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PDL-1+ Neutrophils mediate susceptibility during endotoxemia in Metabolically Dysfunctional-Associated Fatty Liver Disease

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eLife Assessment

This **valuable** study highlights the key role of NK cells and PD-L1+ neutrophils in worsening sepsis responses in the context of MASH (metabolic dysfunction-associated steatohepatitis). It focused on the role of neutrophils in mediating this effect, which is based on a choline-deficient high-fat diet model of various knockouts or selective ablation of immune cell types. While the data presented are of great interest, there are concerns around the reliability of the strength of the evidence provided, which is currently considered **incomplete**. The study may be of interest to researchers in immunopathological disease mechanisms once confirmatory studies have been completed.

[Editors' note: the authors no longer have access to the original flow cytometry data and plan to compile new datasets in the future.]

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Abstract

Metabolically Dysfunctional-Associated Fatty Liver Disease (MAFLD) is a pathological condition affecting many individuals worldwide. Patients with MAFLD are more susceptible to systemic inflammation, including endotoxemia, which accelerates the progressive liver damage. However, the immunological mechanisms that trigger the hyper-inflammatory responses in individuals with MAFLD remain unknown. In the present study, we reported that short-term HFCD (Choline Deficient High Fat Diet)-fed mice, which did not show significant signs of hepatic damage and inflammation in the first two weeks, are more susceptible to a non-severe sepsis-like systemic inflammation induced by LPS challenge. Mechanistically, endotoxemic mice show an excessive accumulation of NK-producing IFN- γ cells in liver tissue, which trigger the recruitment and polarization of a distinct subset of neutrophils, characterized by high expression of PD-L1 and massive release of TNF α . Remarkably, genetic inhibition of IFN- γ or pharmacological blockade of PD-L1 effectively modulated the excessive recruitment of these neutrophils to the liver and TNF- α production, thereby preventing hepatic damage and reducing the severity of host mortality. Thus, these results support the design of novel effective strategies to control hyperinflammatory responses in septic HFCD patients and consequently improve their survival.

Introduction

Metabolically-dysfunction-associated fatty liver disease (MAFLD), previously described as non-alcoholic fatty liver disease (NAFLD), is a metabolic liver dysfunction defined as an abnormal accumulation of fat in the liver without secondary triggers such as alcohol consumption. MAFLD has become a common chronic liver disease in the world population and has a high prevalence varying from simple steatosis to non-alcoholic steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (1–3). Despite its high prevalence and increasing cause of liver-related mortality worldwide, MAFLD is still underestimated, and there are no approved pharmacological or preventive strategies for these medical conditions (4, 5).

The MAFLD is a highly complex condition that involves a “multi-hit” process, in which different stimuli act together to promote fat accumulation and progressive liver damage (6). Although the pathogenesis of MAFLD is multifactorial, inflammation has now been considered a key factor in the progression of advanced hepatic conditions (7–9). In the presence of obesity and lipotoxicity, the immune system is activated in the liver, with a corresponding degree of inflammation triggered by the “wound healing response”. Since these inflammatory signs persist for a long period, monocytes and neutrophils, as well as T CD4, T CD8, and natural killer (NK), can be recruited to the liver, releasing a massive secretion of pro-inflammatory cytokines, including interferon-gamma (IFN- γ) and tumor necrosis factor (TNF α) (7, 10). Likewise, in chronic diseases, these cytokines are also present in systemic and uncontrolled inflammatory processes, such as sepsis (11). Sepsis is a syndrome characterized by a life-threatening dysfunction due to complex interactions between pathogens and the host recruitment of immune cells, resulting in a systemic hyper-inflammatory state followed by extensive vital organ damage, including in the liver (12, 13). Sepsis has also been considered the leading cause of death in intensive care units. In endotoxemia, a well-established sepsis model, this massive production of cytokines provokes uncontrolled recruitment of innate immune cells, mainly monocytes and neutrophils, to vital organs, including the liver, where they induce extensive and lethal damage (11, 14, 15). The majority of data regarding endotoxemia pathogenesis has focused only on the interaction between the infection and the host immune system, and only a few studies have addressed one or more clinical conditions that are considered risk factors leading to endotoxemia aggravation, such as senescence and previous immunosuppressive comorbidities (16–18). Thus, understanding how these clinical conditions can modify the host immune response and consequently affect the outcome of sepsis is crucial to detecting the risks and designing novel preventive and therapeutic approaches to control exacerbated inflammatory responses. Recently, some population studies, including large-scale data from cohorts with biopsy-confirmed individuals, have evidenced a higher incidence of severe infections in MAFLD patients, suggesting that previous hepatic conditions can modify the host immune response, leading to sepsis aggravation (19). However, although a relationship between elevated risk of sepsis in MAFLD patients exists, the underlying immunological mechanisms involved in the progression and exacerbation of systemic and/or local (hepatic) inflammation in these individuals have not been identified yet.

To understand the impact of MAFLD on liver damage during endotoxemia, we used a known murine model of MAFLD and later challenged these animals with a moderate dose of lipopolysaccharide (LPS). We demonstrated that endotoxemia exacerbated the hepatic inflammatory response and mortality in Choline Deficient High Fat Diet (HFCD)-fed mice. The LPS-activated hepatic NK cells secrete IFN- γ , which induces massive recruitment and polarization of a specific subpopulation of PD-L1-expressing neutrophils. This subpopulation of neutrophils releases a high concentration of TNF α , which mediates the liver lesion and increases the mortality of the animals. In fact, biological treatments promoting PD-L1 neutralization, or neutrophil depletion, reduced the TNF- α concentration, the liver inflammatory response and organ pathology, and increased the survival in endotoxemic HFCD fed mice. Similar results were observed with the use of genetic knockout TNF- α receptor mice. Thus, our findings describe the potential mechanism involved in the exacerbation of neutrophil functions in MAFLD mice and suggest a viable approach to the development of individual interventions capable of mitigating the hyperinflammation observed in these pathological conditions.

Results

MAFLD mice are more susceptible to LPS-induced systemic inflammation

To study MAFLD as a pre-condition associated with increasing risk of exacerbation of inflammation induced by endotoxemia, we employed a murine model of MAFLD in which the mice were fed for two weeks with a choline-deficient high-fat HFCD diet, a well-known liver disease model (Fig. 1A). As a result, we used a two-week diet period during which no changes in lymphocyte numbers were observed because the HFCD, which is choline-deficient, leads to a reduction in lymphocyte numbers in the animals (20). Mice fed HFCD showed no increase in liver weight and collagen deposition as evidenced by picrosirius staining (Fig. S1A and Fig. S1C). The HFCD mice challenged with LPS (10 mg/kg; intraperitoneally) triggered an elevated susceptibility with an index of mortality of 100% 24 h after the challenge (Fig. 1B). In our experimental condition, this dose of LPS provoked moderate (below 40%, 4 days after LPS challenge) mortality in chow-fed mice (control group) (Fig. 1B). Compared with the control group, the HFCD animals challenged with LPS showed elevated liver damage, evidenced by an increase in plasma alanine aminotransferase (ALT) levels (a known marker of liver injury) and histopathological alteration, characterized by leukocyte infiltration and edema (Fig. 1C, 1E). Compared with control mice treated with PBS, the HFCD animals also treated with PBS did not show significant signs of hepatic damage and local inflammatory response, evidenced by the absence of leukocyte infiltration (Fig. 1C and 1E). Animals subjected to the HFCD diet showed reduced blood glucose levels with or without LPS challenge (Fig. S1B). Remarkably, compared to the Chow group, HFCD mice exposed to LPS did not show greater changes in other organs commonly affected by endotoxemia, such as the kidneys (Figure 1D), indicating that a pathological inflammatory condition is increased mainly in liver tissue compared to other organs. To assess how MAFLD modulates the immune response in the hepatic microenvironment of human patients, we reanalyzed previously published bulk RNA-seq data from patients with MAFLD to identify potential targets related to the inflammatory process (21). Firstly, we performed analyses of genes differentially expressed in leukocyte samples from patients with MAFLD and healthy controls. We observed that 5240 genes are upregulated, such as *EIF4H*, *TMEM*, *MOB1A*, *SERBP1*, *TM9SF1*, *HINT3*, *ARF3*, *CHTF8*, and *PDIA6*, while 5806 genes, as *FOSB*, *KAT2A*, *SNRNP70*, *CCNL2*, *AMY2B*, *EXD3*, and *EBNA1BP2*, are downregulated. Interestingly, we found that the *CD274* gene (encoding PD-L1) and the *IFNGR2* gene, corresponding to the IFN- γ receptor, are among the upregulated genes (Fig. 2A). Furthermore, after conducting an additional enrichment analysis of the 5240 upregulated genes using Gene Ontology (GO), we detected an increase in several pathways associated with inflammatory processes, such as phagocytosis, leukocyte migration, immune response to bacterial components, and signaling pathways involving IL-1 β , TNF- α , and IFN- γ (Fig. 2B).

Because IFN- γ and TNF- α signaling pathways are upregulated in MAFLD patients, we reanalyzed previously published single-cell RNA-seq data from liver samples from mice fed with a 60% high-fat diet, another known MAFLD model (22). We noticed an increase in immune cells in the hepatic tissue, such as monocytes, neutrophils, dendritic cells, T cells, and NK cells (Fig. 2C and 2D). Interestingly, we observed a specific upregulation of *CD274* and TNF- α genes in monocytes and neutrophils, while T cells and NK cells showed increased IFN- γ expression (Fig. 2E), suggesting that the cytokines produced by these specific cells may be involved in the hyperinflammation observed during MAFLD.

