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Leptin Receptor Q223R Polymorphism Influences Neutrophil Mobilization after *Clostridium difficile* Infection

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Abstract

Clostridium difficile is the leading cause of nosocomial infections in the U.S. Clinical disease outcomes after *C. difficile* infection (CDI) are dependent on intensity of host inflammatory responses. Specifically, peak peripheral white blood cell (WBC) count >20×10^9/L is an indicator of adverse outcomes in CDI patients, and is associated with higher 30-day mortality. We show that homozygosity for a common single nucleotide polymorphism (Q to R mutation in leptin receptor that is present in up to 50% of people), significantly increases the risk of having peak peripheral WBC count >20×10^9/L (odds ratio=4.7; $p = 0.0024$) in CDI patients. In a murine model of CDI, we demonstrate that mice homozygous for the same SNP (RR mice) have more blood and tissue leukocytes (specifically neutrophils), exaggerated tissue inflammation and higher mortality as compared to control mice, despite similar pathogen burden. Further, we show that neutrophilia in RR mice is mediated by gut microbiota-directed expression of CXC chemokine receptor 2 (CXCR2) which promotes the release of neutrophils from bone marrow reservoir. Overall these studies provide novel mechanistic insights into the role of human genetic polymorphisms and gut microbiota in regulating the fundamental biological process of CDI-induced neutrophilia.

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Introduction

Clostridium difficile is the most common cause of hospital-acquired infections in the U.S.[1] and is ranked in the top 3 urgent public health threats by the CDC (www.cdc.gov)[2]. Host genetic factors can alter the risk of acquiring *C. difficile*: a common single nucleotide polymorphism (SNP) in the interleukin-8 gene increases susceptibility to *C. difficile* colitis[3], and we have previously shown that a non-synonymous SNP, rs1137101, on the extra cellular domain of leptin receptor (LEPR) increases the risk of *C. difficile* diagnosis[4]. We found that individuals homozygous for the derived allele G, which encodes for Arginine (R) at position 223 (RR genotype) were at a higher risk of being diagnosed with CDI (odds ratio 3.03), as compared to individuals either homozygous or heterozygous for the ancestral allele A, which encodes for Glutamine (Q, QQ or QR genotype) at the same position[4]. Q223R is a common SNP in humans with a mean allelic frequency of ~50% in European-Americans and 40-60% in African-Americans (http://hgdp.uchicago.edu). Notably, RR genotype has been associated with more severe disease and worse outcomes after intra-abdominal infections: increased risk of amoebic colitis in children[5], increased incidence of amoebic liver abscess[5] and higher mortality after secondary bacterial peritonitis in adults[6]. But the influence of *LEPR* SNP on CDI clinical disease severity and the mechanisms that could alter infectious disease outcomes in individuals with RR genotype remain unknown.

Indigenous gut microbial flora and host inflammatory responses are key determinants of clinical disease outcomes after CDI[7-9]. Alterations in gut microbial communities (dysbiosis), typically caused by antibiotic exposure, create an ideal niche for initial *C. difficile* spore colonization and germination[10]. After spore germination, the vegetative *C. difficile* bacteria produce an enterotoxin and a cytotoxin (toxins A and B), which are the major *C. difficile* virulence determinants that cause colonic tissue damage. The inflammatory response elicited by *C. difficile* and its toxins is predominated by neutrophils and is characterized by peripheral blood leukocytosis, immune cell infiltration into colonic tissue and production of many pro-inflammatory mediators[11]. Neutrophils are the primary innate effector cells in the host response to *C. difficile* but neutrophil responses in CDI need to be tightly regulated since both an over-exuberant neutrophil response after infection or neutropenia can be deleterious to the host. For example, peripheral white blood cell (WBC) count $>20 \times 10^9$/L (mainly comprised on blood neutrophils), is an independent risk factor for increased 30-day mortality[9], and patients with severe CDI develop pseudomembranous colitis, which is characterized by excessive colonic neutrophil infiltration and tissue damage[12]. The importance of neutrophils in CDI pathogenesis is further highlighted by studies in animal models where depletion of neutrophils protects colonic tissue from injury after *C. difficile* toxin challenge[13, 14]. However, complete lack of neutrophils is also not favorable to the host: neutropenic patients have higher incidence of CDI (both initial and recurrent)[15, 16], and in murine models, antibody-mediated neutrophil depletion prior to infection with *C. difficile* (instead of intoxication) results in higher mortality due to increased translocation of gut bacterial pathobionts from colonic lumen to intra-abdominal organs[17]. Taken together, these studies highlight the importance of indigenous gut microbial flora and the dichotomous nature of host neutrophil response in CDI pathogenesis that can affect clinical disease outcomes.

In this study, we investigated the innate immune responses to *C. difficile* in the context of *LEPR* Q to R mutation in humans and mice. We show that CDI patients homozygous for the derived LEPR allele (RR genotype) had higher peripheral blood leukocytosis, compared to patients homozygous or heterozygous for the ancestral allele (QQ/QR genotype). We modeled human CDI in mice with the same SNP and show that similar to patients, mice homozygous for the derived LEPR allele (RR mice) had exaggerated systemic leukocytosis and neutrophilia. In addition, RR mice had higher tissue neutrophils and inflammatory cytokine expression, increased colonic tissue damage and higher mortality, as compared to control QQ mice, despite equivalent *C. difficile* burden. Our studies elucidate the complex interplay between host genetics, immunity and gut microbiome and show that the enhanced neutrophilia in RR mice is mediated by gut microbiota-directed increase in cell surface expression of neutrophil-recruiting CXC chemokine receptor 2 (CXCR2) that promotes the release of bone marrow neutrophils in response to *C. difficile*.

Results

Individuals with RR genotype have higher peak peripheral blood leukocytosis after CDI

We have previously reported that individuals with RR genotype had higher risk of CDI diagnosis[4]. Here, we investigated the host response to *C. difficile* in a cohort of CDI patients, and studied the association between *LEPR* Q223R polymorphism and peak peripheral WBC count (highest WBC value between 3 days before and 3 days after *C. difficile* diagnosis). Patients with RR genotype were more likely to have a peak WBC count of $>20 \times 10^9$/L in the peri-diagnosis time frame [odds ratio = 5.41; p =0.0023; multivariate logistic regression analysis with adjustment for age, race and gender], compared to patients with QQ/QR genotype (Table 1 and Supplementary table 1). Peripheral blood leukocytosis (predominated by neutrophils) is commonly seen in CDI, and magnitude of leukocytosis predicts clinical disease outcomes[9]. The peak peripheral WBC count cut-off value and peri-diagnosis reference time frame used in our analysis was based on previous studies which show that WBC count of $>20 \times 10^9$/L between 5 days before to 3 days after *C. difficile* diagnosis is an independent predictor of increased 30-day mortality[9, 18]. Our data thus indicate that individuals with RR genotype are at an increased risk of having worse outcomes after CDI.

