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Malnutrition drives infection susceptibility and dysregulated myelopoiesis that persists after refeeding intervention

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eLife Assessment

This **important** work advances our understanding of the impact of malnutrition on hematopoiesis and subsequently infection susceptibility. Support for the overall claims is **convincing** in some respects and **incomplete** in terms of identifying mechanism as highlighted by reviewers. This work will be of general interest to those in the fields of hematopoiesis, malnutrition, and dietary influence on immunity.

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Abstract

Undernutrition is one of the largest persistent global health crises, with nearly 1 billion people facing severe food insecurity. Infectious disease represents the main underlying cause of morbidity and mortality for malnourished individuals, with infection during malnutrition representing the leading cause of childhood mortality worldwide. In the face of this complex challenge, simple refeeding protocols have remained the primary treatment strategy. Although an association between undernutrition and infection susceptibility has been appreciated for over a century, the underlying mechanisms remain poorly understood and the extent to which refeeding intervention is sufficient to reverse nutritionally acquired immunodeficiency is unclear. Here we investigate how malnutrition leads to immune dysfunction and the ability of refeeding to repair it. We find that chronic malnutrition induced through prolonged dietary restriction (40% reduction in food intake) severely impairs the ability of mice to control a sub-lethal *Listeria monocytogenes* infection. Malnourished mice exhibit blunted immune cell expansion, impaired immune function, and accelerated contraction prior to pathogen clearance. While this defect is global, we find that

myelopoiesis is uniquely impacted, resulting in reduced neutrophil and monocyte numbers prior to and post-infection. Upon refeeding, we observe that mice recover body mass, size, cellularity across all major immune organs, the capacity to undergo normal immune cell expansion in response to infection, and a restoration in T cell responses. Despite this broad improvement, refed mice remain susceptible to *Listeria* infection, uncoupling global lymphoid atrophy from immunodeficiency. We find peripheral neutrophil and monocyte numbers fail to fully recover and refed mice are unable to undergo normal emergency myelopoiesis. Altogether, this work identifies dysregulated myelopoiesis as a link between prior nutritional state and immunocompetency. We believe these findings raise the possibility that exposure to food scarcity should be treated as an immunologic variable, even post-recovery, with considerations for how patient medical history and public health policy.

Introduction

Almost 500 million adults and over 200 million children are affected by undernutrition worldwide, with over half of all childhood deaths linked to undernutrition (United Nations, 2021; UNICEF, 1998). In the past 5 years, these numbers worsened due to economic instability ushered by COVID-19 pandemic, and they are projected to further increase throughout the impending climate crisis (Mbow et al., 2019; United Nations, 2022). In patients with undernutrition, the largest contributor to morbidity and mortality is infectious disease (Fan et al., 2022; Rice et al., 2000). It has been demonstrated that undernutrition alone puts patients, especially children, at a higher risk of developing long-lasting disability or dying from an infection (Antwi 2011; Bhargava, 2016; Bourke et al., 2016; Caulfield et al., 2004; Rice et al., 2000; Schlaudecker et al., 2011; Sinha et al., 2021). In addition to increased susceptibility to infection, chronically undernourished individuals have been reported to have impaired barrier function, atrophy of immune organs, and less effective responses to vaccines (Bhattacharjee et al., 2021; Bourke et al., 2016; Collins and Belkaid, 2020; Prendergast, 2015). In keeping with this, undernutrition is the leading cause of secondary immunodeficiency in the world (Chinen and Shearer, 2010). Considering the geographical overlap between areas with high prevalence of undernutrition and infectious disease and the persistent nature of the global undernutrition crisis, it is critical to investigate how undernutrition causes immunodeficiency and contributes to poor infection outcomes (Mbow et al., 2019; Murray et al., 1996; Pelletier, 1994; Roberts, 2017; Rohr et al., 2019; Sinha et al., 2021; UNICEF-WHO-World Bank JME Working Group, 2021).

Despite the first link between undernutrition and poor infection outcomes being posited over a century ago, the cellular mechanisms underlying undernutrition-induced immunodeficiency remain poorly resolved (Bourke et al., 2019, 2016; Hess, 1932; Newsholme, 1908). The primary explanation given for this dysfunction has centered on defects in lymphocyte biology. Patient data and animal studies suggest that T, B, and NK cells are reduced in the periphery of patients with undernutrition and experimental animals (Campbell et al., 2020; Cason et al., 1986; Contreras et al., 2018; Howard et al., 1999; Nájera et al., 2004; Saha et al., 1977; Schattner et al., 1990; Yang et al., 2009). Moreover, animal models of short-term fasting and protein deficiency have been found to impair T cell expansion, cytokine production, and memory recall responses (Chatraw et al., 2008; Iyer et al., 2012; Mangheri et al., 1992; Procaccini et al., 2012; Saucillo et al, 2014; Taylor et al., 2013). While the contribution of lymphocyte dysfunction to nutritionally acquired immunodeficiency is well established, the impact of prolonged undernutrition on other immune cell populations and the role they play in disease susceptibility is not well understood.

Prior studies have reported disparate impacts of undernutrition on immunity and infection, with some finding dietary or caloric restriction enhances inflammation or immunity, while others observing a loss in immune function (Bhattacharjee et al., 2021; Campbell et al., 2020; Chatraw et al., 2008; Collins et al., 2019; Han et al., 2023; Hasegawa et al., 2012; Iyer et al.,

2012; Palma et al., 2021; Pena-cruz et al, 1989; Piccio et al., 2008; Starr et al., 2016; Sun et al., 2001; Taylor et al., 2013). These discrepancies likely result from whether animals are subjected to caloric restriction alone or if there is also corresponding limitation in key micro- and macronutrients and whether the restriction in food intake is administered as a short-term fast or is sustained (Cerqueira and Kowaltowski, 2010; Contreras et al., 2018; Meydani et al., 2016; Palma et al., 2021; Yan et al., 2021). To this end, caloric and nutrient restriction diets significantly differ in weight loss patterns, physiology, and nutritional state (Cerqueira and Kowaltowski, 2010). While both diets are associated with reduced inflammation, caloric restriction is understood to support both lifespan and health span, in stark contrast to chronic undernutrition (Collins and Belkaid, 2020; Contreras et al., 2018; Goldberg et al., 2015; Green et al., 2021; Hasegawa et al., 2012; Howard et al., 1999; Jordan et al., 2019; Piccio et al., 2008; Spadaro et al., 2022; Sun et al., 2001; Yang et al., 2009).

Beyond our mechanistic understanding of nutritionally acquired immunodeficiency, there is a limited knowledge on whether this dysfunction is reversible (Contreras et al., 2018; Schattner et al., 1990; Vaisman et al., 2004). Because current treatment guidelines for patients with undernutrition utilize refeeding protocols that focus on weight gain as the indicator of recovery, the effects of refeeding on the immune system are understudied (Ashworth et al., 2003). Moreover, whether prior exposure to malnutrition has durable effects on the ability to control infection following refeeding remains unknown.

Here, we investigate the impact of nutritionally acquired immunodeficiency on the immune responses to *Listeria monocytogenes* infection and the ability of refeeding intervention to restore immune competency. We employ a chronic murine dietary restriction model and demonstrate that it effectively recapitulates the hallmarks of human undernutrition. We find that chronic undernutrition results in a specific atrophy of immune organs that is not mirrored in essential organs, such as the liver and kidney. This loss of lymphoid tissue is accompanied by a broad reduction in both innate and adaptive immune cell compartments. Undernourished mice fail to control sub-lethal *L. monocytogenes* infection, either succumbing to disease or failing to clear pathogen long-term. We find that while these mice undergo the initial phase of immune cell expansion following infection, they fail to sustain it and the response rapidly undergoes contraction. Accordingly, we observe that T cells in these mice display muted expansion, accelerated contraction, and impaired effector function. We further find that undernutrition selectively impairs steady-state and emergency myelopoiesis, resulting in reduced neutrophil and monocyte abundance prior to and post-infection. Finally, we demonstrate that while refeeding protocols are sufficient to restore body mass, growth, and reverse global lymphoid atrophy, refed mice remain more susceptible to infection even months after recovering size and peripheral immune cell numbers. In contrast to the ability of refeeding to reverse the effects of undernutrition on lymphoid numbers, we go on to show refed mice display impaired emergency myelopoiesis as well as neutrophil and monocyte abundance. Altogether, our work identifies dysregulated myelopoiesis as a major factor contributing to increased susceptibility to infection during chronic undernutrition. Furthermore, we for the first time demonstrate that refeeding protocols are not sufficient to reverse defects in the ability of mice to control infection or dysregulated myelopoiesis, despite outwardly displaying a recovery from malnutrition. We believe these findings have important implications for global public health policy and medical standards of care not only for treating people actively experiencing chronic malnutrition, but also for individuals who have experienced and recovered from food scarcity as part of their life history.

Results

Sustained dietary restriction recapitulates the hallmarks of nutritionally acquired immunodeficiency

To investigate the relationship of undernutrition and immunodeficiency, we began by adopting a faithful model of chronic malnutrition. In patients, undernutrition is associated with: (1) a consistent weight loss of 10% or more compared to the age-appropriate body weight that occurs in under 6 months and persists for months to years; (2) stunting of growth; and (3) a change in body composition associated with weight loss (Institute of Medicine, 2000; Maleta, 2006; Martins et al., 2011). While short-term fasting and macronutrient or caloric restriction models have been previously used, we elected to employ a 40% reduced diet (40RD) model, which provides both the weight loss as well as micro- and macronutrient deficiency over extended periods of time (Cerqueira and Kowaltowski, 2010). For the 40RD system, the average food intake of C57Bl6 mice was measured at baseline consumption. After a week of baseline measurements, mice were randomly assigned to either the *ad libitum* fed (AL) or the 40RD group. 40% of the average food intake by weight was removed from the diet of 40RD mice (**Fig. 1a**). Animal weight, body length, and body condition score (BCS) were then regularly surveyed to measure wasting, stunting, and body composition, respectively. Highlighting the ability of the model to reliably generate a state of moderate undernutrition, 40RD mice consistently lost 10% of their initial body weight (IBW) in 4 weeks (**Fig. 1b**) and achieved an average 20% loss of IBW relative to the AL group (**Fig. 1c**). At the same time, BCSs decreased significantly in 40RD mice compared to the AL group over the course of 4 weeks (**Fig. 1d**). This decrease in weight and conditioning was further accompanied by delayed growth and stunting in 40RD mice, as measured by body length (**Fig. 1e**).