Next, based on the results of increased IFN- γ and TNF- α signaling in bulk RNAseq and single-cell RNA sequencing analyses in hepatic samples of MAFLD patients and animals, we investigated the role of these cytokines in the susceptibility of endotoxemic MAFLD mice. Initially, we observed an exacerbated hepatic production of both cytokines induced by endotoxin challenge in the HFCD-fed group compared to the control mice treated with PBS or LPS (Fig. 3A and 3B). Furthermore, we also observed that IFN- $\gamma^{-/-}$ and TNFR1R2 $\alpha^{-/-}$ mice with MAFLD challenged with LPS did not

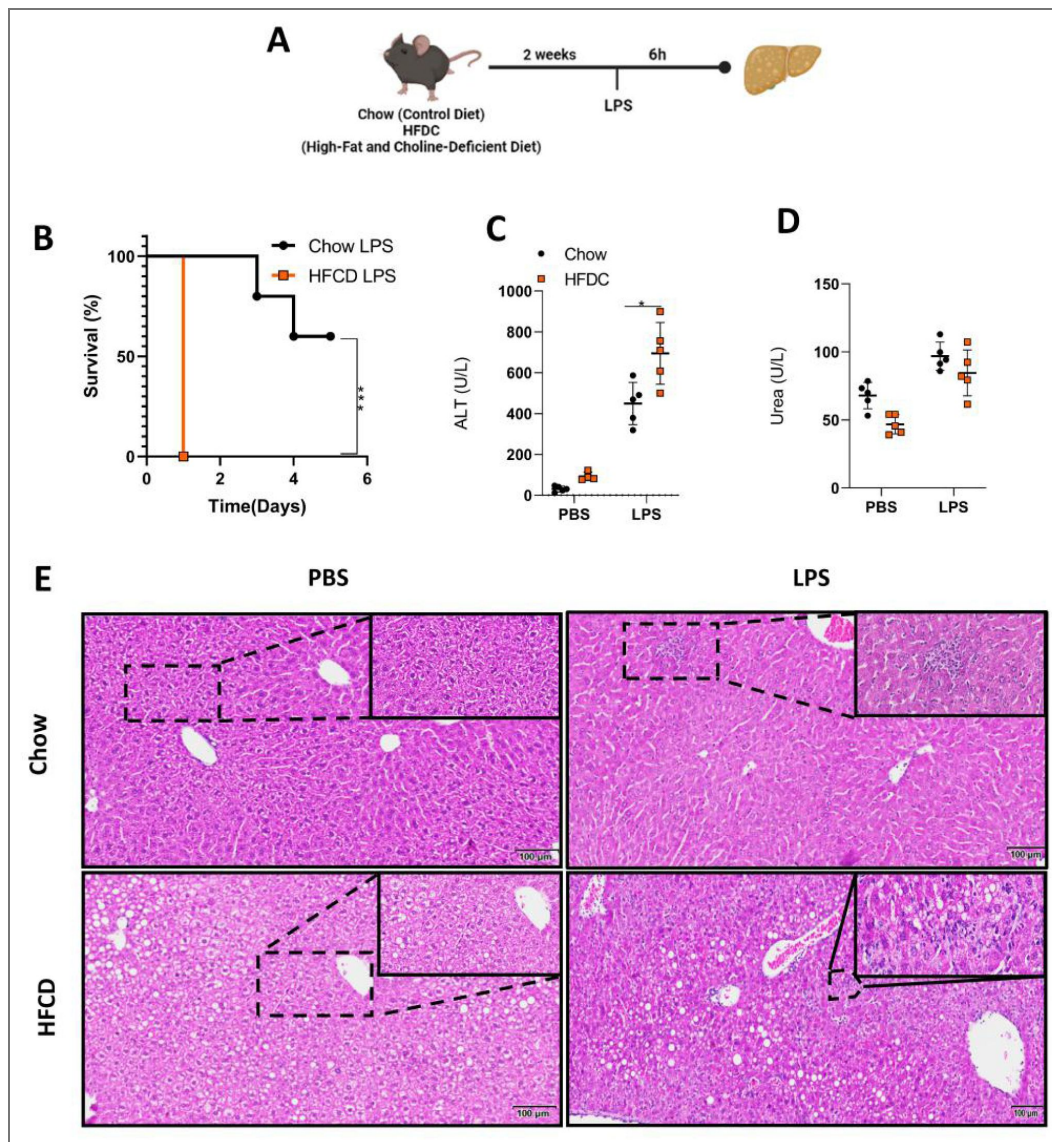


Figure 1. Mice fed with a high-fat and choline-deficient (HFCD) diet develop Metabolically Dysfunctional-Associated Fatty Liver Disease (MAFLD) and are more susceptible to endotoxemia.

(A) Schematic illustration of the experimental design. (B) Survival curves of mice with MAFLD (n=5) and Chow diet (controls) (n=5) which were injected intraperitoneally with LPS (10 mg/kg) and the survival rates were determined daily for 7 days (C and D) Serum ALT and Urea levels from mice with MAFLD and Chow, 6 hours after LPS (10 mg/kg) and PBS administration (n=5). (E) Effect of LPS inoculation on histopathology (H&E stain) of liver from mice with MAFLD or controls (Chow diet). The results are expressed as the mean $\pm$ standard deviation (SD) from one representative experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined with the following p-values: *p < 0.05, **p < 0.01, ***p < 0.001. Each experiment was independently repeated 2 times to ensure reproducibility of the findings.

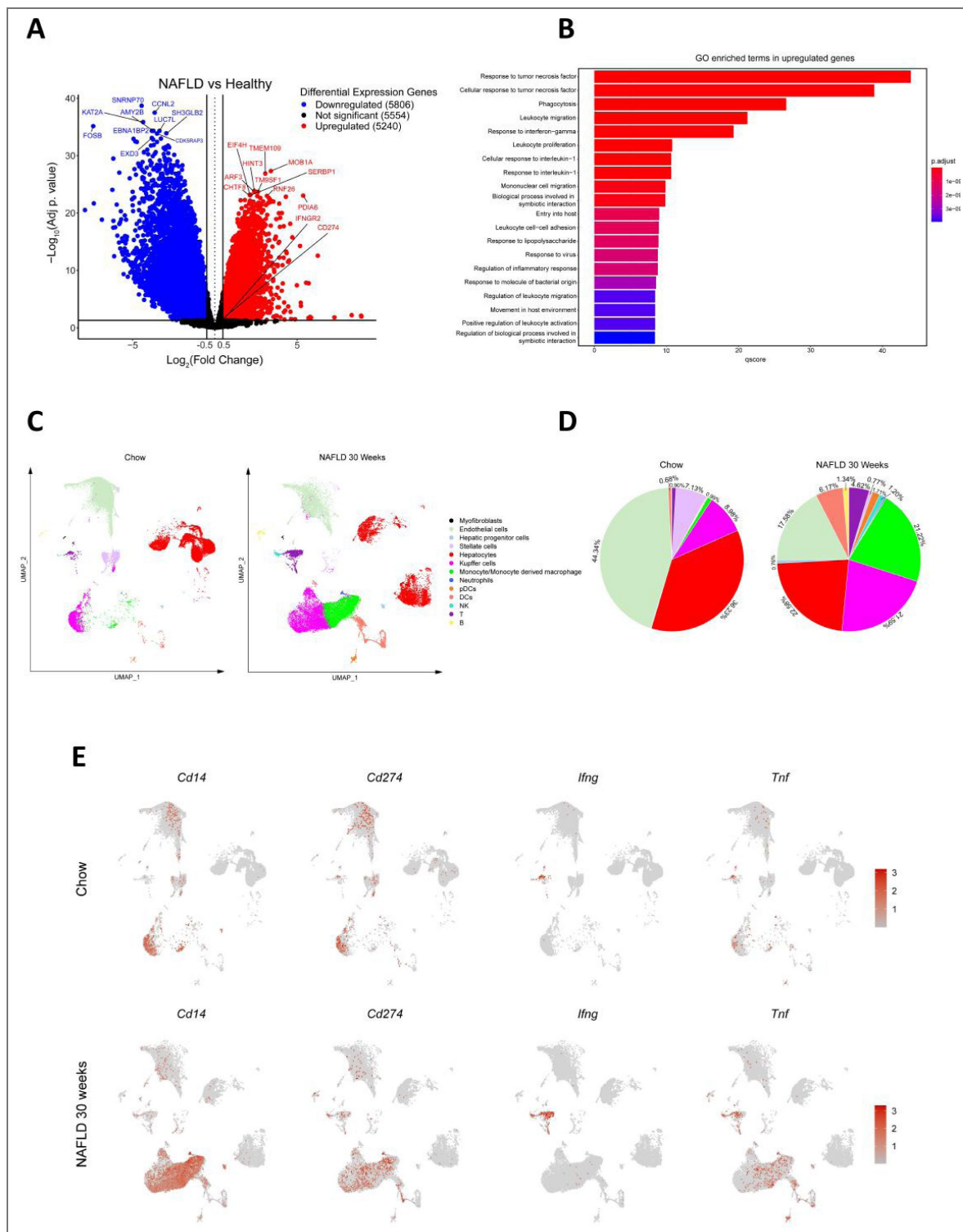


Figure 2. The expression of genes from the inflammatory pathways of IFN- γ and TNF- α is increased during the development of MAFLD in mice and humans.

(A) Volcano plots illustrating the fold change and P-value for gene expression comparisons between livers of MAFLD and healthy patients. Genes of interest are highlighted on the volcano charts. (B) Gene Ontology (GO) functional analysis of differentially expressed genes (DEGs). GO enrichment analysis of DEGs was conducted using DAVID, presenting the 20 most significantly ($P < 0.05$) enriched GO terms in biological process, molecular function, and cellular component categories. All adjusted statistically significant values are presented as negative log-transformed base 10 values. (C) UMAP plots depicting immune cells from the livers of mice subjected to 30 weeks of HFCD diet compared to Chow diet. (D) Frequency distribution of immune cell populations. (E) Expression levels of CD14, CD274, IFN- γ , and TNF- α genes within hepatic immune cell populations.

show an increase in the enzyme ALT, a liver lesion marker (Fig. 3C [↗](#) and 3D [↗](#)). In addition, endotoxemic MAFLD TNFR1R2^{-/-} and IFN- γ ^{-/-} mice exhibited reduced leukocyte infiltration in the liver tissue and improved survival (Fig. 3E [↗](#), 3F [↗](#), and 3G), respectively, as compared to endotoxemic WT mice. Furthermore, splenocytes from HFCD-fed IFN-deficient animals exhibited increased IL-6 and TNF- α production upon *in vitro* LPS stimulation, indicating a response pattern similar to that of control animals (Fig. 2SA [↗](#) and Fig. 2SB [↗](#)). Together, our data demonstrate that MAFLD predisposes to the exacerbation of cytokine production, such as IFN- γ and TNF- α , which mediate an elevated index of mortality in endotoxemic mice.