Mice with RR genotype have higher blood and tissue neutrophilia after CDI

To investigate how LEPR Q223R SNP influences host WBC response to *C. difficile*, we used a murine model of CDI. Age- and gender- matched QQ and RR mice were pre-treated with cefoperazone for 9 days in drinking water, followed by regular water without antibiotics for 2 days as previously described[19], and then challenged with purified *C. difficile* spores by oro-gastric gavage (Figure 1a). Mice were sacrificed at day 1 and day 3 after infection to analyze peripheral blood and tissue immune responses. *C. difficile* challenge increased total WBC and neutrophil counts in both QQ and RR mice compared to their respective sham-challenged controls, but the cell numbers were significantly higher in RR mice ($p<0.05$; Figure 1b). Differential blood count revealed that the surge in WBCs in RR mice was due to increased neutrophils (Figure 1c), and circulating monocytes and eosinophils were similar in both groups (Figure 1d-e). By day 3 after infection, QQ and RR

mice had comparable total (and differential) WBC counts (Figure 1b-e). Antibiotic pre-treated QQ and RR mice had similar number of circulating total and differential WBCs (Figure 1b-e).

We next examined the immune cell infiltrate in cecum and colon of QQ and RR mice by flow cytometry of lamina propria cells. Prior to infection, both QQ and RR mice had very few immune cells in the lamina propria (Figure 2a). By day 1 of *C. difficile* challenge, a large number of immune cells were recruited to the colon in both groups of mice, but similar to peripheral blood, RR mice had significantly higher number of total immune cells ($CD45.2^+$) and neutrophils ($CD45.2^+CD11b^+Ly6G^+$) per gram of cecal tissue ($p=0.017$; Figure 2a-b). As seen in peripheral blood, the number of monocytes ($CD45.2^+CD11b^+Ly6G^-Ly6C^+$) and eosinophils ($CD45.2^+CD11b^+Ly6C^{low}SiglecF^+$) in tissue was comparable between QQ and RR mice (Figure 2c-d). By day 3 after infection, and concurrent with improvement in clinical disease, the total number of immune cells, neutrophils and monocytes in cecal lamina propria of RR mice decreased significantly but the number of tissue neutrophils and monocytes in QQ mice was similar to day 1 levels.

RR mice have higher tissue inflammation and decreased survival after CDI

Within 1 day after *C. difficile* challenge, RR mice had higher expression of pro-inflammatory cytokines: interferon-gamma (IFNγ), macrophage migration inhibitory factor (MIF), tumor necrosis factor alpha (TNF-a) and interleukin-6 (IL-6) in cecum ($*p<0.05$; Figure 3a) and significantly more colonic tissue damage ($*p<0.05$; Figure 3b-c), as compared to control QQ mice. Specifically RR mice had more inflammatory cells and submucosal edema (Figure 3d-f and Supplementary Figure S1). This enhanced inflammation was not due to higher pathogen burden, since the number of *C. difficile* colony forming units (CFU) per gram of cecal content and levels of *C. difficile* toxins were comparable in QQ and RR mice at all time points after infection (Figure 4a-b). Notably, we saw mortality only in RR mice (across 6 different experiments) and mice died between 36 to 60h after CDI (Figure 4c and Supplementary Figure S2a). By day 3 after infection, all QQ mice and the surviving RR mice had improved histology scores (Figure 3c-f) and better clinical disease (improved weight and reduced diarrhea; Figure 4d and Supplementary Figure S2b). Overall, our data from patients with CDI and from the mouse model reveal that host genetics plays a role in regulating the systemic and tissue immune response, and clearly shows that RR genotype is associated with an exaggerated immune response and tissue damage to the same number of *C. difficile* bacteria.

Q223R polymorphism does not alter leptin levels or neutrophil functional capacity

Leptin is known to affect immune cell functions such as generation of reactive oxygen species, inflammatory cytokines/chemokines production, and phagocytic capacity of neutrophils, monocytes and macrophages[20], thus we measured leptin levels and neutrophil functions in QQ and RR mice. Total leptin in plasma and tissue was similar between the two groups of mice (plasma; Figure 5a, Tissue; Supplementary Figure S3a). But the amount of bioavailable leptin is regulated by soluble LEPR (sLEPR) which binds to free leptin[21] and decreases overall leptin signaling, thus we measured sLEPR in plasma of QQ and RR mice. We saw no difference in sLEPR concentration in uninfected or *C. difficile*-infected QQ and

RR mice (Uninfected; Supplementary Figure S3b, Infected; Figure 5b); suggesting that Q223R polymorphism does not impact leptin signaling upstream of LEPR. *In vitro* transfection studies have shown that signaling from LEPR mutant allele (RR) leads to diminished signal transducer and activator of transcription 3 (STAT3) signaling[22], and a lack of leptin-STAT3 signaling reduces phagocytic ability of alveolar macrophages[23], but the impact of Q223R polymorphism on neutrophil functions is not known.

Thus, we tested the functional status of sorted QQ and RR neutrophils. Magnetic bead sorted colonic neutrophils from *C. difficile*-infected QQ and RR mice phagocytosed similar proportions of *Escherichia coli* bioparticles and generated comparable levels of reactive oxygen species (ROS) in response to *ex vivo* N-Formyl methionyl-leucyl-phenylalanine (fMLP) stimulation (Figure 5c-d). Arguably, tissue neutrophils from *C. difficile*-infected mice are already exposed to an inflammatory environment and may have impaired ability to respond to a second *ex-vivo* stimulus, thus we examined phagocytosis and generation of ROS in purified bone marrow neutrophils from naïve QQ and RR mice. Similar to *ex vivo* isolated neutrophils, the functional capacity of bone marrow neutrophils from QQ and RR mice was not different after *in vitro* challenge with fMLP (Supplementary Figure S3c-d). Myeloperoxidase (MPO) is a component of neutrophil azurophilic granules which is secreted upon neutrophil activation. While extracellular MPO activity causes tissue damage, intracellular MPO activity represents the number of neutrophils in tissue[24]. At day 1 of infection, colonic tissue lysates (includes both intracellular and extracellular MPO activity) from RR mice had significantly high MPO activity (Figure 5e) compared to QQ tissue. However, difference in MPO activity was lost when normalized against total neutrophil numbers indicating that there is no difference in MPO activity on a per cell basis between QQ and RR neutrophils (Figure 5f). Taken together, these experiments showed that LEPR Q to R SNP does not alter the functional capacity of neutrophils.