Beyond these gross changes in body condition, patients with undernutrition also exhibit severe lymphoid organ atrophy (Beisel, 1996; Bourke et al., 2016b). In keeping with this, we observed a reduction in spleen, thymus, and lymph node size and mass in mice given the 40RD diet, while organs such as the liver and kidney were unchanged, suggesting a specific effect of malnutrition on the immune system (**Fig. 1f**). We observed a corresponding reduction in the cellularity of the spleen and thymus, with a more modest loss of cellularity observed in the bone marrow (**Fig. 1g**). Collectively, our findings demonstrate that the 40RD model reproduces the lymphoid atrophy found in chronically patients with undernutrition as well as other models of undernutrition in rodents (Beisel, 1996; Bourke et al., 2016b; Cason et al., 1986; Contreras et al., 2018; Yang et al., 2009).

Chronic malnutrition results in a failure to control sub-lethal L. monocytogenes infection

We next sought to investigate how malnutrition impacted immune responses to infection. While the link between poor infection outcomes and undernutrition has been extensively documented, the immunologic mechanisms resulting in poor infection resolution remain unknown (Bourke et al., 2019, 2016b; Dubos, 1955; Ishikawa et al., 2012; Rice et al., 2000b). To address this, we turned to the Ovalbumin expressing *Listeria monocytogenes* (Lm-Ova) system, a classic model of bacterial infection that permits the tracking of adaptive immunity, innate immunity, and bacterial burden. Both 40RD and AL mice were infected with a sublethal dose of Lm-Ova and then infection progression, pathogen clearance, and immune responses were assessed at 5, 8, and 14 days post-infection (**Fig. 2a**). Whereas all mice in the AL group survived, nearly half of the 40RD mice became moribund and required euthanasia (**Fig. 2b**). Even amongst surviving mice, the 40RD group displayed higher clinical scores than the AL group throughout the course of infection (**Fig. 2c**). Consistent with this, 40RD mice failed to clear bacteria at the rate of the AL group, with some mice exhibiting persistent infection (**Fig. 2d**). Amongst the AL group, 48.6% and 86.7% of the mice were able to clear bacteria by day 5 and day 8 post-infection, respectively, with all mice resolving infection by day 14. In contrast, 40RD mice exhibited delayed clearance kinetics with only a third of the mice becoming pathogen-free by day 14 (**Fig. 2d**).

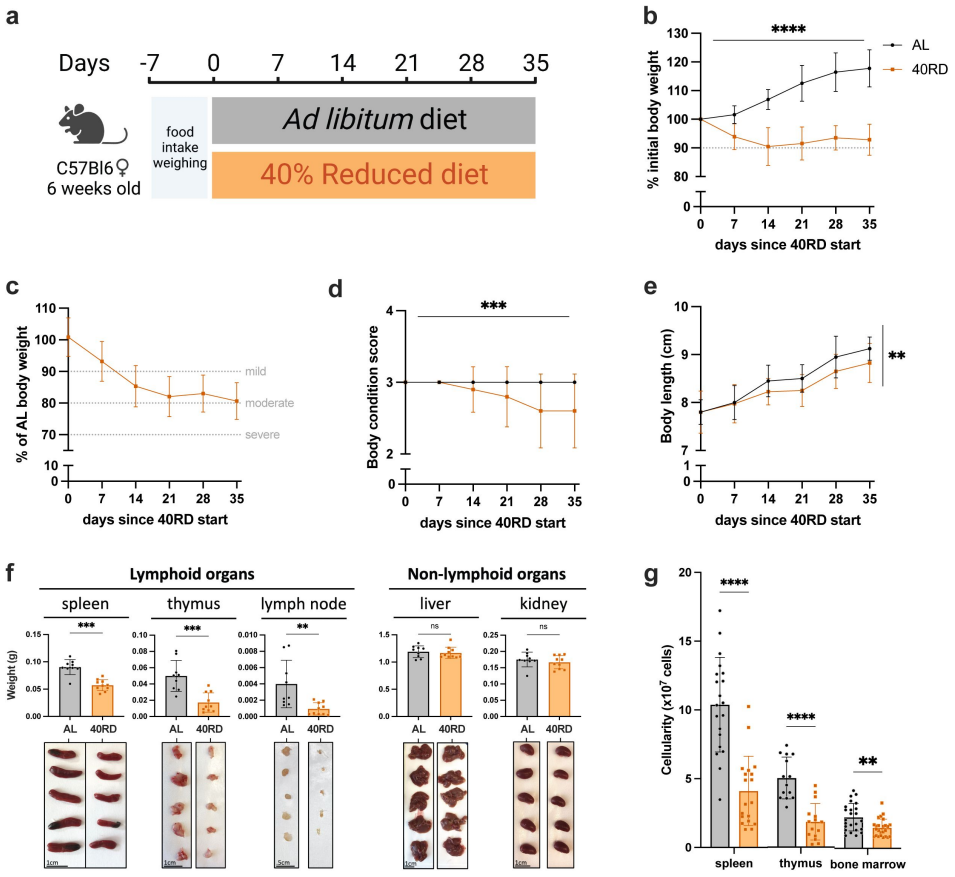

Figure 1.

Sustained dietary restriction recapitulates the hallmarks of nutritionally acquired immunodeficiency.

(a) Schematic of experimental design for 40% reduced diet (40RD, orange) in comparison to control *ad libitum* (AL, black) diet. (b) Body weight of AL and 40RD mice as a percentage of initial body weight over time (n=50). The dotted line represents 10% of initial body weight lost. (c) Body weight of 40RD mice as a percentage of age matched average AL body weight over time (n=50). Each dotted line represents clinical designations of undernutrition severity. (d) Body condition score of AL and 40RD mice over time (n=10). (e) Body length of AL and 40RD mice over time, measured from the nose tip to the base of the tail (n=10). (f) Comparative weights of AL and 40RD lymphoid and non-lymphoid tissues (n=10) with representative photos of the corresponding organs. Scale bars 1 cm (0.5 cm for lymph nodes). (g) Total live cell counts for whole spleen (n=15), thymus (n=15), and bone marrow (n=10). Statistics: (b-g) Plotted as mean ± SD; (b,d) simple linear regression with slope comparisons; (e) simple linear regression with elevation comparison; and (f,g) two-tailed Mann-Whitney test.

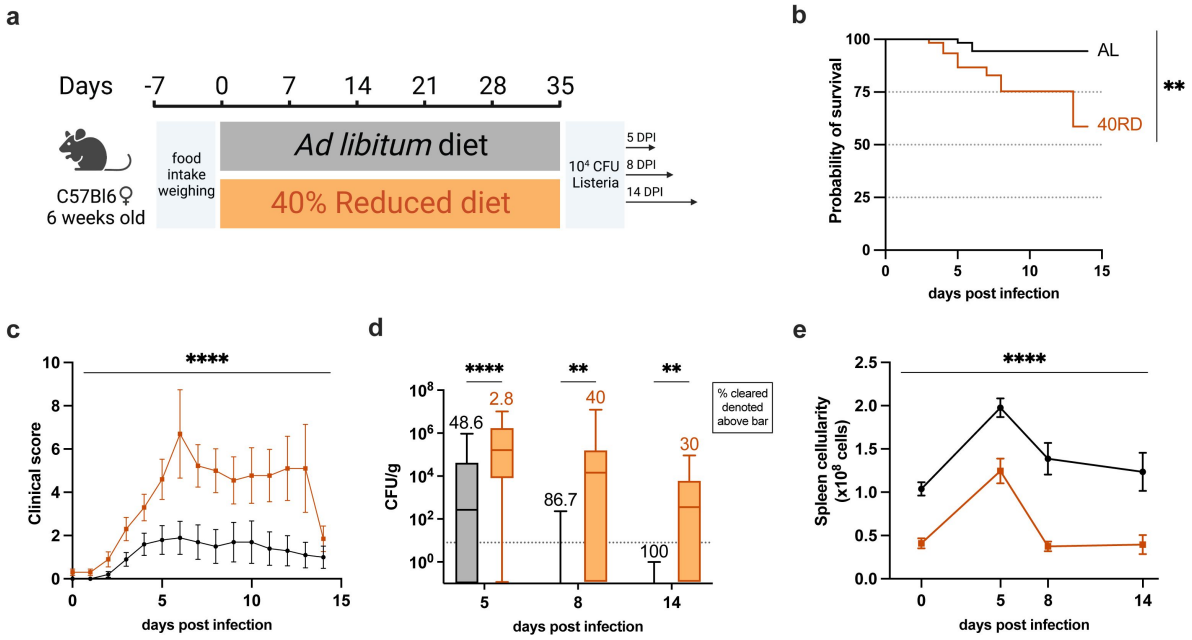

Figure 2.

Chronic malnutrition results in a failure to control sub-lethal *L. monocytogenes* infection.

(a) Schematic of Lm infection (10^4 CFUs per mouse) experimental design in AL (orange) and 40RD (black) mice. Mice were maintained on the corresponding diet throughout the course of the infection. (b) Probability of survival for infected AL and 40RD mice over time. The curves represent pooled data from 3 experimental groups: 5DPI (n=25), 8DPI (n=15), and 14DPI (n=10). Statistics done via log-rank test. (c) Clinical score for infected AL and 40RD mice over time from 14DPI group. Plotted as mean ± SEM; statistics done via mixed-effect two-way ANOVA analysis. (d) Pathogen burden in liver tissue of AL and 40RD mice. Percentage of mice that cleared the pathogen on a given day is represented as numbers above corresponding bars. The dotted line represents the limit of detection. Plotted as box and min to max whiskers; statistics done via two-tailed Mann-Whitney test for each time point. (e) Total splenocyte counts for infected AL and 40RD mice over time. Uninfected spleen cell counts same as used for **Figure 1g**. Plotted as mean ± SEM; statistics done via mixed-effect two-way ANOVA analysis.

These observations prompted us to ask whether 40RD mice could mount a normal immune response to infection. To begin addressing this, we examined immune cell expansion in AL and 40RD mice throughout the course of infection. We found that while splenocyte numbers increased at early time points following infection in 40RD mice, the magnitude and duration of this expansion was significantly lower compared to AL controls (**Fig. 2e**). Thus, chronic malnutrition is sufficient to not only induce immune atrophy, but further abrogates the capacity of mice to mount and sustain an immune response to infection.

Chronic malnutrition diminishes T cell expansion and function while accelerating contraction during infection

Considering the broad defects observed in infected, chronically undernourished mice, we next aimed to investigate the effects malnutrition had on specific immune cell populations during this response. As malnourished patients are known to exhibit the reduction in lymphocyte numbers, we assessed whether 40RD mice exhibited signs of lymphopenia following infection (Cason et al., 1986; Nájera et al., 2004; Saha et al., 1977). We observed that both prior to and after infection, 40RD mice displayed reduced numbers of B cells, CD4 T cells, and CD8 T cells compared to controls (Supplemental 1). Despite this overall loss in lymphocyte number, the relative frequency of each population was either unchanged or elevated, indicating that while malnutrition leads to a global reduction in immune cell numbers, lymphocytes are less impacted than other immune cell populations (Supplemental 1).