MAFLD induces the recruitment of NK cells that are sources of IFN- γ production in endotoxemia

During the inflammatory process, it is already established that CD4 and CD8 T cells are important sources of IFN- γ (23). Herein, we observed a selective increase in IFN- γ production by CD8, but not CD4, T cells during endotoxemia (Fig. 4A [↗](#), Fig. 4B [↗](#), Fig. S3A [↗](#), Fig. S3B [↗](#)), respectively. In light of this, we next investigated the involvement of lymphoid cells in the high susceptibility of endotoxemic mice with MAFLD. For this, we submitted animals with the absence of T and B cells (*Rag1*^{-/-} mice) to the MAFLD protocol, and after challenging them with LPS and the survival rate was assessed. Interestingly, *Rag1*-deficient animals under the HFCD remained susceptible to the LPS challenge (Fig. 4C [↗](#)) with exacerbation of liver injury (Fig. 4D [↗](#)), suggesting that these T and B cells do not contribute to susceptibility to the LPS challenge.

Recent studies have demonstrated that NK cells are other IFN- γ -producing cells recruited to the hepatic tissue during MAFLD and that they are also involved in systemic inflammatory responses, including sepsis (24–29). We observed that the number of NK cells increased in the liver tissue of PBS-treated MAFLD mice compared with HFCD fed a control diet (Fig. 4E [↗](#)). LPS challenge increased the accumulation of NK1.1+CD3⁻ NK cells in the liver tissue of MAFLD mice and the absence of NK1.1+CD3⁺ NKT cells (Fig. S3C [↗](#) and 4E [↗](#)). Furthermore, our cytometric analyses demonstrated that NK cells infiltrated in the liver tissue secrete high levels of IFN- γ (Fig. S3D [↗](#) and 4F [↗](#)). Next, to confirm the pathological involvement of NK cells during hyperinflammation in MAFLD, we depleted these cells by treating animals with anti-NK antibody 1.1, one day before the LPS challenge (Fig. S4 [↗](#)). Interestingly, the pharmacological depletion of NK cells in MAFLD animals provoked an increased survival index (Fig. 4G [↗](#)). Furthermore, when NK cells were depleted, MAFLD animals exhibited reduced hepatic damage (Fig. 4H [↗](#)), IFN- γ levels (Fig. 4I [↗](#)), and TNF- α levels (Fig. 4J [↗](#)). Together, these results suggest that NK cells are responsible for susceptibility via IFN- γ production in MAFLD during endotoxemia.

Neutrophils are responsible for susceptibility during hyperinflammation in MAFLD

During MAFLD development, innate immune cells, such as monocytes and neutrophils, are the first leukocytes recruited to the liver (24, 30). With this in mind, mice with MAFLD were challenged with LPS, and the infiltration of these cells in the liver was quantified. During the initial inflammatory phase, six hours after the LPS challenge, we observed that monocytes were recruited to the site (Fig. 5A [↗](#) and 5B [↗](#)). Since inflammatory monocytes emigrate from the bone marrow towards the periphery via CCR2 and then migrate to inflamed tissues, differentiating into monocyte-derived macrophages (30, 31), we investigated the functional role of monocytes in MAFLD, using CCR2-deficient mice (Fig. 5C [↗](#)). Interestingly, animals deficient in monocyte migration (CCR2^{-/-}) showed a high mortality rate compared to wild type after LPS challenge and neutrophil migration was not altered (Fig. 5SA [↗](#) and Fig. 5SB [↗](#)). Moreover, CCR2^{-/-} animals subjected to the respective diets had leukocyte CD45⁺ infiltration similar to that observed in the LPS challenge control mice (Fig. 5SC [↗](#) and Fig. 5SD [↗](#)). Furthermore, the survival rate of MAFLD CCR2^{-/-} mice challenged with LPS was not different of MAFLD wild type mice also challenged with LPS (Fig. 5SE [↗](#)). Together, the results suggest that monocytes do not contribute to susceptibility in endotoxemic animals with MAFLD.

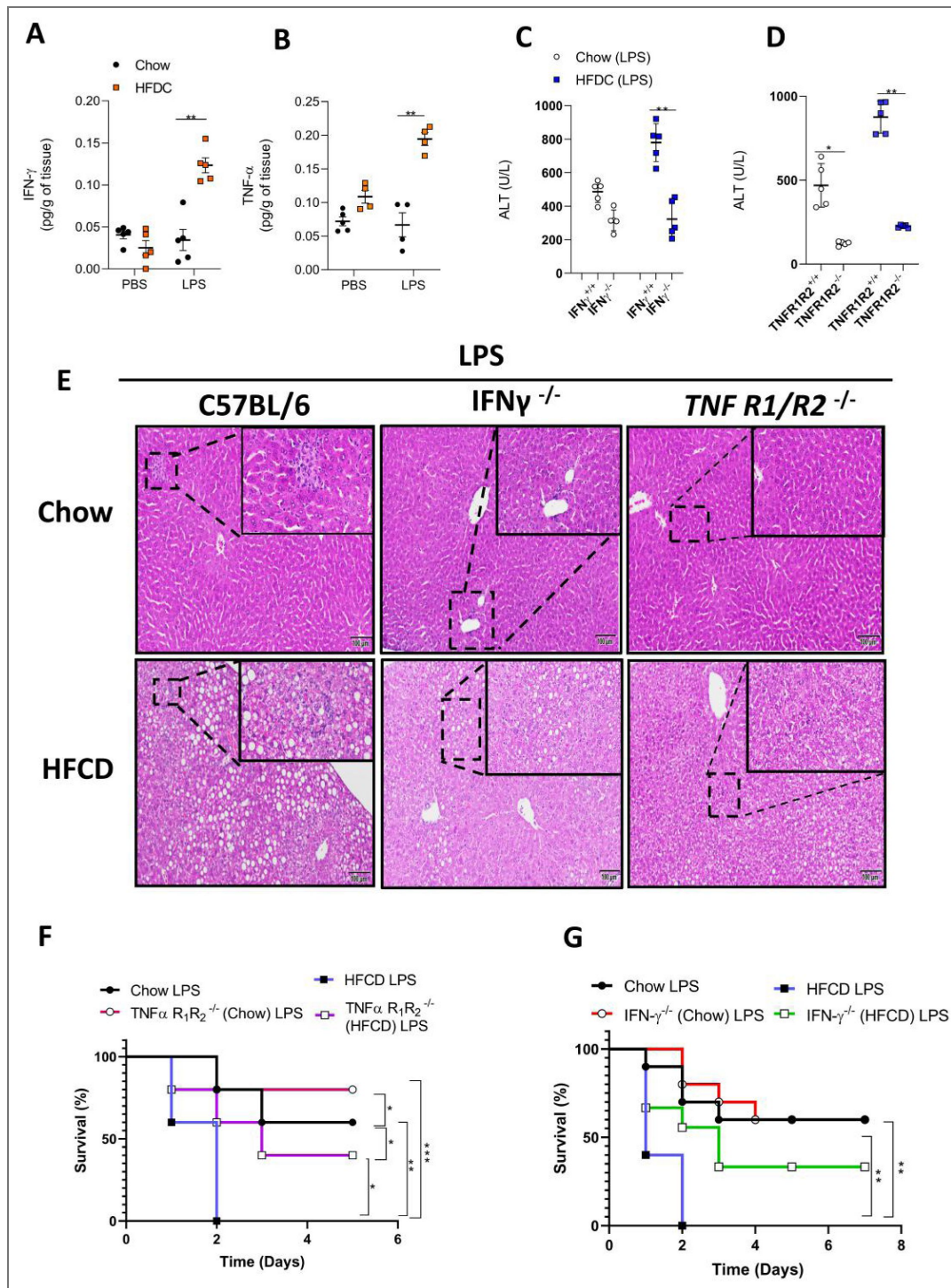


Figure 3. TNF- α and IFN- γ participate in susceptibility in mice with MAFLD to endotoxemia.

(A) IFN- γ and (B) TNF- α secretion in the tissue livers of Chow and MAFLD mice injected with LPS (10mg/kg) (n = 5). (C) Serum ALT levels from mice with MAFLD and Chow and/or knockout of IFN γ (IFN γ ^{-/-}) and (D) TNF α (TNFR1R2^{-/-}), 6 hours after 10 mg/kg LPS and PBS administration (n=5). (E) Effect of LPS inoculation on liver histopathology from mice with MAFLD and Chow and/or knockout of IFN γ ^{-/-} and TNF- α (TNFR1R2^{-/-}). (F) Survival curves of mice with MAFLD (n=5) and Chow (n=5) and/or knockout of IFN- γ (IFN γ ^{-/-}) and (G) TNF- α (TNFR1R2^{-/-}), after intraperitoneal inoculation of LPS (10mg/kg). The results are expressed as the mean $\pm$ standard deviation (SD) from one representative experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined with the following p-values: *p < 0.05, **p < 0.01, ***p < 0.001. Each experiment was independently repeated 2 times to ensure reproducibility of the findings.