RR mice have increased expression of CXC chemokine receptor 2 (CXCR2) on bone marrow and blood neutrophils

Infectious challenge induces a rapid release of neutrophils from bone marrow reservoir and migration to peripheral blood and inflamed tissue[25]. In the tissues, neutrophils control pathogen and regulate inflammation, but a large number of cells also die in the process[26]. Therefore, higher neutrophil numbers observed in RR mice could be due to decreased cell death in tissues or increased cellular release from bone marrow. We found that there was no difference in cell death between QQ and RR neutrophils without infection and after CDI (Figure 6a). However, 1 day after *C. difficile* challenge, and concurrent to increased neutrophils in blood and tissue, we found a significant reduction in number of neutrophils in the bone marrow of RR mice ($p=0.03$; Figure 6b) indicating enhanced neutrophil release from bone marrow after CDI. Thus, we examined the factors involved in neutrophil mobilization from bone marrow.

Neutrophil release from bone marrow is directed by binding of chemokines to chemokine receptors. CXC chemokines (CXCL1 and CXCL2) bind to their cognate receptor CXCR2 and induce neutrophil mobilization from bone marrow to peripheral blood and to colonic tissue[27, 28]. CDI increased CXCL1 and CXCL2 expression (day 1 after infection) in both

QQ and RR mice, as compared to sham-infected controls, but the levels of these chemokines were similar in QQ and RR mice both in plasma and in cecal tissue (Plasma; Figure 6c, Tissue; Supplementary Figure S3e). Levels of other neutrophil recruiting cytokines, GM-CSF and G-CSF, were also comparable in the two groups of mice (Figure 6c and Supplementary Figure S3e).

We thus postulated that expression of chemokine receptors on the cell surface could influence egress of neutrophils from bone marrow. While binding of CXCL1 and CXCL2 to CXCR2 augments cellular release from bone marrow, CXC chemokine receptor 4 (CXCR4) interaction to its ligands promotes cellular retention in bone marrow[28]. Expression of CXCR4 on bone marrow neutrophils was similar between QQ and RR mice (Figure 6d). However, compared to QQ mice, CXCR2 expression was higher on bone marrow and blood neutrophils of RR mice after antibiotic treatment alone and subsequent CDI (Figure 6e-f). In the case of bone marrow neutrophils, the effect of antibiotics on CXCR2 expression was compounded by CDI (Figure 6e). Consistent with more neutrophils (and not monocytes) in RR mice, the increased expression of CXCR2 was seen only on RR neutrophils, and not on bone marrow monocytes (Figure 6g). To study the role of CXCR2 in regulating neutrophil recruitment to cecum after CDI, we used a selective non-peptide inhibitor that blocks CXCR2 signaling (SB225002). Within 1 day of *C. difficile* challenge, MPO activity was induced in cecal tissue of mice (*$p<0.05$; Figure 6h). Blocking CXCR2 signaling by intra-peritoneal injection of SB225002 (1mg/kg/day) for 2 days prior to CDI reduced MPO activity in cecal tissue as compared to vehicle treated controls (**$p<0.01$; Figure 6h). Our data thus demonstrate a critical role for CXCR2 signaling in neutrophil recruitment to colonic tissue after CDI and show that LEPR SNP influences CXCR2 expression on bone marrow and blood neutrophils during the course of antibiotics and after CDI.

Differential dysbiosis of gut microbiota regulates bone marrow neutrophil CXCR2 expression

Antibiotic-induced dysbiosis was sufficient to induce CXCR2 expression in RR mice (Figure 4e). We thus determined the microbial composition of cecal contents in QQ and RR mice (both prior to antibiotics, and after antibiotics and CDI) by shot-gun sequencing. Before antibiotics, RR mice had a slightly more diverse flora compared to QQ mice and cefoperazone treatment reduced the microbial diversity in both groups of mice (Figure 7a). Notably, the phylum composition was significantly different between QQ and RR mice ($p<0.05$; Figure 7b): before antibiotics, RR mice had relatively fewer Bacteroidetes and more Firmicutes than QQ mice, and after cefoperazone Bacteroidetes abundance decreased further in RR mice (Figure 7b). While CDI decreased Bacteroidetes in QQ mice, this phylum was almost completely lost in *C. difficile*-infected RR mice (Figure 7b-c). To determine the role of microbiota in regulating CXCR2 expression, we matched the microbial flora of QQ and RR mice by daily bedding exchange as previously described[29]. Reciprocal transfer of microbial flora between QQ and RR mice during antibiotic pre-treatment resulted in similar levels of CXCR2 expression on QQ and RR bone marrow neutrophils, both after antibiotic treatment and after subsequent CDI (Figure 7d-e). These findings indicate that antibiotic-induced changes in composition of gut microbiota direct CXCR2 expression on bone marrow neutrophils.

Discussion

The most important finding of our studies is that a common SNP that is present in up to 50% of humans influences CDI-induced neutrophil responses in both humans and mice, and the mechanism of enhanced neutrophilia associated with the LEPR SNP is dependent on gut microbiota-induced expression of a neutrophil-recruiting chemokine receptor (CXCR2). Host genetics, immunity and gut microbiome are intricately linked. Genetic make-up influences immune responses to infections and microbiota composition[30, 31], microbiota re-programs host genes by epigenetic modifications[32] and shapes immunity, and the immune system in turn alters composition of the gut microbial communities[33, 34]. Notably, all of these variables clearly influence CDI pathogenesis and outcomes as well[35]. This study thus improves our understanding of how interactions between host genome and gut microbiome influence neutrophil response to *C. difficile* and impact CDI pathogenesis and clinical disease outcomes.

The exaggerated neutrophilic response after CDI associated with RR genotype was seen in both humans and mice. The WBC cut-off value of 20×10^9/L that we used for our association studies has previously been shown to predict increased 30-day mortality in patients with CDI[9], and suggests that RR patients are likely to have higher mortality after CDI. While we did not have mortality data from our patient cohort, mortality was indeed seen in RR mice. Overall mortality seen in our animal studies was modest but this likely due to the genetic background of QQ and RR mice (129J). In multiple studies using the same infection protocol, we have seen that mice on C57Bl/6 genetic background are more susceptible to CDI and have higher mortality. Regardless, across 6 different experiments and total N = 86 mice, only RR mice succumbed to infection, and all QQ mice survived. Additionally, the inflammatory response (as measured by cytokine expression and blood and tissue neutrophils) was clearly exaggerated in RR mice, compared to QQ mice. We did not see any difference in *C. difficile* (or its toxin) in the cecal contents of QQ and RR mice, but it is known that an over-exuberant neutrophil response can cause collateral host damage[36] and increase mortality after CDI[9]. Thus, our data indicate that the tissue damage and survival disadvantage seen in RR mice was due to host-derived factors, i.e. enhanced inflammation and surge in neutrophil infiltration. Of note, the surviving RR mice have slightly faster disease resolution as compared to QQ mice: decrease in tissue neutrophil infiltrate in RR mice on day 3 compared to day 1 after infection (Figure 3c), and faster weight gain (Figure 4d). This observation raises the intriguing possibility that a threshold effect based on host genetic polymorphism and magnitude of inflammatory response exists, and can influence CDI outcomes. Too much inflammation early in the course of disease can increase mortality but if the host survives the initial insult, outcomes are better (as seen in RR mice). On the other hand, slightly lower level inflammation which is persistent leads to better overall survival but longer duration of clinical disease (as seen in QQ mice).