Considering the role CD8 T cells play in resolving intracellular bacterial infections and the failure of 40RD mice to clear infection at later time points, we next examined whether the kinetics of T cell expansion and contraction were similarly impacted. To do so, we tracked T cell function throughout the course of infection, on day 5 (early response), day 8 (peak response), and day 14 (contraction phase) post-infection (Qiu et al., 2018; Zenewicz and Shen, 2007). We found that the antigen-experienced ($CD44^{+}$) and short-lived effector ($CD127^{low}KLRG1^{high}$) $CD8^{+}$ T cell numbers were significantly reduced throughout the infection (**Fig. 3a,b**). These effector-like populations expanded minimally during early and peak response and contracted quickly by day 14, prior to pathogen clearance in 40RD mice. In addition to these defects in expansion/contraction dynamics, we further tested whether chronic malnutrition also impaired T cells function. We thus evaluated both the frequency and per-cell cytokine producing capacity of T cells at day 8 post-infection. We observed that T cells from undernourished mice displayed both a decreased frequency of cytokine producing cells and a decreased capacity for producing cytokines on a per-cell basis (**Fig. 3c-e**). Altogether, these findings suggest that impaired T cell function along with insufficient T cell expansion and premature contraction prior to pathogen clearance contribute to the failure of chronically undernourished mice in controlling *L. monocytogenes* infection.

Chronically malnourished mice display dysregulated myelopoiesis

The observation that 40RD mice exhibited elevated pathogen burden at early time points prior to the peak of the adaptive immune response between days 5 to 8 prompted us to investigate whether innate immune responses were similarly impaired. While total bone marrow cellularity was equivalent between 40RD and AL groups, examination of specific innate immune populations in bone marrow revealed that steady-state neutrophil and monocyte levels were significantly lower in undernourished mice, both in absolute numbers and relative abundance (**Fig. 1g** & **Fig. 4a-d**; Supplemental 2). In keeping with this, we observed that splenic neutrophil and monocyte abundance were significantly lower in 40RD mice at steady-state and underwent impaired expansion following infection, with splenic monocyte frequency elevated at steady state and comparable to control mice post-infection (**Fig. 4b-d**; Supplemental 2). These steady-state changes could be observed early after mice were placed on the 40RD diet, with total peripheral blood cellularity and neutrophil abundance significantly reduced by 1 week post-dietary restriction (Supplemental 2e,f).

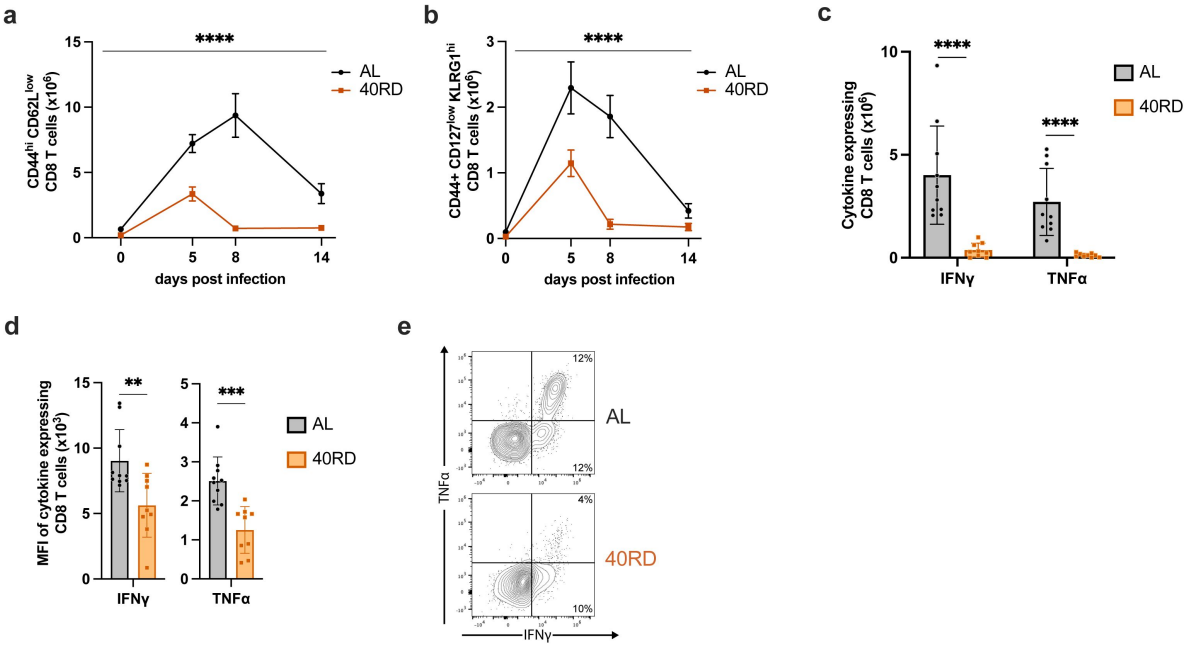

Figure 3.

Chronic malnutrition diminishes T cell expansion and function while accelerating contraction during infection.

AL and 40RD mice were infected with Lm at 10^4 CFUs per mouse. Splenocytes were counted and flow cytometry was performed at days 0, 5, 8, and 14 post-infection to evaluate the total cell number of (a) antigen-experienced $CD8^+$ T cells gated on live single $CD8^+$ $CD44^{hi}$ $CD62L^{low}$ and (b) short lived effector cells further gated on $KLRG1^{hi}$ $CD127^{low}$. Plotted as mean ± SEM; statistics done via mixed-effect two-way ANOVA analysis. Splenocytes from AL (n=10) and 40RD (n=10) mice were harvest at day 8 post-infection and stimulated ex vivo with OVA peptide for 6 hours. Intracellular flow cytometry was performed to quantify (c) total cell number of and (d) mean fluorescence intensity (MFI) of antigen-experienced $CD8^+$ T cells expressing IFNγ and TNFα. Plotted as mean ± SD; statistics done via two-tailed Mann-Whitney test for each cytokine. (e) Representative flow cytometry data of (c,d), with average frequencies shown.

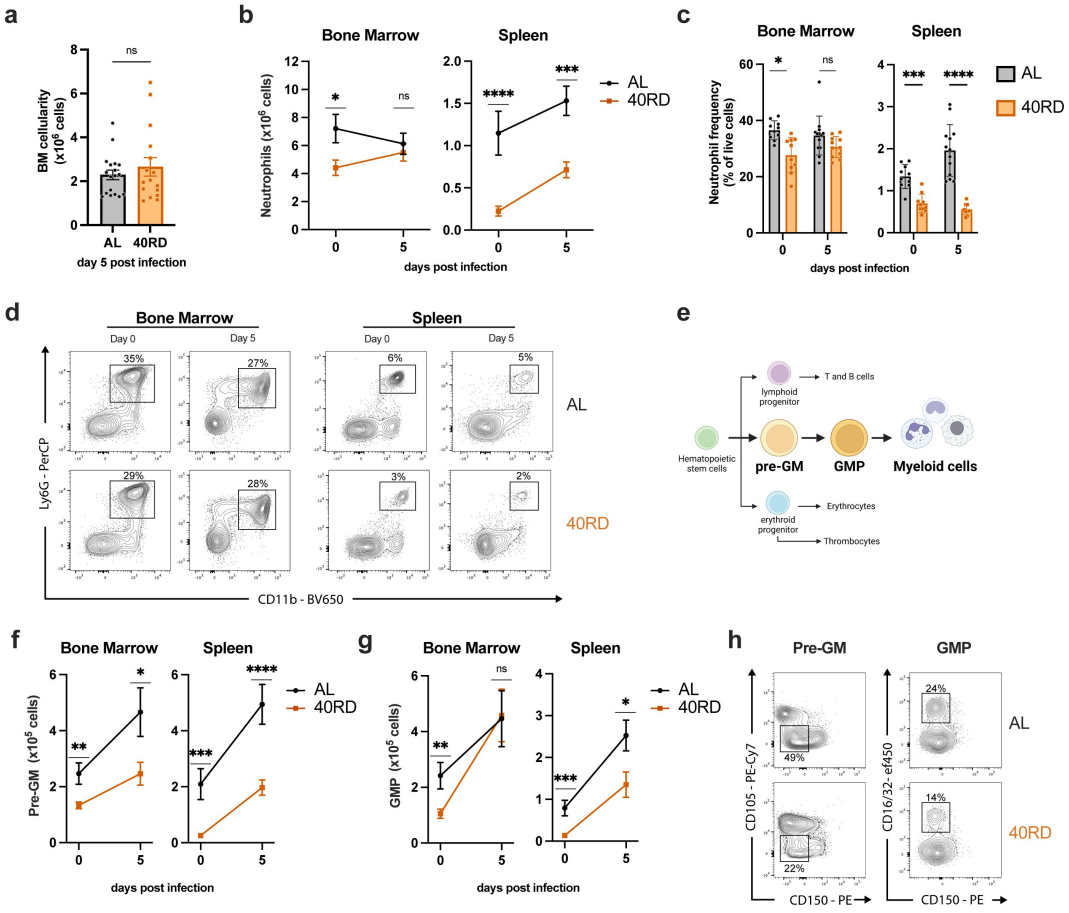

Figure 4.

Chronically malnourished mice display dysregulated myelopoiesis.

(a) Total bone marrow cell counts from day 5 post-infection AL (n=18) and 40RD (n=16) mice. Bone marrow cells and splenocytes were counted and flow cytometry was performed at days 0 (n=10 for both AL and 40RD) and 5 post Lm infection in AL and 40 RD mice to evaluate (b) the total cell number of neutrophils and (c) the relative frequency of neutrophils among live cells. (d) Representative flow cytometry data for the results in (b,c), with average frequencies shown. (e) A simplified schematic representation of myelopoiesis showing pre-GM and GMP as key progenitors in granulocyte/monocyte lineage. Bone marrow cells and splenocytes were counted and flow cytometry was performed at days 0 and 5 post Lm infection in AL and 40 RD mice to evaluate the total cell number of (f) pre-GM cells (Lineage$^-$ Sca1$^-$ CD117$^+$ CD150$^-$ CD16/32$^-$ CD105$^-$) and (g) GMP cells (Lineage$^-$ Sca1$^-$ CD117$^+$ CD150$^-$ CD16/32$^+$). (h) Representative flow cytometry results for the myeloid progenitor data in (f,g), with average frequencies shown. Statistics: (a-c,f,g) Plotted as mean ± SEM; statistics done via two-tailed Mann-Whitney test for each time point.