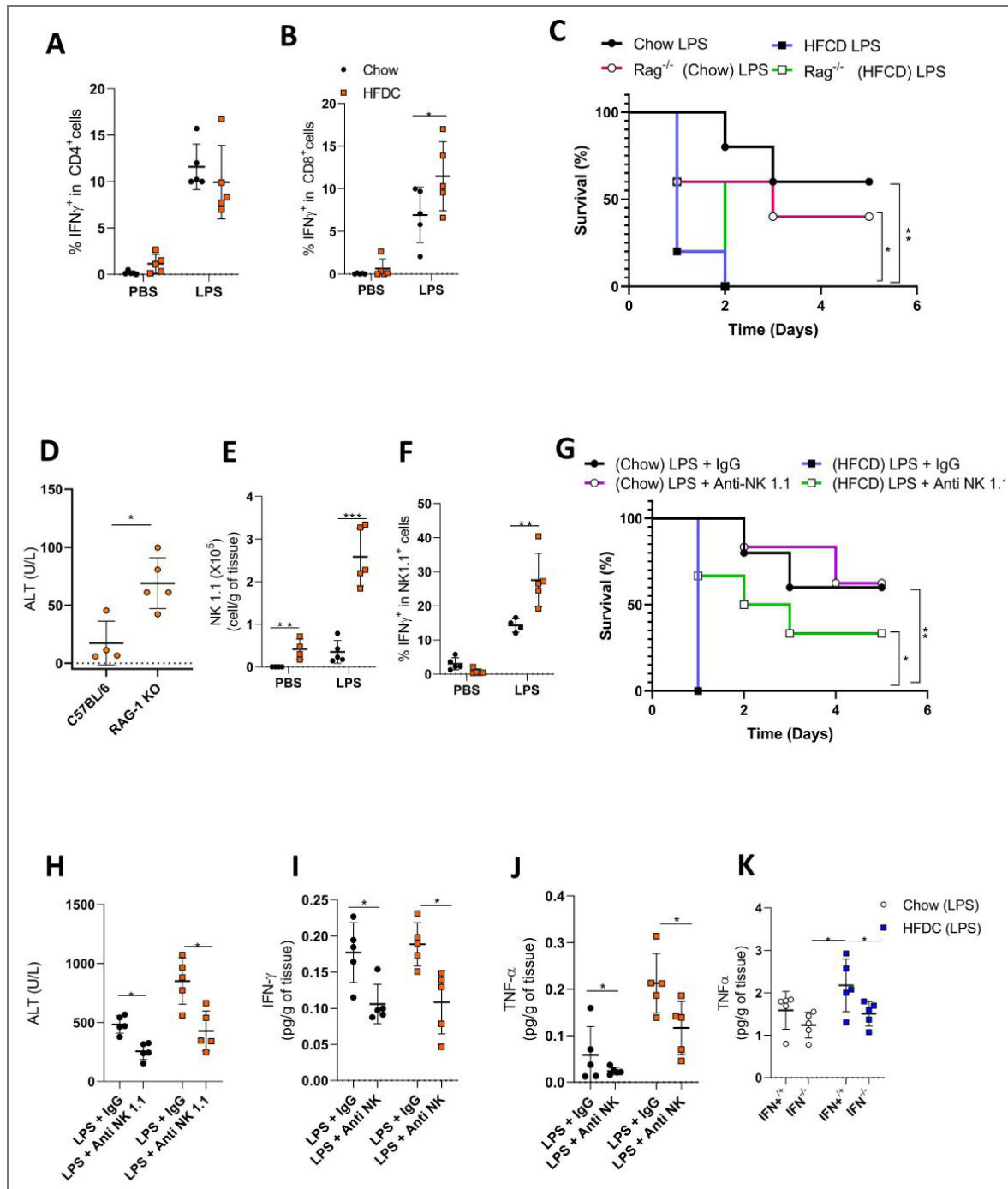


Figure 4. IFN- γ secreted by NK cells increases susceptibility of animals with MAFLD to endotoxemia.

(A) CD4 IFN- γ ⁺ and (B) CD8 IFN- γ ⁺ T cell frequency in animals with MAFLD and Chow 6h after LPS inoculation. (C) displays survival curves of mice with MAFLD (n=5) and Chow (n=5) and T-cell knockout (RAG-/-). (D) Serum ALT levels from mice with MAFLD in knockout of Rag-/-, 6 hours after 10 mg/kg LPS (n=5). (E) and (F) represent the number of cells and frequency of NK IFN- γ ⁺ or IFN- γ ⁻ in animals with MAFLD and Chow 6h after LPS inoculation (G) The survival curves of mice with MAFLD (n=5) and Chow (n=5) and/or depleted NK cells (Anti NK 1.1), 6 days after intraperitoneal inoculation of LPS (10mg/kg) (H) presents the serum ALT levels from mice with MAFLD and Chow of mice with MAFLD (n=5) and Chow (n=5) and/or depleted NK cells (Anti NK 1.1), 6 hours after 10 mg/kg LPS or PBS administration (n=5). (I) and (J) depict IFN- γ and TNF- α secretion in the livers of Chow and MAFLD mice injected with LPS (10mg/kg) (n=5), respectively. (K) shows TNF- α secretion in the livers of Chow and MAFLD and/or knockout of IFN- γ (IFN- γ ^{-/-}) mice injected with LPS (10mg/kg) (n=5). The results are expressed as the mean $\pm$ standard deviation (SD) from one representative experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined with the following p-values: *p < 0.05, **p < 0.01, ***p < 0.001. Each experiment was independently repeated 2 times to ensure reproducibility of the findings.

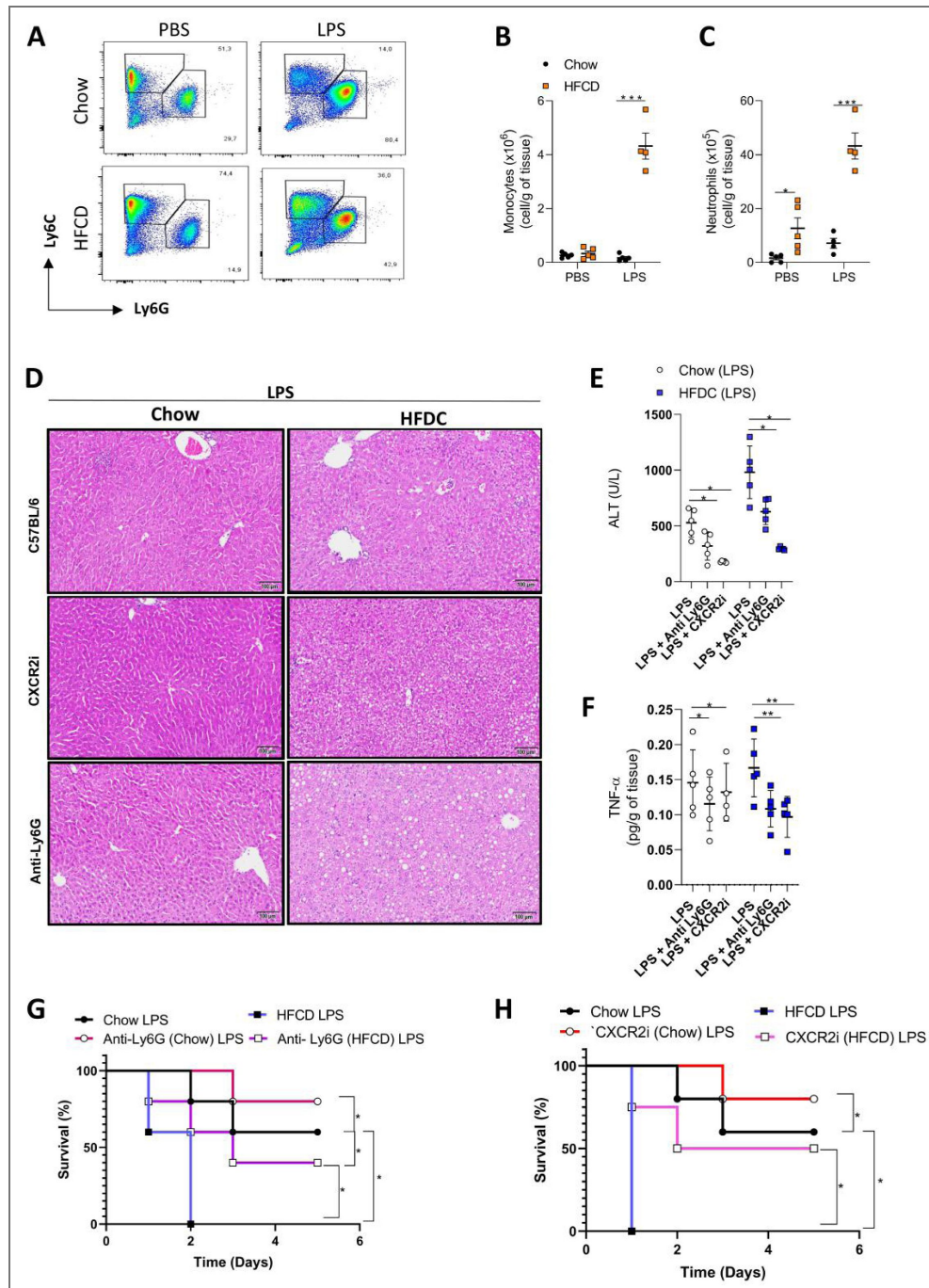


Figure 5. Neutrophils but not monocytes mediate susceptibility of animals with MAFLD to endotoxemia

(A) Representative flow cytometry plot showing monocytes (CD45+CD11b+MHC-Ly6C+Ly6G-) and neutrophils (CD45+CD11b+MHC-Ly6C+Ly6G+) and a number of monocytes (B) and neutrophils (C) in animals with MAFLD and Chow after LPS inoculation. (D) Effect of LPS inoculation on liver histopathology of mice with MAFLD and Chow treated with a CXCR2 inhibitor (CXCR2i) and neutrophil-depleted animals (anti-Ly6G). (E) Serum ALT levels of mice with MAFLD and Chow and/or CXCR2i and anti-Ly6G, 6 hours after administration of 10 mg/kg of LPS and PBS (n=5). (F) TNF-α secretion in the liver of Chow and MAFLD mice injected with LPS (10mg/kg) (n = 5). (G) Survival curves of mice with MAFLD (n=5) and Chow (n=5) treated with anti-Ly6G or (H) CXCR2i, and the survival rates were analysed daily for 6 days after intraperitoneal administration of LPS (10mg/kg). The results are expressed as the mean ± standard deviation (SD) from one representative experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined with the following p-values: *p < 0.05, **p < 0.01, ***p < 0.001. Each experiment was independently repeated 2 times to ensure reproducibility of the findings.