At the mechanistic level, we found that CXCR2 signaling pathway is critical for colonic tissue recruitment of neutrophils after CDI, and the higher CXCR2 expression on RR neutrophils was mediated by gut microbiota. However, CXCR2 inhibition did not alter CDI disease severity during the early stage of infection (day 1 after challenge). Despite a complete lack of neutrophil infiltration to cecal tissue, mice with CXCR2 blocakge had

similar disease expression (as measured by weight loss and reduction in colon length, supplementary Figure S4), as compared to vehicle treated control mice. Since both neutrophilia and neutropenia in patients (and mice) is associated with adverse outcomes after CDI[15-17, 37], this result is not entirely unexpected, and in fact underscores an important role of optimal neutrophil numbers in determining CDI outcomes. Thus, to define the role of CXCR2 in modulating neutrophil hoemostasis and impacting CDI susceptibility and disease severity, appropriate titration of the levels of CXCR2 inhibitor (and/or blocking antibody) will be needed.

A growing body of evidence indicates that gut microbiota influences inflammatory responses[38] including neutrophil production, migration and function[39-41]. Our studies add to the current literature and show that antibiotic- and CDI- induced dysbiosis (specifically in the context of RR genotype) enhances CXCR2 on cell surface of bone marrow and blood neutrophils, and influences neutrophil migration from bone marrow. Prior to antibiotics, composition of gut microbial community was already different between QQ and RR mice, and RR mice had relatively higher abundance of Firmicutes phylum and lower abundance of Bacteroidetes phylum (Figure 7c). The increased ratio of Firmicutes to Bacteroidetes in RR mice was amplified after CDI (Figure 7c). Notably, exchange of microbiota between QQ and RR mice reversed the antibiotic- and CDI- induced differences in neutrophil CXCR2 expression. Thus, our data indicate that microbiota plays a key role in regulating CXCR2 expression are in concert with previous reports in animal models which show that dysbiosis in pregnant mice can impact bone marrow CXCR2 expression in offspring[40]. But additionally our results highlight a critical role of host genetic polymorphism in regulating gut microbiota-mediated alteration in neutrophil chemokine receptor expression.

Somewhat contrary to our observations, a recent report has shown evidence of CXCR2 downregulation on blood neutrophils subsequent to antibiotic treatment[42]. However, we used a single antibiotic, cefoperazone while a cocktail of antibiotics comprising of ampicillin, neomycin, metronidazole and vancomycin was used by Watanabe. *et al*[42], which could contribute to opposing results. In fact, it is known that different antibiotic regimes can induce distinct perturbations in gut microbiota structure[43], and contrasting the findings from our study to Schubert *et al*[43] suggests that use of multiple antibiotic cocktail induces a more profound disruption of microbial communities as compared to cefoperazone alone. While antibiotic treatment decreases the overall microbial diversity and reduces the abundance of many major bacterial phyla, proportion of some bacterial communities also increases. For example, Verrucomicrobia phyla is retained after cefoperazone treatment and its proportion is higher in RR mice (Figure 7b) but this phylum is completely abolished by metronidazole[43] (one of the antibiotic used by Watanabe *et al*). Of note, *Akkermansia muciniphila* is the predominant commensal species present in Verrucomicrobia phyla, and presence of *A. muciniphila* in microbiota enhances gut inflammation (including pro-inflammatory cytokine production and immune cells recruitment) and colonic tissue damage after *Salmonella* infection in mice[44]. Thus it is possible that the microbial communities which survive (or increase in relative proportion) after specific antibiotics are responsible for differences in neutrophil CXCR2 expression after CDI. While transfer of “whole bacterial communities” by bedding exchange supports an important role for gut microbiota in regulating CXCR2 expression, shotgun sequencing data presented here has a relatively

smaller sample size (n = 3). Thus, a potential limitation of our study is the inability to establish associations between individual bacterial genera (or species) and neutrophil CXCR2 expression. Future studies with a higher sample size will be needed to identify specific microbial communities that regulate CXCR2 expression and promote neutrophil trafficking from bone marrow in RR mice.

In sum, our data support a scenario whereby host genetic cues (homozygosity for the mutant *LEPR* SNP, RR genotype) alters microbiota prior to antibiotics. Antibiotics further disrupt the microbiota structure and change the microbial composition that induces CXCR2 expression on bone marrow neutrophils of RR mice, and primes them for release. A secondary insult after antibiotics with *C. difficile* further increases CXCR2 expression and in conjunction with increased chemokines (CXCL1 and 2), manifests as increased neutrophil numbers in peripheral blood and tissue of RR mice. There is no impact on pathogen burden but the exaggerated inflammation in RR mice can then lead to increased mortality. Our study provides novel insights into how antibiotic-induced dysbiosis alters host neutrophil responses in the context of a specific SNP and influences clinical disease outcomes. Of note, both *LEPR* Q223R SNP and antibiotic-mediated dysbiosis are very common in our patient populations, and CXCR2-induced neutrophil trafficking from bone marrow to blood and tissue is a fundamental biological response to both infectious (e.g. influenza virus[45]), and inflammatory (e.g. DSS colitis[46]) stimuli. Thus, our study has important implications beyond CDI as well, and *LEPR* Q223R SNP has the potential to be used as a genetic biomarker to predict neutrophil responses to various diseases.

Methods

Human Data Collection and Analysis

Discarded blood clot samples were collected from 156 consecutive *C. difficile* positive patients at the UVA Medical Center, and DNA was extracted using Qiagen DNeasy Blood and Tissue kit, followed by LEPR SNP typing at the UVA bioinformatics core. Of these patients, clinical data (including gender, ethnicity and daily WBC counts for the same admission as the CDI diagnosis) was available from 129 adults (age >18yrs) in the UVA clinical database repository (CDR). Since clinical data was collected retrospectively, availability of daily WBC counts was at the discretion of ordering physician, and we did not have daily blood count values for all patients. Thus, to get a peak WBC value for association analysis, we included data only from patients who had a minimum of 4 consecutive daily WBC count values. Thus, 19 patients were excluded and we had N = 110 as our final study population. A more rigorous analysis where only patients with a minimum of 5 consecutive daily WBC counts (N = 96) were included did not change our results. Patient data collection and analysis was approved by the University of Virginia Institutional Review Board (IRB-HSR #16926).