Neutrophil and monocyte production is maintained in the bone marrow during steady-state and through emergency myelopoiesis in both bone marrow and spleen upon infection (Janssen et al., 2016; Kim, 2010; Manz and Boettcher, 2014; Yvan-Charvet and Ng, 2019). We thus evaluated how undernutrition affected the key myeloid progenitor populations, pre-granulocyte/monocytes (pre-GM) and granulocyte/monocyte progenitors (GMP) (**Fig. 4e**). Consistent with a loss of mature neutrophils and monocytes, we found that myelopoiesis was significantly impaired in undernourished mice. Within the bone marrow, pre-GM and GMP cells were present at lower numbers in steady-state 40RD mice (**Fig. 4f,g**). Upon infection, these bone marrow progenitor populations were capable of undergoing expansion, with pre-GM cells failing to reach AL levels whereas GMP cells reached comparable numbers (**Fig. 4f-h**). These defects were more exaggerated in the spleen, where 40RD mice displayed diminished numbers and frequency of pre-GM and GMP cells both pre-and post-infection (**Fig. 4f-h**). Altogether, we find that undernutrition impairs steady-state myelopoiesis and extramedullary emergency myelopoiesis, blunting the innate immune response against a bacterial pathogen.

Refeeding intervention reverses wasting, stunting, and global immune atrophy

One of the primary strategies taken to support patients with undernutrition with health complications, including infections, is to refeed them by slowly increasing caloric intake to levels expected for their age group (Ashworth et al., 2003). Despite the widespread employment, the effects of this intervention on immune dysfunction are not known. To address this, we developed a refeeding protocol that safely reintroduces *ad libitum* feeding to 40RD mice to test whether defects in immune responses can be rescued through weight gain and nutrient availability. Undernourished mice undergoing a refeeding protocol (RF) were maintained on a standard 40RD diet for 4 weeks or until 10% BWL. Then, the RF mice were given 10% extra food by weight every two days for a week. On the last day of the refeeding protocol, the RF mice were given *ad libitum* access to feed. Mice were maintained for an additional 6-8 weeks on the *ad libitum* feed until they reached a normal weight range for their age (**Fig. 5a**). The RF group was able to regain IBW during the refeed period and continued to gain weight at an accelerated pace for the first month (**Fig. 5b**). After the initial increase in weight, the weight gain pace of RF mice slowed down to the same rate as the AL group (**Fig. 5c**). We further observed that while RF mice were able to recover growth, the mice maintained a modest but significantly shorter body length than AL controls for their age (**Fig. 5d**), in keeping with persistent stunting common in patients with undernutrition (Ashworth et al., 2003).

With the refeeding protocol established, we set out to test whether restoring body mass was sufficient to reverse the lymphoid atrophy found in 40RD mice. Paralleling recovery in weight, we found that cellularity in the spleen, thymus, and bone marrow were comparable between AL and RF groups (**Fig. 5e**). Moreover, we observed that the weight of lymphoid organs has returned to the AL levels in the RF group (**Fig. 5f**). Together, these findings indicate that refeeding intervention is sufficient to restore global lymphoid atrophy.

Refeeding intervention fails to restore immunocompetency and normal myelopoiesis

After observing successful reversal of the lymphoid atrophy in RF mice, we tested whether refeeding would be sufficient to restore their ability to control *L. monocytogenes* infection. To test this, we infected age-matched AL, 40RD, and RF mice with a sub-lethal dose of Lm-OVA (**Fig. 6a**). While refeeding was able to limit morbidity compared to 40RD mice, a portion of RF animals succumbed to infection whereas all AL mice remained viable (**Fig. 5b**). Consistent with this, we observed that mice with ongoing malnutrition maintained the highest pathogen burdens and that RF mice were unable to resolve infection with the same kinetics as AL mice, with less than half RF

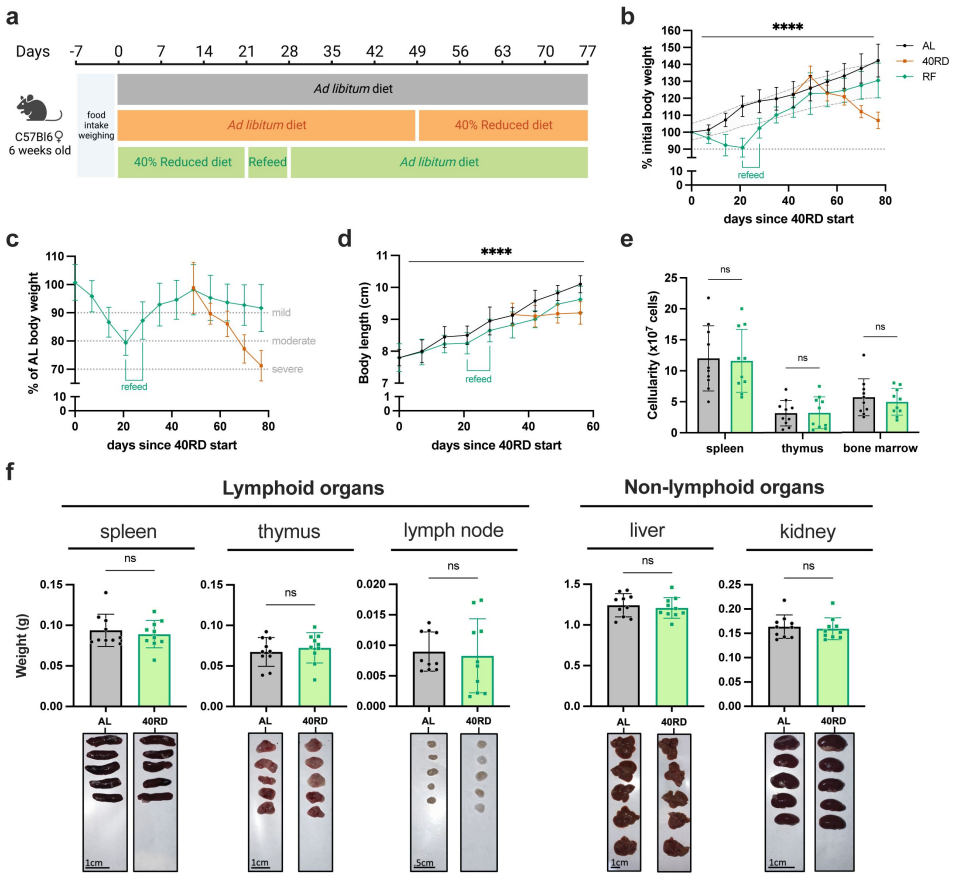

Figure 5.

Refeeding intervention reverses wasting, stunting, and global immune atrophy.

(a) Schematic of the experimental design for refeeding intervention (RF) in comparison to age-matched 40RD and control AL diet. (b) Body weight of AL (n=25), 40RD (n=10), and RF (n=25) mice as a percentage of their initial body weight over time. The dotted line represents 10% of initial body weight lost, and the shaded area represents the normal weight range for age-matched female C57Bl6 mice(C). (c) Body weight of 40RD (n=10) and RF (n=25) mice as a percentage of age matched average AL body weight over time. Each dotted line represents clinical designations of undernutrition severity. (d) Body length of AL and 40RD mice over time, measured from the nose tip to the base of the tail (n=10). (e) Total cell counts for whole spleen, thymus, and bone marrow from AL and RF mice (n=10). (f) Comparative weights of AL and RF lymphoid and non-lymphoid tissues (n=10) with representative photos of the corresponding organs. Scale bars 1 cm (0.5 cm for lymph nodes). Statistics: (b-f) Plotted as mean ± SD; (b,d) simple linear regression with slope comparisons; and (e,f) two-tailed Mann-Whitney test.

mice clearing bacteria (**Fig. 5c**). These data indicate that despite refeeding being sufficient to reverse malnutrition-induced lymphoid atrophy, prior exposure to chronic undernutrition leads to persistent susceptibility to infection.

Having observed this durable impairment in immune protection, we next aimed to identify the specific facets of malnutrition-induced immune dysfunction that failed to recover in RF mice. In keeping with the steady-state recovery observed, post-infection splenic and bone marrow cellularity were equivalent between RF and AL mice (**Fig. 6d**). Moreover, we found that T and B cell numbers were restored in RF mice and all lymphocyte populations underwent comparable expansion to AL controls after infection (Supplemental 3). Similarly, we observed no differences in the number of antigen-experienced T cells in the spleen nor T cell functional capacity (**Fig. 6e-g**). Thus, refeeding permits recovery of lymphocyte number, lymphocyte expansion capacity, and T cell function in response to infection, suggesting lymphocyte dysfunction is unlikely to be responsible for the persistent susceptibility observed.

In contrast to the restoration of the lymphocyte response, we observed sustained defects in the myeloid compartment. RF mice displayed impaired peripheral expansion of neutrophil and monocyte populations after infection, both by frequency and number, with neutrophil number diminished in steady-state RF mice as well (**Fig 6i,j**; Supplemental 4a,b). Within the bone marrow, we observed a reduced frequency in steady-state monocyte and neutrophil abundance as well as reduced total neutrophil numbers, however both populations expanded upon infection, reaching control numbers (Supplemental 4c-f). Having seen this, we next asked whether RF mice exhibited a persistent impairment in either central or emergency myelopoiesis. Whereas myeloid progenitor populations were not perturbed in the bone marrow, we found that RF mice exhibited significantly reduced frequency and numbers of splenic pre-GM and GMP cells following infection, with the GMP population also diminished in steady-state mice (**Fig. 6j-l**); Supplemental 5). Altogether, our findings demonstrate that refeeding intervention uncouples a global recovery in lymphoid atrophy from an enduring impairment in emergency myelopoiesis, resulting in lasting susceptibility to infection.

Discussion

Here we employ a murine model of chronic undernutrition that phenocopies many of the hallmarks of human undernutrition (stunting, wasting, and changes in body composition) to characterize how chronic malnutrition leads to nutritionally acquired immunodeficiency. We demonstrate that sustained malnutrition results in global lymphoid atrophy, with loss of both innate and adaptive immune cell populations. Additionally, we identified a unique and severe impairment in neutrophil abundance and myelopoiesis, with a similar but more modest effect on monocytes. Our data suggests that the combination of diminished homeostatic levels of these critical myeloid populations along with impaired emergency myelopoiesis leads to poor control of early *L. monocytogenes* infection, while a blunted T cell response to infection and their premature contraction results in lack of effective infection resolution. We then tested whether a refeeding intervention could restore protective immunity to infection. We found that restoring food access permitted mice to regain weight and size as well as reverse many signs of immunodeficiency. Refeeding was sufficient to recover lymphoid organ atrophy, the abundance of most circulating lymphocytes, and T cell cytokine production. Nonetheless, refed mice continued to display increased susceptibility to *L. monocytogenes* infection along with sustained defects in neutrophil and monocyte abundance and emergency myelopoiesis. These data demonstrate that exposure to chronic undernutrition can result in lasting changes to specific compartments of the immune system, with long-term implications of the health of the individual even after recovering growth and size.