In addition to monocytes, granulocytes are innate immune cells recruited to the hepatic tissue during the development of MAFLD (19). We observed that high levels of neutrophils are present in the hepatic tissue of animals with MAFLD and that their recruitment is exacerbated in the hepatic microenvironment in endotoxemic mice (Fig. 5A and 5C). It is known that neutrophils are recruited to the inflammatory focus via CXCR2 (32). To understand the involvement of these receptors and neutrophils in MAFLD during endotoxemia, MAFLD animals were pre-treated one day before the LPS challenge with CXCR2i or with anti-Ly6G, which depletes these cells throughout the organism. Interestingly, after both treatments, we observed a reduction of the leukocyte infiltration into the hepatic tissue (Fig. 5D) and a decrease in hepatic lesions (ALT) (Fig. 5E) and TNF- α levels (Fig. 5F). Confirming the participation of granulocytes in susceptibility after the LPS challenge in MAFLD, animals treated with CXCR2i and anti-Ly6G showed an improvement in their survival (Fig. 5G and 5H).

IFN- γ induces CD274 expression during endotoxemia in animals with MAFLD

IFN- γ is a cytokine that plays a crucial role in the T and NK cell-mediated immune response (27–29, 33–35). Recent studies have shown that this inflammatory cytokine also induces the expression of checkpoint molecules, such as programmed death-ligand 1 (PD-L1), in different immune cells, including neutrophils (30, 36, 37). Analyzing datasets from mice with MAFLD, we observed the upregulation of the CD274 (PD-L1) gene and TNF- α in neutrophils and IFN- γ in NK cells (Fig. 1C, Fig1D, and Fig. 1E). Considering these findings, we investigated whether IFN- γ induces the expression of PD-L1 in neutrophils in MAFLD mice during endotoxemia. First, we observed that only the MAFLD diet caused a significant increase in PD-L1 expression in CD45+CD11b+ leukocytes after LPS challenge (Fig. 5C). We observed that within this population, neutrophils predominate in their expression when compared to monocytes (Fig. 6A–6D). Furthermore, PD-L1+ neutrophils showed an exacerbated migration of PD-L1+ neutrophils towards the liver (Fig. 6A and 6B). Next, to confirm the role of IFN- γ in the expression of PD-L1, we investigated whether IFN- γ is capable of increasing the expression of PD-L1 in neutrophils after the LPS challenge of MAFLD. Mice with MAFLD lacking IFN- γ were challenged with LPS and assessed for PD-L1 expression in granulocytes. Interestingly, in the absence of IFN- γ , neutrophils do not express PD-L1, and their infiltration in the liver was attenuated (Fig. 6C and 6D).

As demonstrated previously in sepsis, PD-L1 neutrophils showed reduced rates of apoptosis (36). Therefore, we cultured neutrophils in the presence of IFN- γ and/or LPS and assessed their apoptosis rate. Interestingly, in these *in vitro* conditions, neutrophils expressing PD-L1 showed reduced rates of apoptosis (Fig. 6E and 6F), suggesting that PD-L1-expressing neutrophils can survive for long periods in the hepatic environment in MAFLD, accentuating the LPS-induced inflammatory response.

Neutrophils expressing PD-L1+ secrete TNF- α during hyperinflammation in MAFLD

There is also evidence that excessive secretion of TNF- α by immune cells, especially neutrophils, may be associated with liver damage and the progression of MAFLD (36, 37). To investigate a possible link between the expression of PD-L1 by the neutrophils and their ability to express TNF- α , mice undergoing MAFLD were exposed to LPS, and the expression of TNF- α by PD-L1+ neutrophils was assessed. We observed an exacerbated expression of TNF- α by PD-L1+ neutrophils from MAFLD when compared to control animals (Fig. 7A, Fig. 7B, Fig. 7D and Fig8SG) and no increase in TNF- α +PDL1⁻ neutrophils (Fig. 7C). These results illustrate that after exposure of MAFLD animals to LPS, the expression of PD-L1 by neutrophils is linked to heightened TNF- α expression compared to control animals fed standard chow. To assess whether the lack of TNF- α signaling immunomodulates the granulocytic recruitment to the liver tissues in MAFLD animals, TNF receptor R1 and R2 knockout animals (TNF R1R2) were exposed to LPS. It was also observed that the absence of TNF- α did not interfere with the recruitment of neutrophils to the liver tissue (Fig. S8A and Fig. S8C) or the expression of PD-L1 in neutrophils (Fig. S8B and Fig. S8D).

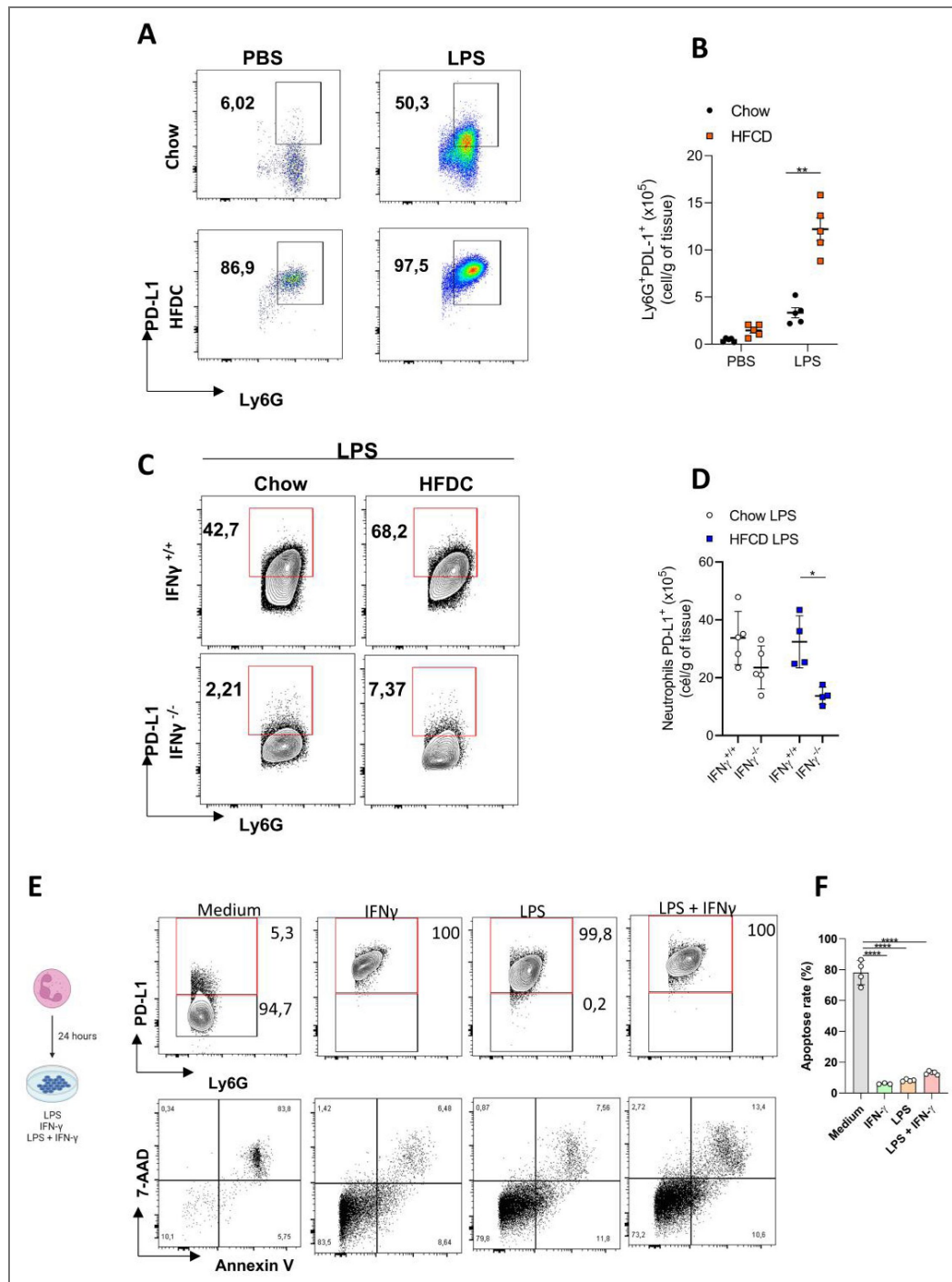


Figure 6. IFN- γ induces PD-L1 expression and reduces apoptosis in mouse neutrophils with MAFLD.

(A) Representative flow cytometry plot showing the expression of PD-L1 in gated neutrophils and (B) the quantification of PD-L1⁺ neutrophils in the liver of mice with MAFLD or that received Chow diet, 6 hours after LPS inoculation (n=5 per group). (C) Representative flow cytometry plot showing the expression of PD-L1 in gated neutrophils and (D) the quantification of PD-L1⁺ neutrophils in the liver of mice with MAFLD and Chow and/or IFN- γ knockout (IFN- γ ^{-/-}) 6 hours after LPS inoculation (n=5 per group). (E) Representative flow cytometry plot showing the expression of PD-L1 and apoptosis (Annexin V + 7-AAD⁺) in gated neutrophils and (F) the frequency of apoptosis in neutrophils cultured in medium, LPS, IFN- γ , or LPS + IFN- γ for 24 hours. The results are expressed as the mean $\pm$ standard deviation (SD) from one representative experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined with the following p-values: *p < 0.05, **p < 0.01, ***p < 0.001. Each experiment was independently repeated 2 times to ensure reproducibility of the findings.