Mouse model of *C. difficile* infection

Mice homozygous for the Q223 or R223 *LEPR* allele (on 129/J background) were maintained and bred at the Department of Laboratory Animal Medicine, University of Cincinnati, accredited by the American Association for Accreditation of Laboratory Animal

Care (Frederick, MD, USA), under pathogen-free conditions in individually ventilated cages. *Clostridium difficile* (VPI 10463) spores were prepared for infection as previously described[11]. Age-matched QQ and RR mice were treated with cefoperazone and challenged with purified *C. difficile* spores (1×10^6/ mouse) by oral gavage as previously described (Figure 1a)[19]. After infection, mice were single caged to prevent cross-infection and were monitored daily. In experiments performed to determine the impact of microbiota on CXCR2 expression, bedding was exchanged every day between QQ and RR mice throughout the course of antibiotics to equilibrate microbiota.

Estimation of *C. difficile* load

Pathogen load of *C. difficile* in mice was determined by plating 1:10 dilutions of cecal contents on beef heart infusion supplement (BHIS) plates containing taurocholate. Plates were incubated anaerobically at 37°C for 24-48h and colonies were counted manually to enumerate colony forming units (CFU) per gram of cecal content. Level of toxins in cecal contents were determined using the *C. difficile* TOXA/B ELISA (TechLab, VA, USA) according to manufacturer's instructions.

Histopathology

Tissue samples were fixed in Bouin's solution (Sigma, MO, USA), washed and dehydrated in 70% ethanol prior to paraffin embedding. Sections were prepared and stained with hematoxylin and eosin (H&E). The slides were examined microscopically and scored for inflammatory cell infiltration, edema, and epithelial disruption. A score of 0 to 4, denoting increasingly severe abnormality, was assigned by pathologist blinded to the identity of the samples.

Cell enumerations and flow cytometry

Peripheral blood was obtained by intra-cardiac puncture and collected in EDTA microtainers (BD Biosciences, CA, USA). Bone marrow cells were harvested by flushing single femur and tibia with ice cold PBS containing 2% fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA). Total and differential cell count was determined using a Hemavet hematology analyzer with veterinary software (Drew Scientific Inc., CT, USA).

Lamina propria cells were isolated and analyzed by flow cytometry as previously described [44] and in supplementary methods. Apoptosis of cecal tissue neutrophils was enumerated using Annexin V/7AAD-apoptosis assay kit (BD biosciences, CA, USA) as per manufacturer's instructions. Flow cytometry of apoptosis assay was performed on a BD FACS Acuri™ C6 flow cytometer equipped with CFlow® Plus (BD Biosciences, CA, USA) software and analyzed using FlowJo V.10 (Tree Star, OR, USA).

Estimation of inflammatory markers and neutrophil functional assays

Leptin, CXCL1, CXCL2, G-CSF and GM-CSF were measured by ELISA following protocols provided by manufacturer (for leptin; Peprotech, NJ, USA, and for all other targets; R&D systems, MN, USA). Preparation of tissue and plasma samples for ELISA is described in supplementary methods. RNA from tissue samples was extracted using Qiagen RNEasy Plus Mini Kit (Qiagen, CA, USA) and expression of inflammatory markers were

estimated by TaqMan qRT PCR. Cells from tissue and bone marrow were also used for functional assays (detailed in supplementary methods).

Microbiota analysis

DNA was extracted from cecal contents using PowerFecal® DNA isolation kit (MO BIO, CA, USA) as per manufacturer's instructions. Library generation was performed using dual NexteraXT adapters followed by sequencing on the NextSeq 500 Illumina platform in the Precision Metagenomic Core Laboratory at CCHMC for 150bp paired end reads with a depth of sequencing targeting 10 million reads per sample. Annotation pipeline analysis for all samples was performed as previously described[47].

Statistical analysis

All statistical analysis were performed using Graph Pad Prism 5.0 software (Graph pad software Corporation, Inc, CA, USA). For comparison of groups, a Student's t-test or ANOVA with Bonferroni correction was used. A ‘p’ value below 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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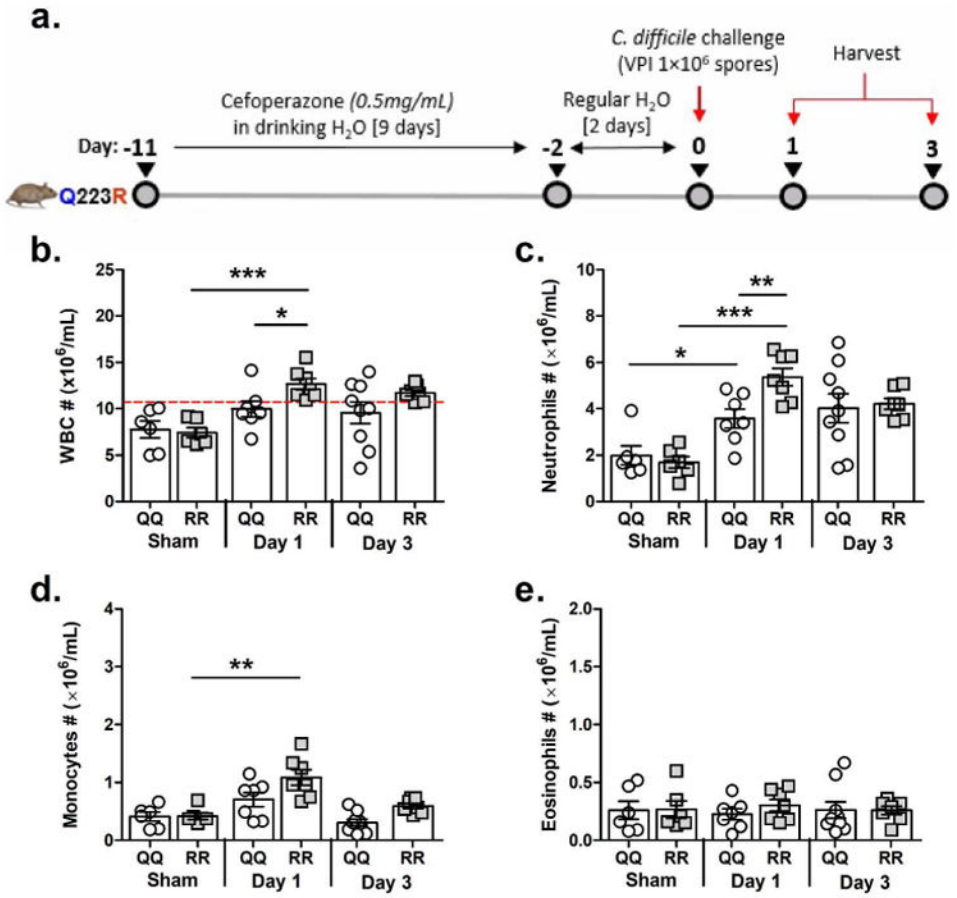

Figure 1.

