets by themselves had clearly effect on luminal iron content and tissue iron stores as expected, we found no effects on general health as reflected by body weights and the immunological parameters lipocalin-2 and calprotectin that we measured at this point. Remarkably, there were profound effects on the gut microbiome composition. Compared to the composition at baseline, all dietary groups had a changed microbiota after 2 weeks, which can probably be explained by the change in diet at the start of the intervention. The gut microbiomes shifted towards an *Allobaculum* dominated profile, which was most apparent for the high-iron diet group. This group also showed a lower relative abundance of the beneficial *Bifidobacteriaceae* and *Lactobacillaceae* families compared to the normal-iron and iron-deficient diets, which is one of the most consistent findings of dietary iron intervention studies so far [11].

As expected, *C. rodentium* challenge clearly resulted in colonic inflammation as observed by colon histopathology and determination of the inflammatory parameters lipocalin-2 and calprotectin. Although iron intervention had only limited effect on the grade of inflammation as determined by histopathological examination, there appeared to be a minor trend towards a higher grade of inflammation with supplementary iron. This needs further confirmation, but it fits with previous studies showing that supplementary iron during IBD exaggerates colitis in animal models and

gastrointestinal complaints in IBD patients [34-39]. Our experiments also indicate that mice on the iron-deficient diet suffered slightly less from the colitis compared to the other groups as reflected by mouse body weights. Interestingly, previous studies showed that non-infected rats on an iron-deficient diet for 5 weeks gained less weight and ate less, while also mice on an iron-deficient diet had a lower body weight compared to control mice after 12 weeks of intervention [40, 41]. This suggests that an iron-deficient diet in a non-inflamed situation tends to decrease weight gain on the longer term, while it may prevent weight loss during intestinal inflammation.

Another systemic effect of the colonic inflammation was an increase in hepcidin levels. Importantly, several infected mice on the iron-deficient diet had lower hepcidin levels compared to uninfected reference mice. These findings are in-line with previous human studies showing that severely anemic infants and children in Africa with elevated inflammatory markers did not always have concurrent elevated hepcidin levels [42, 43]. This suggests that also in mice, a threshold body iron level is required for a physiological Hep-1 upregulation upon intestinal infection. If body iron levels are below this threshold, the iron demand, which asks for low hepcidin, renders these animal non-responsive to the infection stimulus. Analogous to hepcidin, lipocalin-2 has a role in iron homeostasis and is involved in innate immunity by withholding iron from bacterial pathogens [44]. Iron withholding by lipocalin-2 is established by the direct sequestering of iron-containing bacterial siderophores, for example in the gut lumen [11]. It is known that systemic lipocalin-2 levels are elevated in patients with IBD, but the origin of circulating lipocalin-2 is not clear and could be attributed to the activation of both local and distant immune cells [45-47]. Notably, levels of lipocalin-2 (secreted by intestinal epithelial cells and infiltrating immune cells) have also been shown to increase in the inflamed gut [48-50]. It has therefore been suggested that fecal lipocalin-2 can be used as a non-invasive marker for gut inflammation [49]. Remarkably, our study shows that both the iron-deficient diet and the high-iron diet lowered lipocalin-2 production compared to the normal-iron diet and that the effects were very similar on the local and systemic level. Fecal calprotectin has been known as a marker for gut inflammation for a long time [30, 51] and contributes to innate immunity by binding of zinc and manganese, hereby preventing uptake of these micronutrients by bacteria [52]. We here show that the calprotectin response of infected mice on the high-iron diet was blunted, which

was in line with fecal lipocalin-2 levels. As the histopathological score of these mice appeared not to be lower, these findings suggest that calprotectin and lipocalin-2 as a marker for gut inflammation may be less reliable during the provision of a high-iron-supplemented diet. The lower levels of these inflammatory markers may also indicate a reduced innate immune defense. From a functional point-of-view, the consequences of lower lipocalin-2 levels during high-iron conditions might be limited as it is likely that no or less siderophores are produced by intestinal pathogens in that case. In contrast, during iron-limiting conditions, when there likely is siderophore production, it may be advantageous to intestinal pathogens that lipocalin-2 levels are lower. Notably, certain intestinal pathogens can exploit the host lipocalin-2 and calprotectin based defense to their own benefit [22, 53]. It may be envisaged that lower levels of lipocalin-2 and calprotectin abate these exploitation mechanisms. This is however subject for further investigations.