Similarly, the absence of TNF- α receptors in MAFLD during the challenge with LPS did not interfere with the production of IL-10 and IFN- γ (Fig. S8E [↗](#) and Fig. S8F [↗](#)). However, as described above, the absence of the TNF R1R2 receptor reduced liver injury and mortality induced by LPS administration in MAFLD (Fig. 3D [↗](#) and Fig. 3F [↗](#)).

Considering that NK cells present in liver tissue secrete IFN- γ (Fig. 4E [↗](#)) and that in IFN- γ ^{-/-} animals, there is a reduction in TNF- α levels in the liver of mice with MAFLD during endotoxemia (Fig. 4K [↗](#)), we next examined whether PD-L1+TNF α + neutrophils are present in MAFLD animals depleted of NK cells. It was observed that in the absence of NK cells, PD-L1+TNF α + neutrophils were not detected (Fig. 7E [↗](#), Fig. 7F [↗](#), and Fig. 7G [↗](#)), confirming that IFN- γ secreted by NK cells is crucial for inducing PD-L1 expression and TNF- α secretion by neutrophils in the hepatic tissue in MAFLD animals after LPS administration.

Anti-PD-L1 treatment protects animals with MAFLD during hyperinflammation

Next, we investigated the potential role of PD-L1 in TNF- α production by neutrophils and in the lesions in MAFLD mice. Initially, we found that anti-PD-L1, a biological drug inhibiting PD-L1's interaction with PD-1, effectively prevented liver inflammation and tissue damage (Fig. 8A [↗](#), Fig. 8B [↗](#), and Fig. 8F [↗](#)). Additionally, this treatment reduced liver TNF- α levels without affecting IFN- γ (Fig. 8C [↗](#)) and improved host survival (Fig. 8D [↗](#)). Importantly, anti-PD-L1 did not decrease the number of recruited NK cells in all groups studied (Fig. 8I [↗](#) and Fig. 8L [↗](#)). Notably, treatment with anti-PD-L1 successfully blocked LPS-induced neutrophil recruitment and the increased presence of PD-L1-expressing neutrophils in mice on HFCD (Fig. 8J [↗](#) and Fig. 8K [↗](#)), while these parameters remained unchanged in mice fed a standard chow diet (Fig. 8G [↗](#), Fig. 8H [↗](#)). Overall, our findings suggest that anti-PD-L1 therapy primarily suppresses exaggerated TNF- α production by neutrophils in MAFLD mice without affecting the IFN- γ secretory activity of NK cells mechanistically.

Discussion

The pathogenesis of MAFLD and sepsis had been considered two independent conditions, associated with local and systemic inflammatory response, respectively. MAFLD is a chronic liver dysfunction characterized by the buildup of fat in the liver in individuals who do not consume excessive amounts of alcohol. On the other hand, sepsis is an inflammatory state triggered by an infection that leads to damage to vital organs, including the lungs and liver. This occurs when the body's immune response fails to contain the microorganisms at the site of the primary infection⁽⁹⁾. Recent studies demonstrate that the presence of MAFLD is associated with an increased risk of developing sepsis (38, 39), but the exact mechanisms underlying this association are not fully understood. In the present study, we observed that MAFLD mice induced by an HFCD diet are more susceptible to a systemic and hepatic hyper-inflammatory state induced by endotoxemia, confirming in our experimental condition that MAFLD can be considered a crucial risk factor of sepsis progression, even when MAFLD or hepatic inflammation is not yet detected. The LPS administration promotes the polarization of a specific subset of granulocytes expressing PD-L1, which secrete high levels of TNF- α in an NK-derived IFN- γ dependent manner. The released TNF- α plays an important role in the liver lesion. We also observed that CD4+ and CD8+ T cells or monocyte cells do not seem to participate in the mice's susceptibility in this hyper-inflammatory state. In accordance, previous studies implicated NK cells in liver injuries in different MAFLD and sepsis experimental models^(25, 40–44). It was observed that during severe sepsis, NK cell-released IFN- γ play a detrimental role in mediating tissue injury (44). Similarly, in MAFLD, NK cells can accumulate in the liver and release IFN- γ , which contributes to the development of hepatic inflammation and fibrosis⁽⁴⁵⁾. Therefore, these studies, support that the increase in NK cells induced by endotoxemia in MAFLD animals, along with IFN- γ secretion, appears to have a crucial role in the increased susceptibility of these pathological conditions, contributing to disease progression.

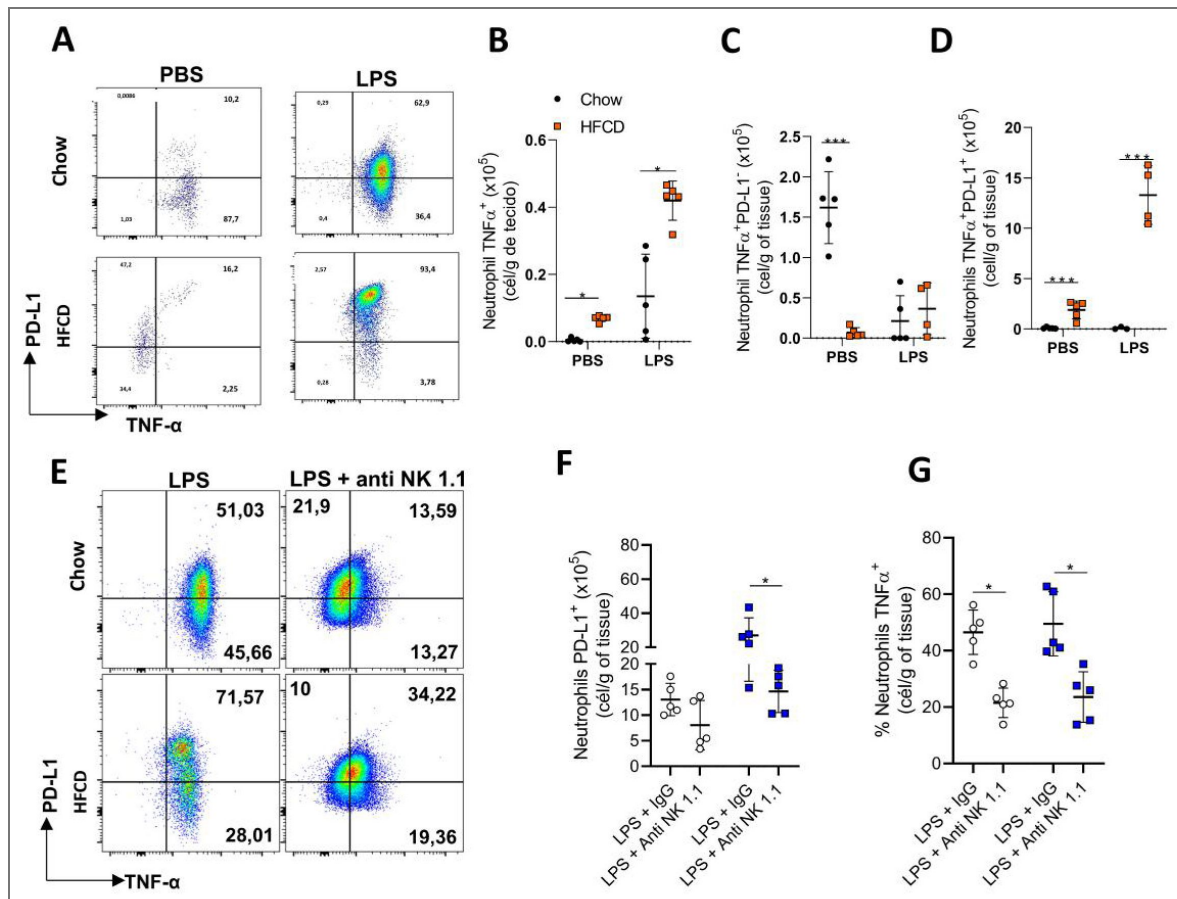


Figure 7. IFN-γ-dependent secretion of TNF-α by PD-L1+ neutrophils induces susceptibility of animals with MAFLD to endotoxemia.

(A) Representative flow cytometry plot showing TNF-α expression in PD-L1+ neutrophils in the liver of animals with MAFLD or fed with Chow diet 6h after LPS inoculation. (B) Number of TNF-α+ neutrophils. (C) Number of TNF-α+PD-L1+ neutrophils. (D) number of TNF-α+PD-L1+ neutrophils. (E) Representative flow cytometry plot showing TNF-α expression in PD-L1+TNF+ neutrophils in the liver of animals with MAFLD and Chow and/or depleted NK cells (anti-NK 1.1) 6h after LPS inoculation. (F) Number of PD-L1+ neutrophils. (G) Frequency of TNF-α+ neutrophils. The results are expressed as the mean ± standard deviation (SD) from one representative experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined with the following p-values: *p < 0.05, **p < 0.01, ***p < 0.001. Each experiment was independently repeated 2 times to ensure reproducibility of the findings.