RR mice have higher blood leukocytosis and neutrophilia after CDI. **(a)** Schematic representation of experimental design of *C.difficile* infection in mice. Age- and gender-matched QQ and RR mice were pre-treated with antibiotics for 9 days in drinking water and challenged with 1×10^6 *C. difficile* (VPI 10463) spores by oro-gastric gavage two days after cessation of antibiotics. Animals are sacrificed at time points indicated to evaluate the peripheral and tissue cellular response dynamics using Hemavet and fluorescent activated cell sorting (FACS) analysis respectively. Plots show number of **(b)** total WBC in peripheral blood (dotted line indicates the maximum of normal range in mice), **(c)** neutrophils in peripheral blood **(d)** monocytes and **(e)** eosinophils per mL of blood. Data shown as mean ± SEM from 6-9 mice/group. (*p<0.05, **p<0.01, ***p<0.001; Students' *t-test*).

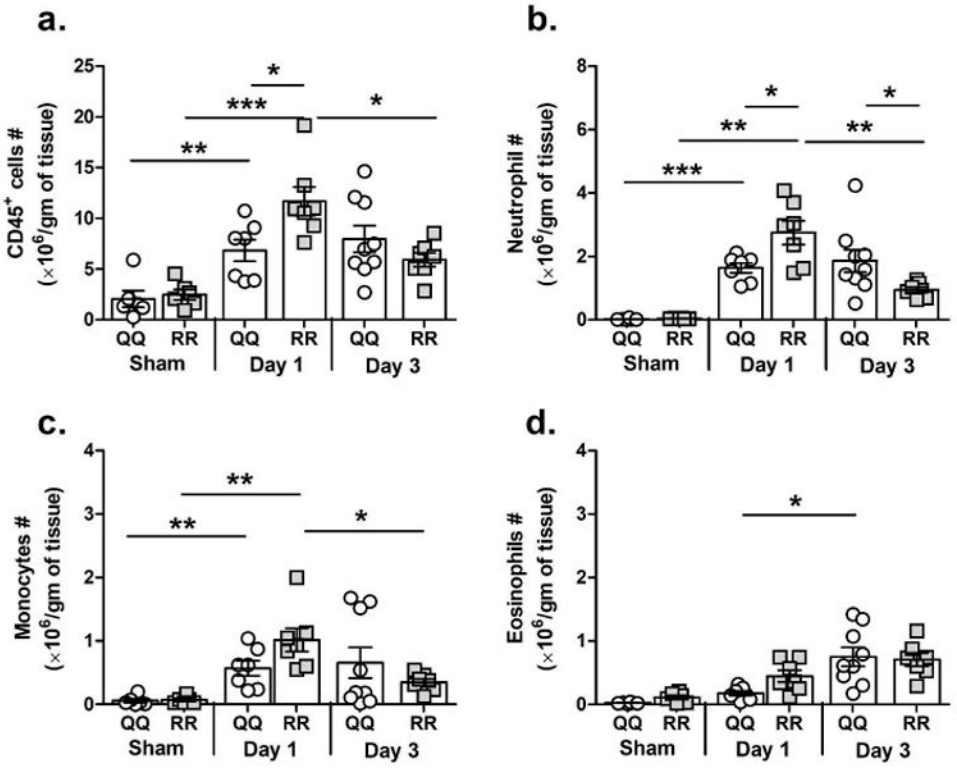

Figure 2.

RR mice have higher tissue leukocytosis and neutrophilia after CDI. (a) $CD45^+$ cells (b) neutrophils (c) monocytes and **(e)** eosinophils per gram of cecal tissue. Data shown as mean ± SEM from 6-9 mice/group. (*p<0.05, **p<0.01, ***p<0.001; Students' *t-test*).

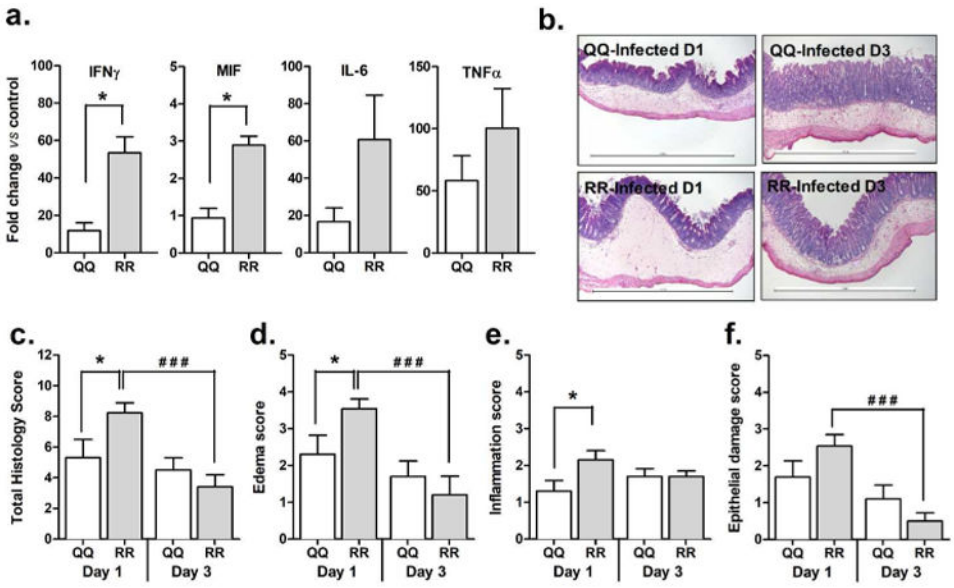

Figure 3.

RR mice have higher inflammation after CDI. **(a)** mRNA expression in cecal tissue represented as fold change vs uninfected controls. Data shown as mean ± SEM from 6 mice/group **(b)** Representative histology of hematoxylin and eosin–stained colon sections at day 1 (D1) and day 3 (D3) of *C. difficile* infection. Scale bar = 1mm. **(c)** Total histology score for (d) submucosal edema, (e) inflammatory cell infiltration and (f) epithelial damage in cecal tissue sections. Data shown as mean ± SEM from 10-13 mice/group. (*$p<0.05$, $^{###}p<0.001$; Students' *t-test*).