It is difficult to explain why both the iron-deficient diet and the high-iron diet lowered lipocalin-2 production compared to the normal-iron diet. We hypothesized that local lipocalin-2 production could be influenced by a combination of luminal iron concentration and bacterial siderophore production as it would make sense that expression of lipocalin-2 is highest when readily available iron is low and when siderophores are being produced. This does however not fit with the lower levels of lipocalin-2 in the iron-deficient group compared to the normal-iron group. In addition, pro-inflammatory cytokine secretion and fecal calprotectin levels generally showed the same trend, pointing at a more general mechanism. As already mentioned, both host iron deficiency and iron overload have been associated with an attenuated immune response [6, 15-17] and it might thus very well be that the iron status of mice contributed to a decrease in levels of pro-inflammatory cytokines, lipocalin-2 and calprotectin. Other explanations could lie in the effects of dietary iron and host iron status on the gut microbiome and whether *C. rodentium* and other intestinal pathogens were able to thrive in the intestinal lumen and trigger the host immune response. Interestingly, it has recently been described that the gut microbiota composition is an important factor in the susceptibility to *C. rodentium* infection and the subsequent immune response [54].

After 2 weeks of infection, *C. rodentium* abundance in the feces was not different among groups, but we cannot exclude that earlier during infection *C. rodentium* thrived best in the colonic

lumen of mice on the normal-iron diet. We nevertheless found profound effects of dietary iron and colitis on the gut microbiome composition, which may have contributed to the effects on the host inflammatory response. *Allobaculum*, that dominated the gut microbiome of mice on the normal-iron and high-iron diet, could play a role. However, very little is known about *Allobaculum* and its effects on the host inflammatory response. Of note, *Enterorhabdus* virtually only appeared after infection and its relative abundance was highest in the normal-iron group, in which also fecal lipocalin-2 and calprotectin levels were highest. Although further research to a potential causal relationship is needed, it could imply that *Enterorhabdus* spp., of which little is known, contributed to the effects on the host inflammatory response. It may be hypothesized that the gut microbiome profile of the mice on the iron-deficient diet was potentially less pathogenic compared to the diets with iron as the relative abundance of the beneficial *Bifidobacteriaceae* and *Lactobacillaceae* was highest in the iron-deficient group. For *Lactobacillaceae* this may be expected as they, unlike most bacteria, do not require iron for their growth, and have previously been shown to decrease upon *C. rodentium* infection [55, 56]. Interestingly, mice on the iron-deficient diet had a *Parabacteriodes* dominated gut microbiota profile. This could have contributed to a lower histopathology score and reduced inflammatory response, as it has been described that the abundance of *Parabacteroides* is lower in patients with IBD than in healthy volunteers [57], and that *Parabacteroides* antigens have been shown to exert anti-inflammatory effects in DSS-treated mice [58]. It has previously been shown that *C. rodentium* infection in mice caused a temporal increase in the relative abundance of *Enterobacteriaceae* (of which *C. rodentium* itself is a member) 1 week after initiation of infection, with a decrease to 2 weeks after infection [59].. In the present study, we only analyzed the fecal gut microbiome 2 weeks after initiation of infection and found indeed a very low relative abundance of potentially pathogenic *Enterobacteriaceae*.