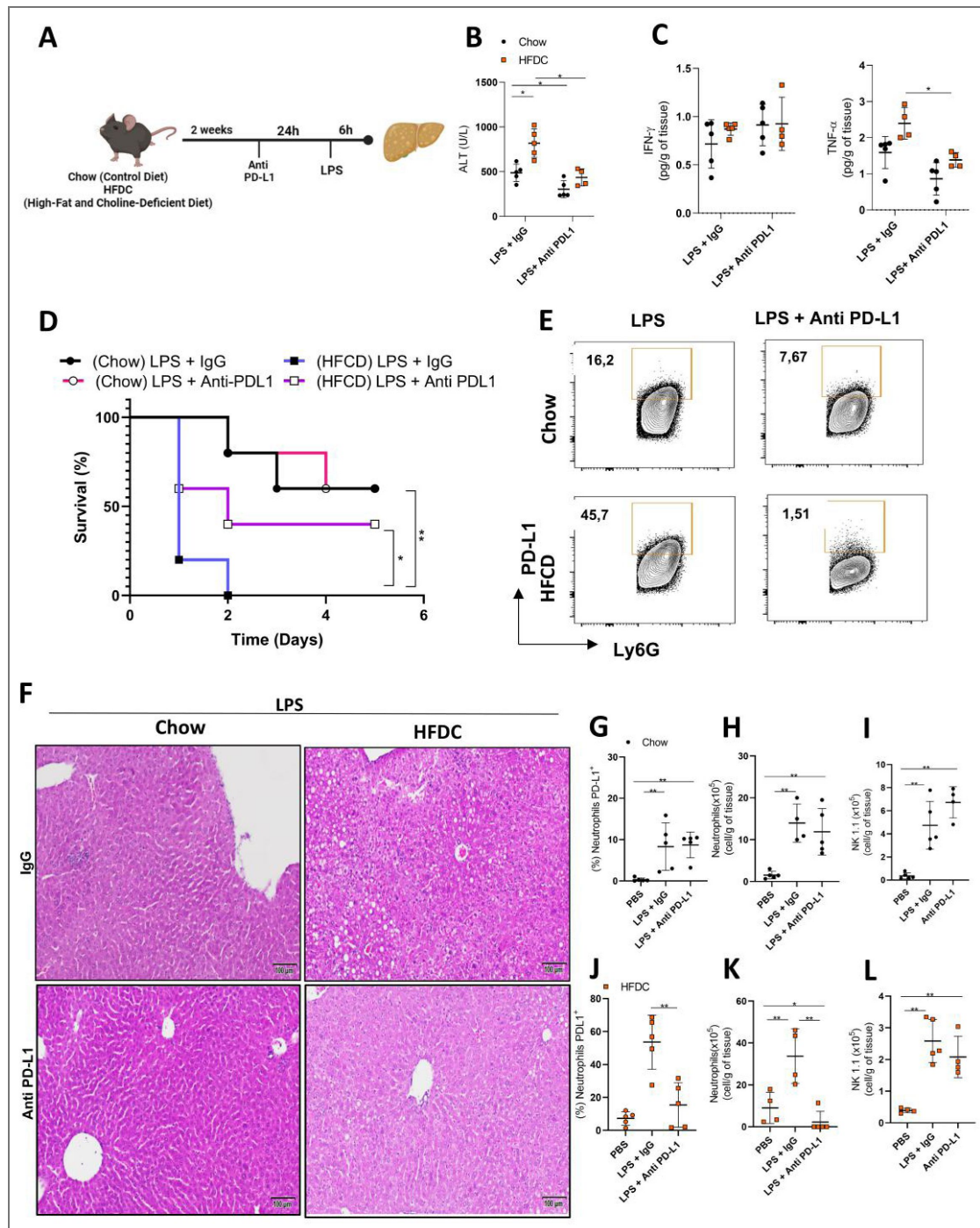


Figure 8. Anti PD-L1 treatment reduces PD-L1 neutrophils in animals with MAFLD during endotoxemia.

(A) Schematic illustration of experimental design. (B) Serum ALT levels of mice with MAFLD or fed with Chow and/or treated with anti-PDL1, 6 hours after administration of 10 mg/kg of LPS or PBS (n=5). (C) IFN- γ and TNF- α secretion in the liver of Chow and MAFLD mice injected with LPS (10mg/kg) treated with anti-PDL1 (n = 5). (D) Survival curves of mice with MAFLD (n=5) and Chow (n=5) treated or not with anti-PD-L1. (E) Representative flow cytometry graph showing TNF- α expression in PD-L1+ neutrophils in the liver of animals with MAFLD and Chow and/or treated with anti-PD-L1 6h after LPS inoculation. (F) Effect of LPS inoculation in liver histopathology of mice with MAFLD and Chow treated or not with anti-PD-L1. (G and J) Frequency of PDL1 neutrophils, (H and K) Number of neutrophils, (I and L) Number of NK cells from animals with MAFLD and Chow and/or treated with anti-PDL1 after 6h of LPS inoculation. The results are expressed as the mean $\pm$ standard deviation (SD) from one representative experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined with the following p-values: *p < 0.05, **p < 0.01, ***p < 0.001. Each experiment was independently repeated 2 times to ensure reproducibility of the findings.

Considering this evidence, we re-analyzed previously published bioinformatic data (21, 22) and observed that, in MAFLD patients, IFN- γ and TNF- α are up-regulated, and that, in high-fat high fructose diet-fed mice, there is up-regulation of IFN- γ in NK cells, and TNF- α and CD274 (PD-L1) in myeloid cells (21, 22), suggesting that similar immune dysfunctions are observed in patients and experimental models of MAFLD. To add experimental information about how the IFN- γ mediates the liver lesion, we demonstrated that in endotoxemic MAFLD animals hepatic neutrophils are important producers of TNF α , and this release is stimulated by NK-derived IFN- γ signaling. In addition, IFN- γ promoted the differentiation of PDL-1-expressing neutrophils, given that in those animals depleted of NK, the neutrophils did not exhibit this activated phenotype. Moreover, the PDL-1-expressing neutrophils showed a reduced apoptosis rate, increasing the retention time of the neutrophils in hepatic tissue. Thus, these events could explain the increase in hepatic injury in MAFLD animals during endotoxemia. According, in the context of infection-triggered inflammation, the participation of IFN- γ in modulating neutrophil pro-inflammatory profiles, including the expression of PD-L1, has been demonstrated (36). Neutrophils, as frontline innate immune cells, control pathogens through phagocytosis and the release of microbial mediators, including cytokines, cytosolic enzymes, free radicals, and NETs (46, 47). However, excessive neutrophil activity can lead to tissue damage, particularly in sepsis, where they contribute significantly to multi-organ damage via the overproduction of inflammatory mediators, including TNF- α (16, 48, 49). Our data demonstrate that during MAFLD, liver neutrophils are major sources of TNF- α production induced by endotoxemia. Confirming the involvement of TNF- α release by PDL-1+ neutrophils stimulated by NK cells-derived IFN- γ in the increase of susceptibility of MAFLD, we demonstrated that the absence of TNF- α signaling or depleted NK cells led to improved survival in mice with MAFLD during endotoxemia. However, the absence of TNF- α signaling did not affect PD-L1 expression in neutrophils. These findings imply that, while TNF- α plays a crucial tissue lesions, its intracellular signaling does not notably impair the expression of PD-L1 in the neutrophils or their migration into liver tissue during MAFLD. To reinforce that neutrophils are the main source of the TNF- α involved in susceptibility of MAFLD by endotoxemia, we evaluate whether the neutralization of these cells with specific antibodies could improve the survival of endotoxemic HFDC-fed mice. Numerous strategies for targeting neutrophils are being investigated in various clinical and experimental settings (12, 50), including the use of CXCR2 inhibitors in patients with NASH (13, 50). Interestingly, we demonstrated that targeting neutrophils with different biological drugs, similar to the inhibition of TNF- α , mitigated inflammation-induced liver lesions.

Recent studies suggest that during the progression of MAFLD, PD-L1 is expressed in both parenchymal and non-parenchymal cells (10, 51). Given that the neutrophils responsible for hepatic damage in endotoxemic MAFLD mice exhibit a specific PD-L1-expressing phenotype, we demonstrated that anti-PD-L1 significantly reduced TNF- α -producing PD-L1+ neutrophils in the liver environment, attenuating endotoxemia-induced inflammation and liver damage, ultimately reducing mortality in MAFLD mice. This highlights the role of PD-L1 expression in neutrophils in promoting a proinflammatory phenotype associated with heightened TNF- α production and susceptibility of endotoxemic HFDC-fed mice. In the context of endotoxemia, recent research indicates that PD-L1-expressing neutrophils also contribute to immunosuppression and increased susceptibility to secondary infections (52) and also contribute to lung injury. Thus, our data support that the inhibition of PD-L1, besides preventing the organ lesions induced by sepsis in MAFLD patients, could also prevent the long-term immunosuppression observed in these patients.

Although macrophages, including Kupffer cells, have been considered a relevant source of cytokines in endotoxemic animals (53, 54), we did not find an important role for these cells expressing CCR2 in the aggravation of endotoxemia, since the absence of these chemokine receptor, which governs the migration and differentiation of inflammatory monocytes into macrophages, did not influence the endotoxemia mortality in animals with MAFLD. However, it's worth noting that we did not extensively investigate the interaction of IFN- γ and TNF- α in the role of monocytes in our model, necessitating further studies to unravel the possible mechanisms of these cells.

Our study presents several limitations that should be acknowledged and discussed. First, we cannot entirely rule out the possibility that our mice deficient in pro-inflammatory components exhibit reduced responsiveness to LPS. However, our *ex vivo* analyses using splenocytes from these animals revealed a preserved cytokine production following LPS stimulation. These results suggest that the *in vivo* differences observed are primarily driven by the MAFLD condition rather than by intrinsic defects in LPS sensitivity. Second, the absence of publicly available single-cell RNA-seq datasets from MAFLD subjects under endotoxemic or septic conditions limited our ability to perform direct translational comparisons. To overcome this, we analyzed existing MAFLD patients and experimental MAFLD datasets, which consistently demonstrated upregulation of IFN- γ and TNF- α inflammatory pathways in MAFLD. In line with these findings, our murine model revealed TNF- α^+ myeloid and IFN- γ^+ NK cell populations, thereby reinforcing the validity and translational relevance of our results.

Overall, our findings establish a biological basis for understanding the progression of endotoxemia in MAFLD, highlighting the dysregulated role of neutrophils in which hepatic metabolic syndrome potentiates the inflammatory effects of granulocytes via NK cells, advocating interventions to mitigate this inflammatory condition.