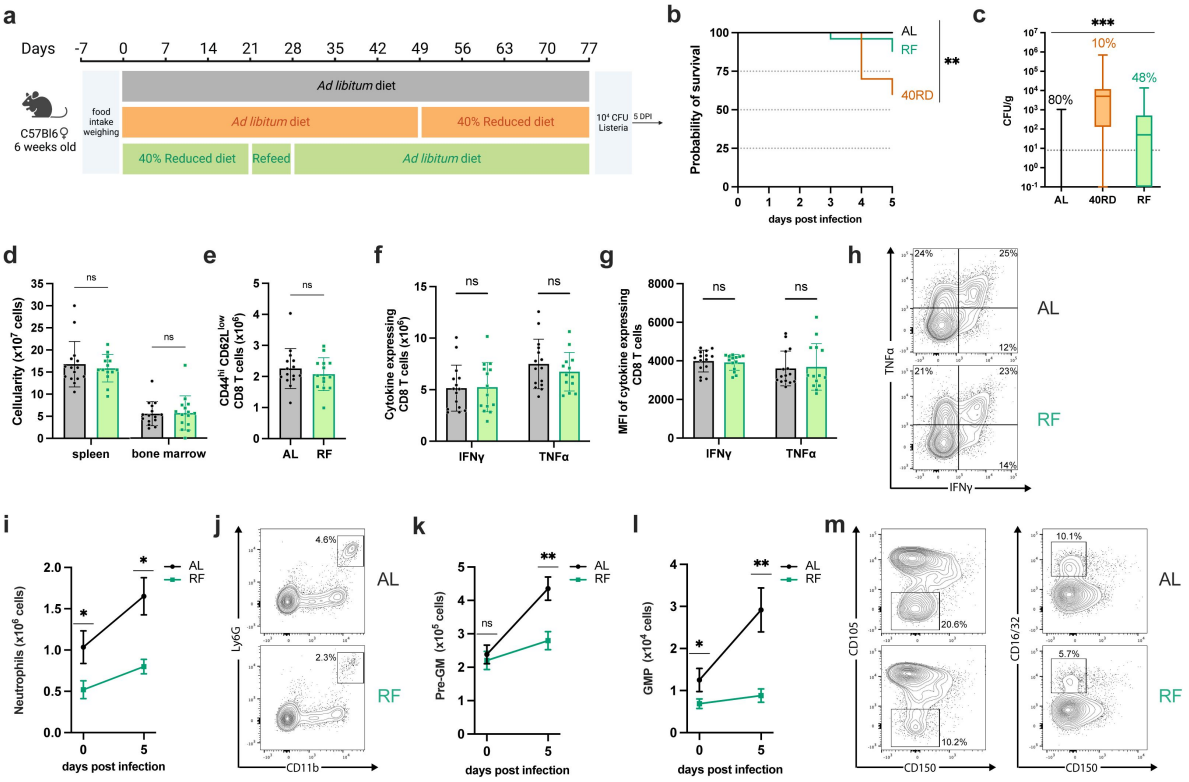

Figure 6.

Refeeding intervention fails to restore immunocompetency and normal myelopoiesis.

(a) Schematic of Lm infection (10^4 CFUs per mouse) experimental outline in AL, 40RD, and RF mice. Mice were maintained on the corresponding diet throughout the course of the infection. (b) Probability of survival for infected AL (n=25), 40RD (n=10), and RF (n=25) mice over time. Statistics done via log-rank test. (c) Pathogen burden in liver tissue of 5 DPI AL (n=25), 40RD (n=10), and RF (n=25) mice. Percentage of mice that cleared the pathogen on a given day is represented as numbers above corresponding bars. The dotted line represents the limit of detection. Plotted as box and min to max whiskers; statistics done via Kruskal-Wallis test. (d) Total splenocyte and bone marrow cell counts for AL (n=15) and RF (n=15) mice at day 5 post-infection. (e) Total cell number of $CD44^+CD8^+$ T cells in AL and RF mice at days 0 and 5 post-infection. Splenocytes from AL and RF mice were harvest at day 5 post-infection and stimulated ex vivo with OVA peptide for 6 hours. Intracellular flow cytometry was performed to quantify (f) total cell number of and (g) mean fluorescence intensity (MFI) of antigen-experienced $CD8^+$ T cells expressing IFNγ and TNFα. (h) Representative flow cytometry results for data in (f,g), with average frequencies shown. (i) Total splenic neutrophils abundance in AL and RF mice at days 0 and 5 post-infection. (j) Representative flow cytometry results for day 5 post-infection neutrophil data in (i), with average frequencies shown. At days 0 and 5 post-infection, spleens from AL and RF were evaluate for the total number of (k) pre-GM cells and (l) GMP cells. (m) Representative flow cytometry results plots for the myeloid progenitor data in (k,l), with average the average frequencies shown. Statistics: (d-g) Plotted as mean ± SD; statistics done via two-tailed Mann-Whitney test for each category. (i,k,l) Plotted as mean ± SEM; statistics done via two-tailed Mann-Whitney test for each time point.

Previous studies have suggested that impaired pathogen responses during undernutrition are driven by lymphocyte loss and functional impairment (Beisel, 1996, 1982; Institute of Medicine, 2000). Low levels of circulating lymphocytes have been well documented in patients with undernutrition and various restrictive diet models (Campbell et al., 2020; Cason et al., 1986; Contreras et al., 2018; Howard et al., 1999; Saha et al., 1977; Schattner et al., 1990; Yang et al., 2009). Our data from the refeeding model suggest that while these defects likely contribute to poor resolution of infection, they are not sufficient to explain why previously malnourished mice remain vulnerable to infection, as these mice recover from lymphoid atrophy and display normal T cell responses. Nonetheless, we find consistent with other groups that undernourished mice exhibit a steady-state reduction in circulating T cells. Upon infection, the T cell compartment is capable of undergoing expansion in malnourished conditions, but the magnitude of this response is blunted and quickly contracts prior to pathogen clearance. We also found that there was reduced abundance of antigen experienced T cells throughout the infection. Additionally, we found that the intrinsic ability of T cells to produce inflammatory cytokines was reduced. In keeping with work demonstrating that T cells from patients with undernutrition display reduced function, we find that T cells from malnourished mice have impaired cytokine producing capacity post-infection. Altogether, our work supports clinical observations and earlier studies delineating the relationship of undernutrition on lymphoid atrophy and T cell dysfunction and has revealed the reversible nature of these defects after refeeding intervention.

In contrast to the recovery found in the adaptive immune cells after refeeding, our work demonstrates that exposure to chronic periods of malnutrition results in durable defects in the myeloid compartment. We find that undernourished mice display impaired myelopoiesis and a loss in abundance in critical peripheral myeloid populations that does not properly recover upon refeeding. Indeed, some small cohort studies have shown that circulating neutrophil numbers are reduced in undernourished children and that hematopoiesis is impacted in anorexic patients (Kohama et al., 2021; Vaisman et al., 2004). Thus, while hematopoiesis and peripheral immune cell numbers are broadly sensitive to an animal's ongoing nutritional status, specific subsets are uniquely sensitive to periods of undernutrition and exhibit nutritional scarring that uncouples their activity from future improvements in dietary intake. These findings raise the possibility that lasting epigenetic changes within the myeloid compartment occur during exposure to undernutrition, akin to what has been observed for trained immunity (Netea et al., 2020). Indeed, a recent study in a small cohort of undernourished children reported changes in H3 acetylation in peripheral blood mononuclear cells (Kupkova et al., 2021).

While this work provides a novel link between myelopoiesis and nutritional state, many questions remain. Our study investigated the effect of chronic undernutrition during *L. monocytogenes* infection, however the extent to which these findings apply more broadly to other models of bacterial infection or innate inflammation are unclear. Moreover, our malnutrition model broadly restricted food intake without regard to the components of diet that are required for immunocompetency nor consideration for potential interactions of the magnitude of food restriction with immunodeficiency and the rate of onset. It will be of interest in future studies to resolve the extent to which the phenotypes observed are due to the gross loss of calories, specific macronutrients, certain micronutrients, and the grade of dietary restriction enforced. In addition, our study focused on the effects of dietary restriction on adult, normal weight mice. Whether similar phenotypes would emerge if obese animals were dietarily restrict and if weight loss was achieved through pharmacologic means (e.g. GLP-1 targeted drugs) is not clear. Likewise, how similar dietary restriction might affect the immune system if it were initiated in juvenile mice is not addressed here. Mechanistically, additional work is needed to address whether the changes in myelopoiesis are due to changes within the hematopoietic compartment, non-hematopoietic cells, and/or to systemic levels of key cytokines and hormones – such as G-CSF, interferons, and leptin – both in malnourished and refed mice. Finally, the known impact dietary restriction has on

microbiota composition and in turn how this might durably impact the immune system is unaccounted for in this work and warrants investigation (Gordon et al., 2012; Blanton et al., 2016).

Altogether our work offers new insight into the contribution of myelopoiesis in nutritionally acquired immunodeficiency and the ability of refeeding intervention to treat these defects. The burden of morbidity and mortality of infections on undernourished individuals is one of the leading global health crises and evidence of impaired vaccine responses in undernourished people is putting in question the world's ability to protect the most vulnerable populations with its most reliable strategies for prevention (Bhattacharjee et al., 2021; Bhargava, 2016; Bourke et al., 2019, 2016; Dubos, 1955; Ishikawa et al., 2012; Prendergast, 2015; Rice et al., 2000; Saha et al., 1977). We provide evidence that even prior exposure to food scarcity may be sufficient to permanently alter future immune responses. This suggests that the current dietary status of an individual may be insufficient information to understand the impact nutrition has on their immune system. Future work delineating the specific components of diet required to sustain immune health in the face of food scarcity will be critical for developing nutritional interventions or broader food supplementation programs for at risk populations. We believe that this work indicates periods of poverty and food insecurity may be an important factor in patient medical history as well as for formulating global health policy in these areas.

Materials and Methods

Mice and diets

All mice were female on a C57Bl6 background maintained at Children's Hospital of Philadelphia (CHOP) animal facility. Mice were either purchased from Jackson laboratories at 6 weeks of age and acclimated in the CHOP facility for 1 week or bred in house from breeders also obtained from Jackson laboratories. All animal experiments were approved by IACUC, and mice were cared for in accordance with IAC 21-001325 protocol. All 40RD and RF experiments were performed with mice between 6 and 8 weeks of age. Mice were housed in groups of 4-5 animals for all experiments. All experiments were performed on age-matched littermate controls. All mice were maintained on LabDiet 5001 from weaning/arrival to facility.