Although our experiments provide leads for the possible mechanisms behind the net effects of the interventions, the design did not allow to dissect the complex interplay of dietary iron, host iron status and the gut microbiome on the host intestinal immune response. To dissect the underlying mechanisms further, and to address our thought-provoking findings, future studies are needed. For example a similar mouse study, but with a larger number of mice to increase power, could be

complemented with non-infected control mice that are more closely followed for 4 weeks. To allow exclusion of host iron status effects, mice that receive oral iron treatment during infection only, can also be included. In germ-free mice, effects of the gut microbiota can be excluded. Our analyses can be extended with measurements of other immune factors such as expression of AMPs, a larger panel of cytokines, and analysis of host gene expression by e.g. microarray. It remains however difficult to investigate the interplay between the gut microbiome and host iron status as both host iron status and dietary iron content have been reported to affect the gut microbiome composition, making it very hard to unravel the effects of host iron status on the gut microbiome composition and vice versa [11].

In summary, our descriptive data suggest the existence of a maximum in the inflammatory response with regard to dietary iron content, with the peak lying around the normal daily intake of iron, something which has not previously been described in literature. Mainly the iron-deficient diet dampened the intestinal inflammatory response, and which also seemed to lower intestinal pathology. The latter was however more evident in our simple nematode gut model, in which iron-limitation restrained pathology as reflected by a prolonged survival of *C*. *elegans* that foraged on the intestinal pathogen *S*. Typhimurium under iron-limitation. Furthermore, it confirms that supplementary iron can enhance the virulence of *S*. Typhimurium as we previously have shown *in vitro* [32]. These findings underscore an undesired combination of a possible increase in the virulence of intestinal pathogens, and a decrease of host intestinal defenses at the same time. This undesired combination may provide intestinal pathogens with increased opportunities to evade the host immune response during oral iron therapy and strengthens the idea that oral iron administration programs in developing countries need to be set up with the highest amount of care, with close monitoring until the remaining questions about the actual effect of iron at the intestinal host-microbiota interface have been unraveled. Future research should also be directed at finding iron formulations that do not affect the gut microbiome to a large extent. Together, our data support the hypothesis that low iron intake is predictive against intestinal infection and inflammation, but also suggest that the clinical outcomes of oral iron administration may highly depend on the iron status, immune status and the gut microbiota composition of children that receive oral iron treatment.

Materials and methods

Animals, iron diets and *Citrobacter rodentium* challenge

The mouse trials were performed in four separate experiments, allowing the analysis of an increased number of different parameters. For all experiments, female, 4-6 weeks old C57BL/6 mice from Jackson Laboratories were group-housed and placed on diets with different concentrations of iron (iron-deficient, normal-iron, and high-iron). The iron-deficient diet (Harlan laboratories) contained 2-6 mg of iron per kg chow. The normal-iron diet (control diet; 45 mg/kg iron) was either obtained pre-prepared from Harlan (experiments 1-3), or was prepared by adding ferrous sulfate to the iron-deficient diet to give a total of 45 mg/kg iron (experiment 4). To obtain the high-iron diet, enough ferrous sulfate was added to either the pre-prepared normal-iron diet (experiments 1-3) or iron-deficient diet (experiment 4) to provide a total of 225 mg iron per kg chow. We note that rodent diets with natural ingredients may contain up to ±200 mg iron per kg chow, but for widely used standardized diets the recommended normal iron content is about 35 mg/kg [60]. Mice in experiments 1 (n=3 per group) and 2 (n=3-4 per group) were placed on the iron diets for 2 weeks after which they were euthanized to investigate the local and systemic effects of dietary iron intervention alone. For animals in experiment 3 (n=5 per group) and experiment 4 (n=5 per group) these two weeks were followed by a challenge with *C. rodentium* (strain DBS100; from the American Type Culture Collection) through oral gavage. To this purpose, bacteria were grown overnight in Luria broth (LB) and resuspended in PBS before administration to the mice (0.2 mL/mouse; ~$5*10^8$ CFUs). Diets were continued during infection. Body weights were monitored during the course of infection and stool samples were sampled at appropriate time points. Animals were euthanized 2 weeks after starting the *C. rodentium* challenge after which colon, liver, spleen, serum/plasma and fecal samples were collected for several analyses. The design of these experiments is depicted in **Figure 1**.