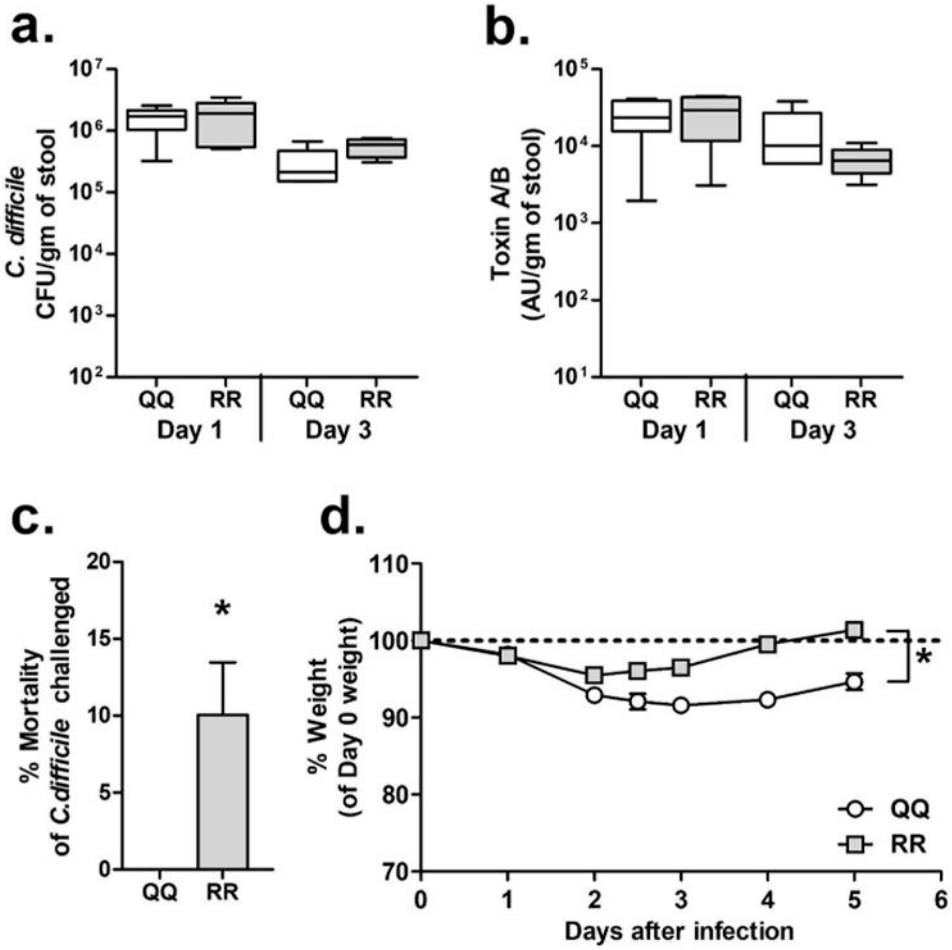

Figure 4.

RR mice have decreased survival irrespective of similar *C.difficile* burden. *C. difficile* **(a)** bacterial colony forming unit (CFU) and (b) toxin A/B per gram of cecal content of *C. difficile*-challenged mice. Data shown as mean ± SEM from 6-9 mice/group **(c)** Percent mortality and **(d)** percent weight change of *C. difficile*-challenged mice. Data shown as mean ± SEM from 6 different experiments with a total n= 42-44 mice per group. (*$p<0.05$; Students' *t-test or ANOVA*).

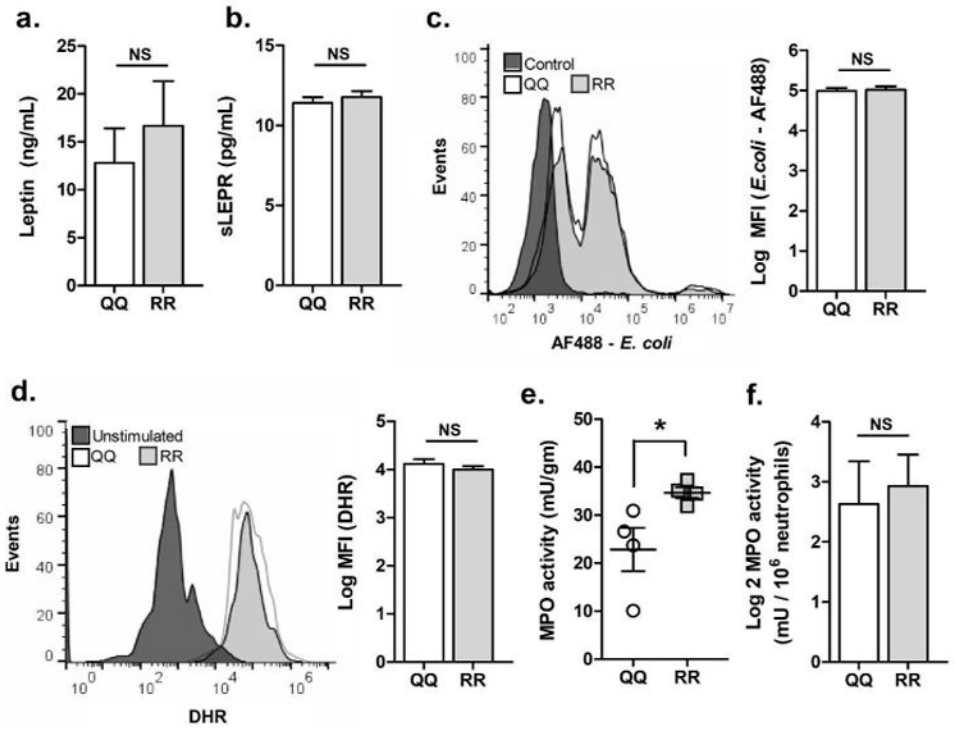

Figure 5.

Q223R polymorphism does not alter leptin levels or neutrophil functional capacity. **(a)** Leptin and **(b)** soluble leptin receptor concentration in plasma on day 1 of CDI. Flow cytometric analysis of **(c)** phagocytosis (*E. coli* AF-488) and **(d)** oxidative burst; DHR-123 in cecal tissue neutrophils after *ex vivo* fMLP stimulation. Myeloperoxidase (MPO) activity in tissue homogenates of *C. difficile*-infected QQ and RR mice on day 1 of infection **(e)** normalized to tissue weight and **(f)** number of tissue neutrophils. Data shown as mean ± SEM from 4-5 mice/group, representative of at least two independent experiments. (*p<0.05; Students' *t-test*).