For the 40RD diet, mice were maintained on *ad libitum* diet until the start of the experiment. One week before the start of the experiment average food intake was measured per 5 mice per day. Average food consumption for all experimental animals between 6 and 8 weeks of age was 4 g/day/mouse. At the start of the experiment, mice were weighed to make sure that all the animals were in the range of ±1.5 g and randomly assigned to an AL or a 40RD group. AL mice were maintained as before with unlimited access to chow. 40RD mice were placed on the 40% reduced diet by weight, resulting in 2.4 g/day/mouse of feed. Consistent weight loss and no competition for food was observed in 40RD mice. 40RD mice lost 10% of their IBW every 4 weeks. If mice lost over 20% of their IBW they were euthanized. During the 40RD diet, experimental animals did not exhibit behavioral or clinical changes and only moderate changed body composition and growth stunting. When all 40RD mice reached 10%-15% IBW loss, they were considered undernourished and utilized for further experiments.

For the RF diet, mice were set up in the identical way to the 40RD diet until they reached 10%-15% IBW loss. At which point they underwent a refeeding intervention developed using Guidelines for the inpatient treatment of severely malnourished children. The refeeding intervention lasted a week and increased the weight of available food to the restricted mice by 10% every 2 days. On the 40RD diet, mice had access to 2.4 g/day/mouse (60% of the initial average food consumption). On day 1 of refeeding intervention, mice had access to 2.8 g/day/mouse (70%). On day 3, they had access to 3.2 g/day/mouse (80%). On day 5, it was 3.6 g/day/mouse (90%). On day 7, mice were given

unrestricted food access. RF mice were then maintained on *ad libitum* feed for a month after they entered normal weight range for their age[88] and their food intake normalized to the AL age-matched controls. At that point mice were considered refed and were utilized for further experiments.

40RD mice and mice on the 1 week-long refeeding intervention were fed daily between 4pm and 6pm to avoid interference with circadian rhythm. Mice on the 40RD and intervention consumed all food provided to them between feeding windows. All mice were observed daily for signs of sickness. All mice were weighed every 2-3 days for 3 times a week total. Food intake for all mice was recorded either daily, if fed daily, or every 2-3 days for 3 times a week total if fed *ad libitum*. Mice were maintained on assigned diets during infection and sepsis experiments. Mice had unlimited access to drinkable water.

Body length, clinical score, and body condition score (BCS)

For 30 40RD mice and 30 age-matched AL controls body length and BCS were recorded weekly. For 25 RF mice and 25 age-matched AL controls body length were recorded weekly. Body length was recorded using standard centimeter ruler from base of the tail to the tip of the nose. The length was recorded in 0.25cm increments. BCS was recorded using IACUC Pain and Distress Recognition Policy guidelines in a blinded manner by the same observer for all time points. BCS was rated on the scale of 1 – 5, as follows: 1) emaciated, prominent skeletal structure and distinct vertebrae segmentation; 2) underconditioned, segmentation of vertebrae, dorsal pelvic bones palpable; 3) well-conditioned, vertebrae and dorsal pelvis not prominent; 4) overconditioned, spine is a continuous column and vertebrae palpable only with firm pressure; 5) obese, mouse smooth and bulky with bone structure that disappears under flesh and subcutaneous fat (Ullman-Culleré and Foltz, 1999).

Clinical score is a sum of 7 parameters ranked on the scale of 0 - normal to 4 - severely impaired. The scores were measured by the same observer at all time points in a blinded manner. For the measurement, an animal was transferred to a new empty cage and allowed to acclimate for 30 seconds. Then, appearance of the body, appearance of the eyes, level of consciousness, and activity were observed. Then, the cage was tapped twice for auditory stimulus and the animal was touched gently on the back for touch stimulus or gently tipped over if no locomotion was observed. Then, the animal was restrained with standard hold, allowed to acclimate for 30 seconds, and its breathing rate and quality were observed and recorded.

Listeria preparation and infection

For all the *Listeria monocytogenes* (Lm) infections, the recombinant Lm strain Lm-OVA, kindly provided by the laboratory of Dr. Hao Shen, was used. Lm were grown to early log phase (OD600 = 0.1) in brain heart infusion media (BHI, BD, cat. 237200) at 37°C, washed in PBS (Corning, cat. 21030CV) and diluted to 10^4 CFUs per 100uL of PBS using our observation that 1 OD unit = 3×10^9 CFUs. The CFU count was confirmed after infection by plating an aliquot of diluted Lm on BHI plate and incubating overnight at 37°C. Experimental animals were temporarily sedated using Isoflurane (Med Vet International, cat. RXISO-250). 100uL of Lm suspension was injected retro-orbitally per mouse. Mice were observed for full anesthesia recovery. All mice were observed for signs of sickness during infection progression. Any mice passed before the endpoint were tested for CFUs in their liver and recorded as “succumbed to infection” if CFUs were detected or “censored” if CFUs were not detected. For 10 40RD mice and 10 age-matched AL controls, clinical scores were recorded daily at the time of infection and for 14 days following infection.

Tissue collection and processing

At the end of the experiment, mice were euthanized by carbon dioxide and subsequent cervical dislocation. Either all or a subset of the following tissues were collected, depending on the type of experiment: spleen, bone marrow, thymus, liver, kidney, and inguinal lymph nodes. For steady state assessment, all the tissues were weighed and photographed except for bone marrow before being processed. Liver and kidney were then disposed of and only spleen, thymus, and bone marrow were processed. For the infection model, only spleen and bone marrow were processed, and liver was used for CFU counting described in the next section. Bone marrow was harvested and homogenized by flushing bones with 25G needle and 10mL of cold PBS. Thymus and spleen were harvested in full, homogenized in PBS and strained through a 70-micron cell filter (Celltreat, cat. 229483). All homogenates were resuspended in 1mL of ACK buffer (Quality Biological, cat. 118-156-101) for 2 minutes to perform red blood cell lysis. The reaction was quenched with 10mL of RPMI 1640 supplemented with 10% FBS (Fisher Scientific, cat. BP9703100), 1% Penicillin-streptomycin (Mediatech, cat. MT30-002-CI), 1% L-Glutamate (Mediatech, cat. 1249), 10mM HEPES (Invitrogen, cat. 15630080), 1mM sodium pyruvate (CCS, cat. 1175), and 0.1% 2-Mercaptoethanol (Life technologies, cat. 21985023). Cells then were counted using a hemocytometer and TrypanBlue (Corning, cat. 25-900-CI).

Liver processing and CFU count

Livers were harvested from Lm infected mice at days 5, 8, and 14-post-infection as described above. Left lateral lobes were collected and weighed. Then, the lobes were homogenized in 1mL of PBS using the Bead Lysis Kit (Next Advance, cat. PINK5E100) and Next Advance Bullet Blender 5E machine for 10 minutes at power 8. The homogenates were serially diluted in PBS between 1:5 and 1:200,000 using serial dilution. The dilutions were plated on BHI plates and incubated at 37oC overnight. A plate with a countable number of CFUs (20 - 100 colonies) was picked per sample. The CFUs were counted by eye and using weight of the lobe and dilution factor, CFUs/g of liver were calculated.

Flow cytometry and cytokine staining

For surface staining, 4×10^6 cells from spleen, thymus, and bone marrow homogenates were washed with PBS and stained with a LIVE/DEAD™ Fixable Aqua Dead Cell Stain (Fisher Scientific, cat. L34966) for 15 minutes at 4°C. Then, cells were washed and stained for 20 minutes at 4°C in 2% Rat Serum (StemCell, cat. 13551) and PBS with the following antibodies based on the type of the panel (details below).

Innate immune cells

anti-MHC II (eBioscience M5/114.15.2, cat. 48-5321-80, 1:200), anti-CD11b (eBioscience M1/70, cat. 64-0112-82), anti-CD19 (BioLegend 6D5, cat. 115506), anti-Ly6G (Biolegend 1A8, cat. 127616), anti-Ly6C (eBioscience HK1.4, cat. 17-5932-82), anti-Siglec F (eBioscience 1RNM44N, cat. 127616), anti-TCRβ (eBioscience H57-597, cat. 61-5961-82), anti-CD11c (eBioscience N418, cat. 61-5961-82), anti-F4/80 (eBioscience BM8, cat. 17-4801-82, 1:200), anti-NK1.1 (eBioscience PK136, cat. 47-5941-82)

Myelopoiesis

anti-C16/32 (eBioscience 93, cat. 14-0161-81), anti-CD3 (eBioscience 145-2c11, cat. 11-0031-85), anti-CD4 (BioLegend GK1.5, cat. 100406), anti-CD8 (Invitrogen 53-6.7, cat. MA1-10303), anti-CD19 (BioLegend 6D5, cat. 115506), anti-CD11b (BioLegend M1/70, cat. 101206), anti-CD11c (eBioscience N418, cat. 11-0114-85), anti-Ter119 (eBioscience TER-110, cat. 11-5921-85), anti-Ly6G/C (eBioscience RB6-8C5, cat. 11-5931-85), anti-B220 (eBioscience RA3-6B2, cat. 11-0452-85), anti-NK1.1 (eBioscience

PK136, cat. 11-5941-85), anti-Sca1 (eBioscience D7, cat. 45-5981-82), CD150 (eBioscience mShed 150, cat. 12-1502-82), CD117 (eBioscience 2B8, cat. 61-1171-82), CD105 (eBioscience MJ7/18, cat. 25-1051-82), CD135 (eBioscience A2F10, cat. 17-1351-82), CD48 (BD HM48.1, cat. 561242, 1:200)

Effector T cells

anti-CD4 (eBioscience GK1.5, cat. 64-0041-82), anti-CD8 (Invitrogen 53-6.7, cat. MA1-10303), anti-CD44 (eBioscience IM7, cat. 45-0441-82), anti-CD127 (eBioscience A7R34, cat. 12-1271-82, 1:200), anti-KLRG1 (eBioscience 2F1, cat. 25-5893-82)

After staining, cells were washed with PBS. If cells were extracted from infected mice, they were also fixed for 30 minutes at 4°C using eBiosciences Intracellular Fixation Buffer (Termo, cat. 88-8824-00).