Full descriptions of the materials and methods, i.e. colon histopathology, iron measurements, ELISA measurements, hepcidin quantification and gut microbiome analysis are available in the **Supplementary materials and methods**.

Nematode viability assay

For nematode infection assays, *Caenorhabditis elegans glp-4(bn2) sek-1*(*km4*), a pathogen-sensitive strain with temperature-sensitive sterility [61] was maintained at 15°C on nematode growth medium, using *E. coli* DH5α (Life Technologies Inc.) as a source of food. Nematodes were age-synchronised by isolating eggs through treatment with hypochlorite/NaOH, and L1 hatchlings were deposited on lawns of *E. coli* DH5a grown on NGM agar. Plates were incubated at 25°C (at this temperature adult nematodes of the strain used do not produce any progeny), and when the nematodes reached the L4 stage they were collected from the plates and washed at least three times using M9 buffer (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl and 0.25 g/L $MgSO_4 \cdot 7H_2O$). Agar plates of Iscove's Modified Dulbecco's Medium (does not contain iron in its formulation) (IMDM, Invitrogen) were prepared as follows. Firstly, IMDM medium was briefly warmed to 45 °C and mixed with 5% (in water) melted ultra pure agar (Difco Noble Agar) that was cooled to 45 °C, to give a final concentration of 1.2% agar. Ferric ammonium citrate to final concentrations of 0, 1, 10 or 100 µmol/L were added and plates (55 mm) were poured immediately. Cultures of *S*. Typhimurium NTB6 [32] or *E. coli* DH5α were grown in IMDM medium with 0.5 µmol/L ferric ammonium citrate until the end of the exponential growth phase. 10 µl of these cultures was used to seed the IMDM-agar plates, which were then incubated for 18 hours at 37°C. 30-40 L4 stage nematodes were deposited on the lawns of *S*. Typhimurium or *E. coli* DH5α. 5 plates were used for each iron concentration. Survival of nematodes during co-incubation was scored regularly during 13 days and was expressed as the LT50 (defined as the time to kill 50% of the population) and the area under the curve (AUC) was determined as another measure for survival time. Observations were carried out using a standard dissecting microscope; nematodes were scored as dead when they lost their normal sigmoidal shape and failed to move in response to gentle touch with a platinum wire. Viability of the bacterial lawns was not affected by iron concentration (data not shown).

Statistics and data representation

Analysis of mouse responses and nematode survival

To compare means, one-way ANOVA with Tukey's post-hoc test (to compare all means) or with Bonferroni's post-hoc test (for comparison of selected means) was performed. To analyze the effect of dietary iron intervention and intestinal infection on body weights, the area under the curve (AUC) for each mouse was determined, after which groups were compared as described above. Colon histopathology data was also analyzed by one-way ANOVA with a post-test for linear trend. The correlation between fecal lipocalin-2 and calprotectin levels was assessed by Pearson correlation test and linear regression was used to plot the best-fit line (with 95% confidence interval). Statistical tests were performed using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA. P-values < 0.05 were considered statistically significant and P-values < 0.10 were considered as an important significance level.

Analysis of 16S rDNA pyrosequencing data

Full descriptions are available in the **Supplementary materials and methods**.

Ethics Statement

All mouse studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital (protocol number 2008N000061, animal welfare assurance number A3596-01).