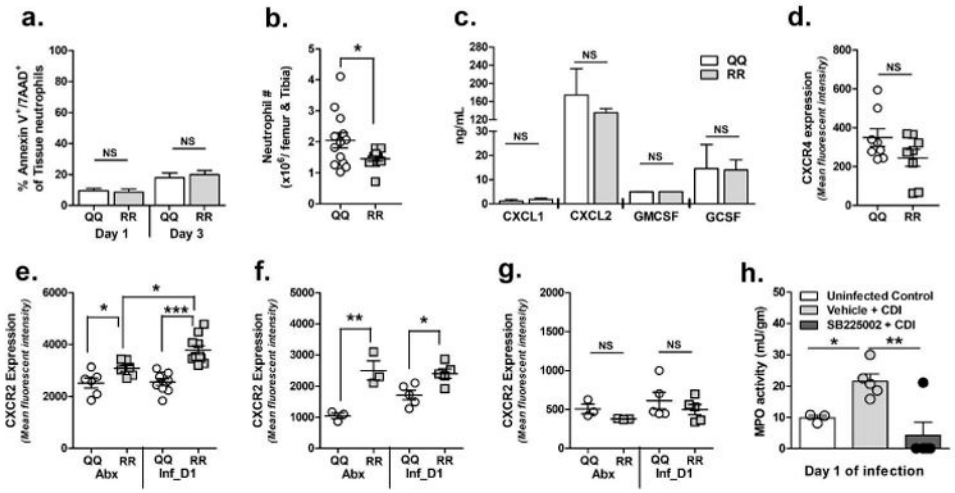

Figure 6.

RR mice have increased expression of CXC chemokine receptor 2 (CXCR2) on neutrophils. QQ and RR mice were challenged with *C. difficile* spores. **(a)** Annexin V^+ $7AAD^+$ (apoptotic) neutrophils in cecal tissue as measured by flow cytometry. **(b)** Percentage of neutrophils in bone marrow cells **(c)** concentration of neutrophil chemokines in plasma, and **(d)** Mean fluorescence intensity (MFI) of CXCR4 on mouse bone marrow neutrophils at day 1 of *C.difficile* infection. MFI of CXCR2 on **(e)** bone marrow neutrophils **(f)** blood neutrophils and **(g)** bone marrow monocytes after cefoperazone treatment (Abx) and at day 1 of CDI (inf_D1). CXCR2 inhibitor (SB225002) was injected intraperitoneally *(1mg/kg/day)* for two days prior to *C. difficile* challenge and **(h)** MPO activity was assessed in cecal tissue homogenates on day 1 of infection. Data shown as mean ± SEM from 3-9 mice/group (*p<0.05, **p<0.01, ***p<0.001; Students' *t-test*).

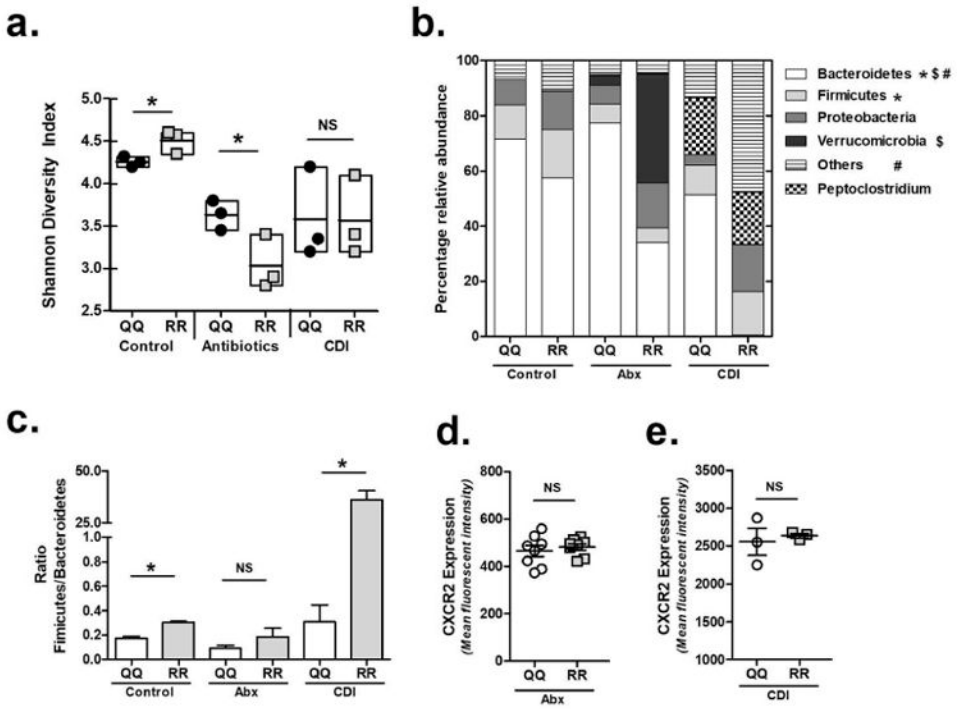

Figure 7.

Differential dysbiosis of gut microbiota regulates bone marrow neutrophil CXCR2 expression. **(a)** Changes in the Shannon diversity indices (SDI), **(b)** percent relative abundance of different phyla, and **(c)** Firmicutes to Bacteroidetes ratio phyla among gut microbiota of mice at baseline, after antibiotics (day 0) and after *C. difficile* infection (day 1) (n = 3 mice/group). Data shown as mean ± SEM.*p<0.05 at baseline, $p<0.05 after cefoperazone treatment, and #p<0.05 at day 1 of CDI (Students' *t test*). Bedding was exchanged daily between QQ and RR mice for the entire duration of antibiotics (9 days) and CXCR2 expression on bone marrow neutrophils were analyzed by flow cytometry **(d)** after antibiotics and **(e)** after *C. difficile* infection (Day 1). Data shown as mean ± SEM from 3-8 mice/group. (*p<0.05; Students' *t-test*).

Table 1

Homozygosity for *LEPR* 223R mutation is associated with increased risk of having peripheral WBC count >20 × 10^9/L between -3 and +3 days from *C. difficile* diagnosis

	Peak peripheral WCC				
Genotype	**>20 × 10^9/L**	**<20 × 10^9/L**	**Odds ratio**	**95% CI**	***P* value**
All patients (N = 110)	# of patients (%)	# of patients (%)			
QQ/QR	12 (14.5)	71 (85.5)	1.0		
RR	12 (44.4)	15 (55.6)	**5.4**	**1.83, 16.04**	**0.0023**

OR, Odds ratio (adjusted for age, race and gender); CI, confidence interval.