For intracellular cytokine staining, 4×10^6 splenocytes from infected mice were plated in 100uL of supplemented RPMI 1640 described above in U-bottom tissue culture treated 96-well plates (Celltreat, cat. 229190). Cells were stimulated with 2 µg/mL of SIINFEKL peptide (Anaspec, cat. AS-60193-5) and 1 mg/mL of Brefeldin A (Invitrogen, cat. inh-bfa) added 1 hour after the peptide for a total of 6 hours at 37°C. After incubation, cells were washed with PBS and stained with L/D aqua dye for 15 minutes at 4°C. Then, cells were washed and stained for 20 minutes at 4°C in 2% Rat Serum and PBS with the following antibodies: anti-CD4 (eBioscience GK1.5, cat. 64-0041-82), anti-CD8 (Invitrogen 53-6.7, cat. MA1-10303), anti-CD44 (eBioscience IM7, cat. 45-0441-82). After staining, cells were washed with PBS and fixed for 30 minutes at 4°C using eBiosciences Intracellular Fixation Buffer. Cells then were washed using eBiosciences Intracellular Permeabilization Buffer (Termo, cat. 88-8824-00) and stained for 30 minutes at room temperature n 2% Rat Serum and Permeabilization Buffer with the following antibodies: anti-TNFα (eBioscience MP6-XT22, cat. 12-7321-82), anti-IL-2 (eBioscience MQ1-17H12, cat. 25-7029-42, 1:200), anti-IFNγ (eBioscience XMG1.2, cat. 17-7311-82).

All antibodies used at 1:300 dilution unless otherwise specified. Regardless of staining, all cells were washed with PBS at the end of the protocol and resuspended in PBS to perform flow cytometry. Stained samples were analyzed on a 4 laser CytoFlex by Beckman Coulter using an automated plate reader. Data were analyzed using FlowJo 10.5.3 software.

Statistics and reproducibility

All experiments were repeated at least 3 independent times. Data was represented either as a mean ± standard deviation (SD) or standard error of mean (SEM). For measurements over time within the same experimental cohort, statistical analysis was done via simple linear regression comparing slopes or elevation. For survival curves, log-rank test was performed. For all other pairwise comparisons, Mann-Whitney test was performed. For all other measurements, statistical analysis was done via non-parametric one-way (Kruskal-Wallis) or two-way (mixed-effect) ANOVA depending on the number of variables. P-values are recorded on the graphs as follows: **** for $p < 0.0001$; *** for $p < 0.001$; ** for $p < 0.01$; * for $p \leq 0.05$; ns for $p > 0.05$. All statistical analysis was performed using GraphPad Prism 9.2.0 software.

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Additional files

Supplemental Figures

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Reviewer #2 (Public review):

Summary:

Sukhina et al. uses a chronic murine dietary restriction model to investigate the cellular mechanisms underlying nutritionally acquired immunodeficiency as well as the consequences of a refeeding intervention. The authors report a substantial impact of undernutrition to the myeloid compartment, which is not rescued by refeeding despite rescue of other phenotypes including lymphocyte levels, and which is associated with maintained partial susceptibility to bacterial infection.

Strengths:

Overall, this is a nicely executed study with an appropriate number of mice, robust phenotypes, and interesting conclusions, and the text is very well written. The authors' conclusions are generally well-supported by their data.

Weaknesses:

There is little evaluation of known critical drivers of myelopoiesis (e.g. PMID 20535209, 26072330, 29218601) over the course of the 40% diet, which would be of interest with regard to comparing this chronic model to other more short-term models of undernutrition.

Further, the microbiota, well-established to be regulated by undernutrition (e.g. PMID 22674549, 27339978, etc.), and also well-established to be a critical regulator of hematopoiesis/myelopoiesis (e.g. PMID 27879260, 27799160, etc.), should be studied in any future explorations using this model.

The authors have recognized these limitations to the study in their discussion.

https://doi.org/10.7554/eLife.101670.2.sa2

Reviewer #3 (Public review):

This communication from Sukhina et al argues that a period of malnutrition (modeled by caloric restriction) causes lasting immune deficiencies (myelopoesis) not rescued by re-feeding. This is a potentially important paper exploring the effects of malnutrition on immunity, which is a clinically important topic. The revised study adds some details with respect to kinetics of immune compartment and body weight changes, but most aspects raised by the referees were deferred experimentally. Several textual changes have been made to avoid over-interpreting their data. My overall assessment of this revised study is similar to my impression before, which is that while the observations are interesting, there is both a lack of mechanistic understanding of the phenomena and a lack of resolution/detail about the phenomena itself.

https://doi.org/10.7554/eLife.101670.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***eLife Assessment***
>
> *This important work advances our understanding of the impact of malnutrition on hematopoiesis and subsequently infection susceptibility. Support for the overall claims is convincing in some respects and incomplete in others as highlighted by reviewers. This work will be of general interest to those in the fields of hematopoiesis, malnutrition, and dietary influence on immunity.*

We would like to thank the editors for agreeing to review our work at eLife. We greatly appreciate them assessing this study as important and of general interest to multiple fields, as well as the opportunity to respond to reviewer comments. Please find our responses to each reviewer below.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *In this study, the authors used a chronic murine dietary restriction model to study the effects of chronic malnutrition on controls of bacterial infection and overall immunity, including cellularity and functions of different immune cell types. They further attempted to determine whether refeeding can revert the infection susceptibility and immunodeficiency. Although refeeding here improves anthropometric deficits, the authors of this study show that this is insufficient to recover the impairments across the immune cell compartments.*
>
> *Strengths:*
>
> *The manuscript is well-written and conceived around a valid scientific question. The data supports the idea that malnutrition contributes to infection susceptibility and causes some immunological changes. The malnourished mouse model also displayed growth and development delays. The work's significance is well justified. Immunological studies in the malnourished cohort (human and mice) are scarce, so this could add valuable information.*
>
> *Weaknesses:*
>
> *The assays on myeloid cells are limited, and the study is descriptive and overstated. The authors claim that "this work identifies a novel cellular link between prior nutritional state and immunocompetency, highlighting dysregulated myelopoiesis as a major." However, after reviewing the entire manuscript, I found no cellular mechanism defining the link between nutritional state and immunocompetency.*

We thank the reviewer for deeming our work significant and noting the importance of the study. We appreciate the referee's point regarding the lack of specific cellular functional data for innate immune cells and have modified the conclusions stated in text to more accurately reflect the results presented.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Sukhina et al. use a chronic murine dietary restriction model to investigate the cellular mechanisms underlying nutritionally acquired immunodeficiency as well as the consequences of a refeeding intervention. The authors report a substantial impact of undernutrition on the myeloid compartment, which is not rescued by refeeding despite rescue of other phenotypes including lymphocyte levels, and which is associated with maintained partial susceptibility to bacterial infection.*
>
> *Strengths:*
>
> *Overall, this is a nicely executed study with appropriate numbers of mice, robust phenotypes, and interesting conclusions, and the text is very well-written. The authors' conclusions are generally well-supported by their data.*
>
> *Weaknesses:*
>
> *There is little evaluation of known critical drivers of myelopoiesis (e.g. PMID 20535209, 26072330, 29218601) over the course of the 40% diet, which would be of interest with regard to comparing this chronic model to other more short-term models of undernutrition.*
>
> *Further, the microbiota, which is well-established to be regulated by undernutrition (e.g. PMID 22674549, 27339978, etc.), and also well-established to be a critical regulator of hematopoiesis/myelopoiesis (e.g. PMID 27879260, 27799160, etc.), is completely ignored here.*

We thank the reviewer for agreeing that the data presented support the stated conclusions and noting the experimental rigor. The referee highlights two important areas for future mechanistic investigation that we agree are of great importance and relevant to the submitted study. We have included further discussion of the potential role cytokines and the microbiota might play in our model.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *Sukhina et al are trying to understand the impacts of malnutrition on immunity. They model malnutrition with a diet switch from ad libitum to 40% caloric restriction (CR) in post-weaned mice. They test impacts on immune function with listeriosis. They then test whether re-feeding corrects these defects and find aspects of emergency myelopoiesis that remain defective after a precedent period of 40% CR. Overall, this is a very interesting observational study on the impacts of sudden prolonged exposure to less caloric intake.*
>
> *Strengths:*
>
> *The study is rigorously done. The observation of lasting defects after a bout of 40% CR is quite interesting. Overall, I think the topic and findings are of interest.*
>
> *Weaknesses:*
>
> *While the observations are interesting, in this reviewer's opinion, there is both a lack of mechanistic understanding of the phenomena and also some lack of resolution/detail*

about the phenomena itself. Addressing the following major issues would be helpful towards aspects of both:

(1) Is it calories, per se, or macro/micronutrients that drive these phenotypes observed with 40% CR. At the least, I would want to see isocaloric diets (primarily protein, fat, or carbs) and then some of the same readouts after 40% CR. Ie does low energy with relatively more eg protein prevent immunosuppression (as is commonly suggested)? Micronutrients would be harder to test experimentally and may be out of the scope of this study. However, it is worth noting that many of the malnutrition-associated diseases are micronutrient deficiencies.

(2) Is immunosuppression a function of a certain weight loss threshold? Or something else? Some idea of either the tempo of immunosuppression (happens at 1, in which weight loss is detected; vs 2-3, when body length and condition appear to diverge; or 5 weeks), or grade of CR (40% vs 60% vs 80%) would be helpful since the mechanism of immunosuppression overall is unclear (but nailing it may be beyond the scope of this communication).

(3) Does an obese mouse that gets 40% CR also become immunodeficient? As it stands, this ad libitum --> 40% CR model perhaps best models problems in the industrial world (as opposed to always being 40% CR from weaning, as might be more common in the developing world), and so modeling an obese person losing a lot of weight from CR (like would be achieved with GLP-1 drugs now) would be valuable to understanding generalizability.

(4) Generalizing this phenomenon as "bacterial" with listeriosis, which is more like a virus in many ways (intracellular phase, requires type I IFN, etc.) and cannot be given by the natural route of infection in mice, may not be most accurate. I would want to see an experiment with E.Coli, or some other bacteria, to test the statement of generalizability (ie is it bacteria, or type I IFN-pathway dominant infections, like viruses). If this is unique listeriosis, it doesn't undermine the story as it is at all, but it would just require some word-smithing.

(5) Previous reports (which the authors cite) implicate Leptin, the levels of which scale with fat mass, as "permissive" of a larger immune compartment (immune compartment as "luxury function" idea). Is their phenotype also leptin-mediated (ie leptin AAV)?

(6) The inability of re-feeding to "rescue" the myeloid compartment is really interesting. Can the authors do a bone marrow transplantation (CR-->ad libitum) to test if this effect is intrinsic to the CR-experienced bone marrow?

(7) Is the defect in emergency myelopoiesis a defect in G-CSF? Ie if the authors injected G-CSF in CR animals, do they equivalently mobilize neutrophils? Does G-CSF supplementation (as one does in humans) rescue host defense against Listeria in the CR or re-feeding paradigms?

We thank the reviewer for considering our work of interest and noting the rigor with which it was conducted. The referee raises several excellent mechanistic hypotheses and follow-up studies to perform. We agree that defining the specific dietary deficiency driving the phenotypes is of great interest. The relative contribution of calories versus macro- and micronutrients is an area we are interested in exploring in future studies, especially given the literature on the role of micronutrients in malnutrition driven wasting as the referee notes. We also agree that it will be key to determine whether non-hematopoietic cells contribute as well as the role of soluble factors such G-CSF and Leptin in mediating the immunodeficiency all warrant further study. Likewise, it will be important to evaluate how malnutrition impacts other models of infection to determine how generalizable these

phenomena are. We have added these points to the discussion section as limitations of this study.