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Disclosure

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Trial profile, tissue iron content and body weight of the mice during the time course of the experiment

A) Mouse trials which were performed in four separate experiments. 4-6 weeks old , female, C57BL6 mice in experiment 1 (n=3 per group) and 2 (n=3-4) were sacrificed after 14 days of dietary iron intervention and were analyzed for serum / stool / tissue iron content, or lipocalin-2. Mice in experiment 3 (n=5) and 4 (n=5) were orally infected with *C. rodentium*, while the diets were continued for another 14 days. Diets contained 2-6 mg Fe/kg (iron-deficient), 45 mg Fe/kg (normal-iron), or 225 mg Fe/kg (high-iron). Samples were analyzed for stool / tissue iron content, hepcidin, lipocalin-2, calprotectin, or colon histopathology. Gut microbiome analysis was performed on mice in experiment 4. **B)** Tissue iron stores (mean + SE) of uninfected mice of experiment 2 after 14 days of dietary iron intervention (n=3-4) and **C)** of infected C57BL6 mice of experiment 4 after 28 days of dietary intervention (n=5). Means without a common letter differ significantly, $p < 0.05$ (Tukey's post-hoc test). **D)** Body weights (mean ±SE) of mice in experiment 4 (n=5) were monitored during 28 days. Until infection at day 14 (indicated by the arrow) body weights were similar, but tended to differ among the dietary groups after infection ($p = 0.085$; one-way ANOVA).

Figure 2. Systemic lipocalin-2 in uninfected and infected mice, and hepcidin (Hep-1) in infected mice and uninfected reference mice

A) Systemic lipocalin-2 levels (mean) during dietary iron intervention in uninfected C57BL6 mice of experiment 1 (serum; n=3), or in infected mice of experiments 3 (serum; n=4-5) and 4 (plasma; n=5)). Solid lines indicate comparisons with significant outcome between dietary groups at one time point (Tukey's post-hoc test). Dashed lines indicate significant comparisons between uninfected and infected mice (only mice of the same dietary intervention group were compared; Bonferroni's post-hoc test). **B)** Hepcidin levels (mean + SE) in infected C57BL6 mice (day 28) in serum of mice in experiment 3 (n=4-5) and in plasma of mice in experiment 4 (n=5). Hepcidin levels in plasma of an

independent group of (uninfected) control mice (C57BL/6 mice on a standard diet) were included to show reference Hep-1 levels in healthy mice (n=7). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3. Colon histopathology and *ex vivo* secretion of pro-inflammatory cytokines by mouse colon explants

A) Histopathological score (mean) of colon sections of infected C57BL6 mice that were on the different iron-diets in experiment 3 (n=3) and experiment 4 (n=4-5) at day 28. There was a tendency for a linear trend from the mean in the iron-deficient group, towards the mean in the high-iron group ($p = 0.087$; post-test for linear trend).

B) Colon explants of infected C57BL6 mice (day 28) in experiment 4 (n=3-5) that were on the different iron-diets and were challenged with *C. rodentium*, were incubated overnight in culture medium, after which secreted pro-inflammatory cytokines IL-6, TNF-α and IL-17 were measured by ELISA. Cytokine levels were normalized for total colon protein. Levels (mean + SE) of IL-6 and TNF-α are on the left y-axis, and IL-17 levels on the right y-axis. Means without a common letter of the same cytokine differ significantly, $p < 0.05$ (n=3-5; Tukey's post-hoc test).

Figure 4. Fecal lipocalin-2 and calprotectin levels in infected and uninfected mice

Fecal lipocalin-2 levels (mean) in uninfected C57BL6 mice on the iron-diets of experiment 4 (day 13; n=4-5), or in infected mice of experiment 3 and 4 (day 27; n=6-10) are depicted in panel **A**. Panel **B** shows fecal calprotectin levels (mean) in uninfected (day 13; n=4-5) and infected (day 27; n=4-5) C57BL6 mice of experiment 4. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Solid lines indicate comparisons with significant outcome between dietary groups at one time point (Tukey's post-hoc test). Dashed lines indicate significant comparisons between uninfected and infected mice (only mice of the same dietary intervention group were compared; Bonferroni's post-hoc test).

C) The (Pearson) correlation between fecal calprotectin and lipocalin-2 levels in C57BL6 mice of experiment 4 (day 13 and day 27), plotted by linear regression (best-fit line with 95% confidence interval), is shown in panel **C**.