Regarding how the phenotypes correspond to the timing of the immunosuppression relative to weight loss, we have performed new kinetics studies to provide some insight into this area. We now find that neutropenia in peripheral blood can be detected after as little as one week of dietary restriction, with neutropenia continuing to decline after prolonged restriction. These findings indicate that the impact on myeloid cell production are indeed rapid and proceed maximum weight loss, though the severity of these phenotypes does increase as malnutrition persists. We wholeheartedly agree with the reviewer that it will be interesting to explore whether starting weight impacts these phenotypes and whether similar findings can be made in obese animals as they are treated for weight loss.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *In this study, the authors used a chronic murine dietary restriction model to study the effects of chronic malnutrition on controls of bacterial infection and overall immunity, including cellularity and functions of different immune cell types. They further attempted to determine whether refeeding can revert the infection susceptibility and immunodeficiency. Although refeeding here improves anthropometric deficits, the authors of this study show that this is insufficient to recover the impairments across the immune cell compartments. The authors claim that "this work identifies a novel cellular link between prior nutritional state and immunocompetency, highlighting dysregulated myelopoiesis as a major." However, after reviewing the entire manuscript, I could not find any cellular mechanism defining the link between nutritional state and immunocompetency. The assays on myeloid cells are limited, and the study is descriptive and overstated.*
>
> *Major concerns:*
>
> *(1) Malnutrition has entirely different effects on adults and children. In this study, 6-8 weeks old C57/Bl6 mice were used that mimic adult malnutrition. I do not understand then why the refeeding strategy for inpatient treatment of severely malnourished children was utilized here.*
>
> *(2) Figure 1g shows BM cellularity is reduced, but the authors claim otherwise in the text.*
>
> *(3) What is the basis of the body condition score in Figure 1d? It will be good to have it in the supplement.*
>
> *(4) Listeria monocytogenes cause systemic infection, so bioload was not determined in tissues beyond the liver.*
>
> *(5) Figure 3; T cell functional assays were limited to CD8 T cells and lymphocytes isolated from the spleen.*
>
> *(6) Why was peripheral cell count not considered? Discrepancies exist with the absolute cell number and relative abundance data, except for the neutrophil and monocyte data, which makes the data difficult to interpret. For example, for B cells, CD4 and CD8 cells.*
>
> *(7) Also, if mice exhibit thymic atrophy, why does % abundance data show otherwise? Overall, the data is confusing to interpret.*
>
> *(8) No functional tests for neutrophil or monocyte function exist to explain the higher bacterial burden in the liver or to connect the numbers with the overall pathogen load*

The rationale for examining both innate and adaptive immunity is not clear-it is even more unclear since the exact timelines for examining both innate and adaptive immunity (D0 and D5) were used.

(9) Figure 2e doesn't make sense - why is spleen cellularity measured when bacterial load is measured in the liver?

(10) Although it is claimed that emergency myelopoiesis is affected, no specific marker for emergency myelopoiesis other than cell numbers was studied.

(11) I suggest including neutrophil effector functions and looking for real markers of granulopoiesis, such as Cebp-b. Since the authors attempted to examine the entirety of immune responses, it is better to measure cell abundance, types, and functions beyond the spleen. Consider the systemic spread of m while measuring bioload.

(12) Minor grammatical errors - please re-read the entire text and correct grammatical errors to improve the flow of the text.

(13) Sample size details missing

(14) Be clear on which marks were used to identify monocytes. Using just CD11b and Ly6G is insufficient for neutrophil quantification.

(15) Also, instead of saying "undernourished patients," say "patients with undernutrition" - change throughout the text. I would recommend numbering citations (as is done for Nature citations) to ease in following the text, as there are areas when there are more than ten citations with author names.

(16) No line numbers are provided

(17) Abstract

- What does accelerated contraction mean?

- "In" is repeated in a sentence

- Be clear that the study is done in a mouse model - saying just "animals" is not sufficient

- Indicate how malnutrition is induced in these mice

(18) Introduction

- "restriction," "immune organs," - what is this referring to?

- You mention lymphoid tissue and innate and adaptive immunity, which doesn't make sense.

Please correct this.

- You mention a lot of lymphoid tissues, i.e. lymphoid mass gain, but how about the bone marrow and spleen, which are responsible for most innate immune compartments?

(19) Results

a) Figure 1

- Why 40% reduced diet?

- It would be interesting to report if the organs are smaller relative to body weight. It makes sense that the organ weight is lower in the 40RD mice, especially since they are

smaller, so the novelty of this data is not apparent (Figure 1f).

- You say, "We observed a corresponding reduction in the cellularity of the spleen and thymus, while the cellularity of the bone marrow was unaffected (Fig. 1g)." however, your BM data is significant, so this statement doesn't reflect the data you present, please correct.

b) Figure 2

- Figure 2d - what tissue is this from, mentioned in the figure? And measure cellularity there. The rationale for why you look only at the spleen here is weak. Also, we would benefit from including the groups without infection here for comparison purposes.

c) Figure 3

- The rationale for why you further looked at T cells is weak, mainly because of the following sentence. "Despite this overall loss in lymphocyte number, the relative frequency of each population was either unchanged or elevated, indicating that while malnutrition leads to a global reduction in immune cell numbers, lymphocytes are less impacted than other immune cell populations (Supplemental 1)." Please explain in the main text.

d) Figure 4

- You say the peak of the adaptive immune response, but you never looked at the peak of adaptive immune - when is this? If you have the data, please show it. You also only show d0 and d5 post-infection data for adaptive immunity, so I am unsure where this statement comes from.

- How did you identify neutrophils and monocytes through flow cytometry? Indicate the markers used. Also, your text does not match your data; please correct it. i.e. monocyte numbers reduced, and relative abundance increased, but your text doesn't say this.

- Show the flow graph first then, followed by the quantification.

- The study would benefit from examining markers of emergency myelopoiesis such as Cebpb through qPCR.

- Although the number of neutrophils is lower in the BM and spleen, how does this relate to increased bacterial load in the liver? This is especially true since you did not quantify neutrophil numbers in the liver.

e) Figure 6

- Some figures are incorrectly labelled.

- For the refeeding data, also include the data from the 40RD group to compare the level of recovery in the outcome measures.

(20) Discussion

- You claim that monocytes are reduced to the same extent as neutrophils, but this is not true.

Please correct.

- Indicate some limitations of your work.

We thank the reviewer for offering these recommendations and the constructive comments.

Several comments raised concerns over the rationale or reasoning behind aspects of the experimental design or the data presented, which we would like to clarify:

• Regarding the refeeding protocol, we apologize for the confusion for the rationale. We based our methodology on the *general* guidelines for refeeding protocols for malnourished people. We elected to increase food intake 10% daily to avoid risk of refeeding syndrome or other complications. Our method is by no means replicates the administration of specific vitamins, minerals, electrolytes, nor precise caloric content as would be given to a human patient. The citation provided offers information from the WHO regarding the complications that can arise during refeeding syndrome, which while it is from a document on pediatric care, we did not mean to imply that our method modeled refeeding intervention for children. We have modified the text to avoid this confusion.

• The reviewer requested more clarity on why we studied both the innate and adaptive immune system as well as why we chose the time points studied. As referenced in the manuscript, prior work has observed that caloric restriction, fasting, and malnutrition all can impact the adaptive immune system. Given these previous findings, we felt it important to evaluate how malnutrition affected adaptive immune cell populations in our model. To this end, we provide data tracking the course of T-cell responses from the start of infection through day 14 at the time that the response undergoes contraction. However, since we find that bacterial burden is not properly controlled at earlier time points (day 5), when it is understood the innate immune system is more critical for mediating pathogen clearance, we elected to better characterize the effect malnutrition had on innate immune populations, something less well described in the literature. As phenotypes both in bacterial burden and within innate immune populations were observable as early as day 5, we chose to focus on that time point rather than later time points when readouts could be further confounded by secondary or compounding effects by the lack of early control of infection. We have tried to make this rationale clear in the text and have made changes to further emphasize this reasoning.

• The reviewer also requested an explaination over why bacterial burden was measured in the liver and the immune response was measured in the spleen. While the reviewer is correct that our model is a systemic infection, it is well appreciated that bacteria rapidly disseminate to the liver and spleen and these organs serve as major sites of infection. Given the central role the spleen plays in organizing both the innate and adaptive immune response in this model, it is common practice in the field to phenotype immune cell populations in the spleen, while using the liver to quantify bacterial burden (see PMID: 37773751 as one example of many). We acknowledge this does not provide the full scope of bacterial infection or the immune response in every potentially affected tissue, but nonetheless believe the interpretation that malnourished and previously malnourished animals do not properly control infection and their immune responses are blunted compared to controls still stands.

The reviewer raised several points about di3erences in the results for cell frequency and absolute number and why these may deviate in some circumstances. For example, the reviewer notes that we observe thymic atrophy yet the frequency of peripheral T-cells does not decline. It should be noted that absolute number can change when frequency does not and vice versa, due to changes in other cell types within the studied population of cells. As in the case of peripheral lymphocytes in our study, the frequency can stay the same or even increase when the absolute number declines (Supplemental 1). This can occur if other populations of cells decrease further, which is indeed the case as the loss of myeloid cells is greater than that of lymphocytes. Hence, we find that the frequency of T and B cells is unchanged or elevated, despite the loss in absolute number of peripheral cell, which is our stated interpretation. We believe this is consistent with our overall observations and is why it is important to report both frequency and absolute number, as we have done.

We have made the requested changes to the text to address the reviewers concerns as noted to improve clarity and accuracy for the description of experiments, results, and overall conclusions drawn in the manuscript. We have also included a discussion of the limitations of our work as well as additional areas for future investigation that remain open.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *Regarding the known drivers of myelopoiesis, can the authors quantify circulating levels of relevant immune cytokines (e.g. type I and II IFNs, GM-CSF, etc.)?*
>
> *Regarding the microbiota (point #2), how dramatically does this undernutrition modulate the microbiota both in terms of absolute load and community composition, and how effectively/quickly is this rescued by refeeding?*

We thank the reviewer for raising these recommendations. We agree that the role of circulating factors like cytokines and growth factors in contributing to the defects in myelopoiesis is of interest and is the focus of future work. Similarly, the impact of malnutrition on the microbiota is of great interest and has been evaluated by other groups in separate studies. How the known impact of malnutrition on the microbiota affects the phenotypes we observe in myelopoiesis is unclear and warrants future investigation. We have added these points to the discussion section as limitations of this study.

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