Figure 5. Phylogenetic diversity of the mouse gut microbiome during iron intervention and intestinal inflammation

The phylogenetic diversity (PD) index of the C57BL6 mice gut microbiomes of the dietary iron groups over time are given (min-max whiskers with median). Statistically significant differences between groups (n=5, except for mice on the iron-deficient diet at day 1 with n=4) at the same time point are indicated with solid lines; comparisons between groups were made at the same time point only (Bonferroni's post-hoc test). Differences within groups over time are indicated with dashed lines (Tukey's post-hoc test). * $p < 0.05$, ** $p < 0.01$.

Figure 6. Multivariate redundancy analysis (RDA) of the microbiota composition and hierarchical microbiome clustering

A & B) RDA was performed using Canoco 5.0. Taxonomic composition at the genus level was used as response data and dietary iron groups over time as explanatory variable. Red symbols represent dietary iron intervention groups at day 1, 13 and 27 (experiment 4; n=5 per group (n=4 for iron-deficient C57BL6 mice at day 1)). Other symbols are the individual samples. The colored lines are envelopes connecting samples of the same group. Length of arrows reflects significance and the direction shows to what group(s) of mice the genus is associated with most. **A)** Classified sample diagram. **B)**: Taxa – metadata biplot. To test the significance of the links between dietary intervention and sample clustering, separate RDAs were done for each individual time point. Clustering by treatment was not significant at baseline ($p = 0.422$), while they significantly differed at day 13 ($p = 0.002$) and at day 27 ($p = 0.002$) (permutation tests).

C) The microbiomes of fecal samples at day 1, day 13 and day 27 (experiment 4; n=5 per group (n=4 for iron-deficient C57BL6 mice at day 1)) were clustered using UPGMA with weighted UniFrac as a distance measure. The figure was generated using iTOL [62]. Sample names with the same color are within the same dietary group at the same time point. Colored bars represent the relative abundance of a bacterial genus (the number of reads assigned to a genus divided by the total number of reads

assigned up to the phylum level) in the sample. Mice within a group had no individual ID (indicated as `x`).

Figure 7. Combined effect of dietary iron and intestinal inflammation on the mouse gut microbiome

Nodes represent taxa; edges link the different taxonomic levels. The fold difference between dietary iron groups at day 27 is calculated as the 2log of the ratio of the relative abundance in the C57BL6 mice on the iron-deficient and normal-iron conditions (**A**) or in the iron-deficient and high-iron conditions (**B**) or in the normal-iron and high-iron conditions (**C**) (0 = no difference between groups, 1 = twice as abundant, etc.). In this explorative analysis, the significance is expressed as the p value of a Mann-Whitney U test, n=5 per group (experiment 4). The node-size corresponds to the relative abundance. Taxa (that is, nodes) most likely to play important roles are therefore brightly colored (a large difference between treatment groups), have a thick border (the effect is significant) and may be relatively large (abundant). Taxa were included in this visualization when the fold difference met a significance level of $p < 0.1$ and when the relative abundance was > 0.05%, or when the taxon had a child (that is, more specific taxonomic classification) meeting this criterion.

[1] These taxa were not detected in one of the dietary groups and have therefore an estimated 2log fold difference of 10.

Figure 8. Survival of *C. elegans* decreases upon bacterial infection with increasing iron levels

Survival of the nematode *C. elegans glp-4(bn2) sek-1*(*km4*) on *S.* Typhimurium NTB6 and *E. coli* DH5α, which was used as a simple *in vivo* gut model, was monitored during 13 days and the LT50 (defined as the time to kill 50% of the population) was determined. Whiskers without a common letter and representing nematode survival on *S.* Typhimurium that was pre-incubated with increasing amounts of iron, differ significantly, $p < 0.01$ (single experiment with n=5; Tukey's post-hoc test). Whiskers are displayed with median and min to max.