Material and methods

Experimental Design

This study was designed to interrogate the pathologic mechanisms in MAFLD during endotoxemia-induced generalized inflammation. The mice were submitted to the HFDC diet for two weeks, and subsequently, LPS administration and the cellular mechanisms were outlined after 6h of the challenge, knowing that no animal succumbed to death (indicated by survival). Furthermore, knockout animals and those treated with pharmacological inhibitors or neutralizing antibodies shared the same control groups (chow and HFCD), as required by the animal ethics committee. Inflammatory infiltrates along with liver lesions were assessed based on H&E-stained tissue sections, and endpoint analyses included flow cytometry.

Animals

The mice used in this study were in the C57BL/6 genetic background and included CCR2^{-/-}, IFN- γ ^{-/-}, and TNFR1R2^{-/-}. Male mice aged 5 to 6 weeks were reared and maintained under specific pathogen-free conditions in the University of São Paulo vivarium, FMRP/USP. All animals received food and water *ad libitum*, at 25°C, with food and water *ad libitum*. Mice were fed a standard low-fat diet (hereinafter referred to as the standard “Chow” diet – 10% of calories derived from fat) or a Choline Deficient High Fat Diet (referred to as “HFCD”, 60% of calories derived from fat and choline-deficient) for 2 weeks for the development of MAFLD. The diets were provided by Rhoister (Araçoiaba da Serra-São Paulo). Care for the mice complied with institutional guidelines on ethics in animal experiments; approved by CETEA (Ethics Committee for Animal Experimentation of the Faculty of Medicine of Ribeirão Preto, approved protocol number 193/2019).

Endotoxemia

Lipopolysaccharide (LPS; Escherichia coli (O111:B4), L2630, Sigma-Aldrich, St. Louis, MO, USA) was administered intraperitoneally (i.p.; 10 mg/kg) in C57BL/6, CCR2^{-/-}, IFN- γ ^{-/-}, and TNFR1R2^{-/-} mice. The HFCD was initiated in 5–6 week-old mice, and LPS was administered after 2 weeks on the diet, meaning that LPS administration occurred when the mice were 7–8 weeks old, with body weights ranging from 22 to 26 g. LPS was previously solubilized in sterile saline and frozen at -70°C. The animals were euthanized 6 hours after LPS administration.

Hepatic non-parenchymal cell isolation

Livers were collected, and 200–300 mg of tissue was taken for flow cytometry analysis. The tissue was minced with surgical scissors and then digested with 1 mg of collagenase II, and 60 U/ml of DNase I in PBS at 37 °C for 45 minutes, shaking at 110 rpm. The digested tissue was then processed

through a 100 μ m cell strainer and centrifuged at 1,500 rpm for 5 minutes. Subsequently, the isolation of non-parenchymal cells was performed by Percoll (GE Healthcare) centrifuged at 2300rpm at 22 °C for 30 minutes, subjected to ACK (red blood cell lysis), and resuspended in 0.5% BSA in PBS for staining. Single-cell suspensions were stained with Zombie Aqua viability stain (Biolegend), blocked with TruStain FcX (Biolegend), and then stained with flow cytometry antibodies when indicated; antibodies are tabulated in Table S1. As animals were already in a hyperinflammatory state, no additional in vitro stimulation was required. Intracellular detection of IFN- γ and TNF- α was conducted using the Foxp3 staining kit (eBioscience). Since both antibodies were conjugated to PE, staining and analyses were performed in separate experiments. Cells were collected on the CANTO II flow cytometer using BD FACS Diva software and then analyzed using FlowJo (USA).

Histology

Mouse livers were fixed in 10% formalin overnight, washed twice with PBS, and placed in 70% ethanol or PBS until processing. For H&E, tissues were embedded in paraffin, sectioned, and stained with H&E at the MGH Histopathology Research Core.

Cytokine quantification

For the detection of IFN- γ , TNF- α , and IL-10 in the liver, tissue samples were weighed and titrated in 1 mL of PBS Complete (Roche Diagnostics, Mannheim, Germany) containing a cocktail of protease inhibitors. Cytokine levels were determined using commercial ELISA kits for IFN- γ , TNF- α , and IL-10 (Biolegend, USA). Wavelength correction and background signals were subtracted from absorbance values

Serum analysis

At the time of sacrifice, blood samples were collected in EDTA collection tubes and subsequently centrifuged to obtain serum. Serum ALT and Urea levels were measured using a colorimetric kit (BioQuant, Heidelberg, Germany) according to the manufacturer's instructions.

Neutrophil targeting

For neutrophil targeting, antibodies/inhibitors were used as follows: Anti-Ly6G (BioXCell, catalog # BP0075) was administered at 500 mg/kg per mouse on day 13 after starting the diet (24h before inoculation of the LPS) and sacrificed on day 14. For survival studies, all mAbs were inoculated once daily for 7 days. CXCR2 inhibitor SB 225002 (Tocris, catalog #2725/10) was injected at 200 mg per dose and programmed as anti-Ly6G. For anti-PD-L1-based neutrophil targeting, anti-PD-L1 (BioXCell, catalog #BE0101) was injected at 12.5 mg/kg ~12h before the LPS challenge and again 1 day after treatment, each time followed by anti-mouse IgG2b (12.5 mg/kg) (BioXCell, catalog no. BE0252). All injections were intraperitoneal.

Neutrophil purification and apoptosis assay

Peripheral blood samples were collected by venipuncture and centrifuged at 450 $\times$ g for plasma separation. The blood cells were then resuspended in Hank's balanced salt solution (Corning; cat. 21-022-CV), and the neutrophil population was isolated by Percoll density gradient (GE Healthcare; cat. 17-5445-01) (72 %, 63%, 54% and 45%). Isolated neutrophils were resuspended in RPMI1640 (Corning; cat. 15-040-CVR) supplemented with 0.5% BSA. Neutrophil purity > 95% was determined by Rosenfeld color Cytospin (Laborclin; cat 620529) To quantify apoptosis, cells were permeabilized with Triton X-100 and stained with propidium iodide. After 10 minutes of incubation at 4 degrees in the dark, neutrophil apoptosis was quantified as PI+ staining on FACSCanto II (Becton Dickinson, Franklin Lakes, NJ, USA). Results were analyzed using Flowjo software (Tree Star, Ashland, OR, USA). Apoptosis was detected as a measure of the hypodiploid peak in PI fluorescence histograms, as described by Nicoletti et al. BD Bioscience, Franklin Lakes, NJ, USA) and Annexin V and 7-AAD double staining (Biolegend, San Diego, USA).

Splenocyte culture

Mice were fed a control chow or HFCD diet for 2 weeks. Animals were euthanized, and spleens were aseptically removed in cold PBS (Gibco) and then mechanically dissociated through a 100- μ m cell strainer to obtain a single-cell suspension. Red blood cells are lysed with ACK buffer for 1–2 min at room temperature, the reaction is quenched with complete RPMI 1640 medium (supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics), and cells are centrifuged and resuspended at the desired density, 1×10^6 cells/mL. Splenocytes are plated in 96-well plates and incubated at 37°C, 5% CO₂. To induce immune activation, cells are stimulated with lipopolysaccharide (E. coli O111:B4) at concentrations ranging from 100 ng/mL to 1 μ g/mL for 24 h, and cytokine dosage was performed for IL-6 and TNF α .

NK cell depletion

For in vivo NK cell depletion, male C57BL/6 mice received NK cell neutralizing antibody (BioXCell, catalog # PK136) or isotype-matched mouse IgG2 α monoclonal antibody control (BioXCell, catalog # BE0252) by intraperitoneal injection (150 μ g in 200 μ L PBS 24h before LPS challenge). To assess survival, the antibodies were inoculated 24 hours before the LPS challenge and then once a day for 7 days.

Single-cell RNA sequencing analysis

We re-analyzed single-cell transcriptomic data (GSE166504) from the mouse liver from mice with MAFLD and their respective control groups (21). The dataset was downloaded, and the RDS file was imported into R (55) environment version v4.2.3 and Seurat v4.1.1 (56) by filtering genes expressed in at least 300 cells. For the pre-processing step, outlier cells were filtered out based on three metrics (library size < 20,000, number of expressed genes between 200 and 4,000, and mitochondrial percentage expression < 0.8). The top 3,000 variable genes were then identified using the ‘best’ method using the FindVariableFeatures function. Percent of mitochondrial genes was regressed out in the scaling step, and Principal Component Analysis (PCA) was performed using the top 3,000 variable genes, and the top 30 PCs were selected for dimension reduction by Uniform Manifold Approximation and Projection (UMAP). Clusters were identified using the author’s annotation. Then, differential gene expression analysis was performed using the FindAllMarkers function in Seurat with default parameters to obtain a list of significant gene markers for each cluster of cells. Visualization of genes illustrating expression levels was performed using R/Seurat commands (DimPlot, FeaturePlot, and DotPlot) using ggplot2 (57) and customized (58) R packages.

RNA sequencing analysis

RNA-seq data were obtained from GSE185051 (59). The R/Bioconductor package edgeR was used to identify differentially expressed genes among the samples, after removing absent features (zero counts in more than 75% of samples) (60). Genes with a fold change of >0.5 were identified as differentially expressed.

Pathway enrichment analysis

The list of differentially expressed genes was enriched using the ClusterProfiler R package (8). Gene ontology (GO) terms in the Biological Processes category with $P < 0.05$ were considered significant. Statistically significant, non-redundant GO-enriched terms were plotted.

Statistics

Statistical significance was determined by two-tailed paired or unpaired Student’s t-test or one-way ANOVA followed by Tukey’s post hoc test. Absolute numbers and percentages were compared using Fisher’s exact test. Spearman rank order correlation (r) was calculated to describe the correlations. $P < 0.05$ was considered statistically significant. Statistical analyses and graphics were performed using the GraphPad Prism 8.4.2 software.

Supplementary figures

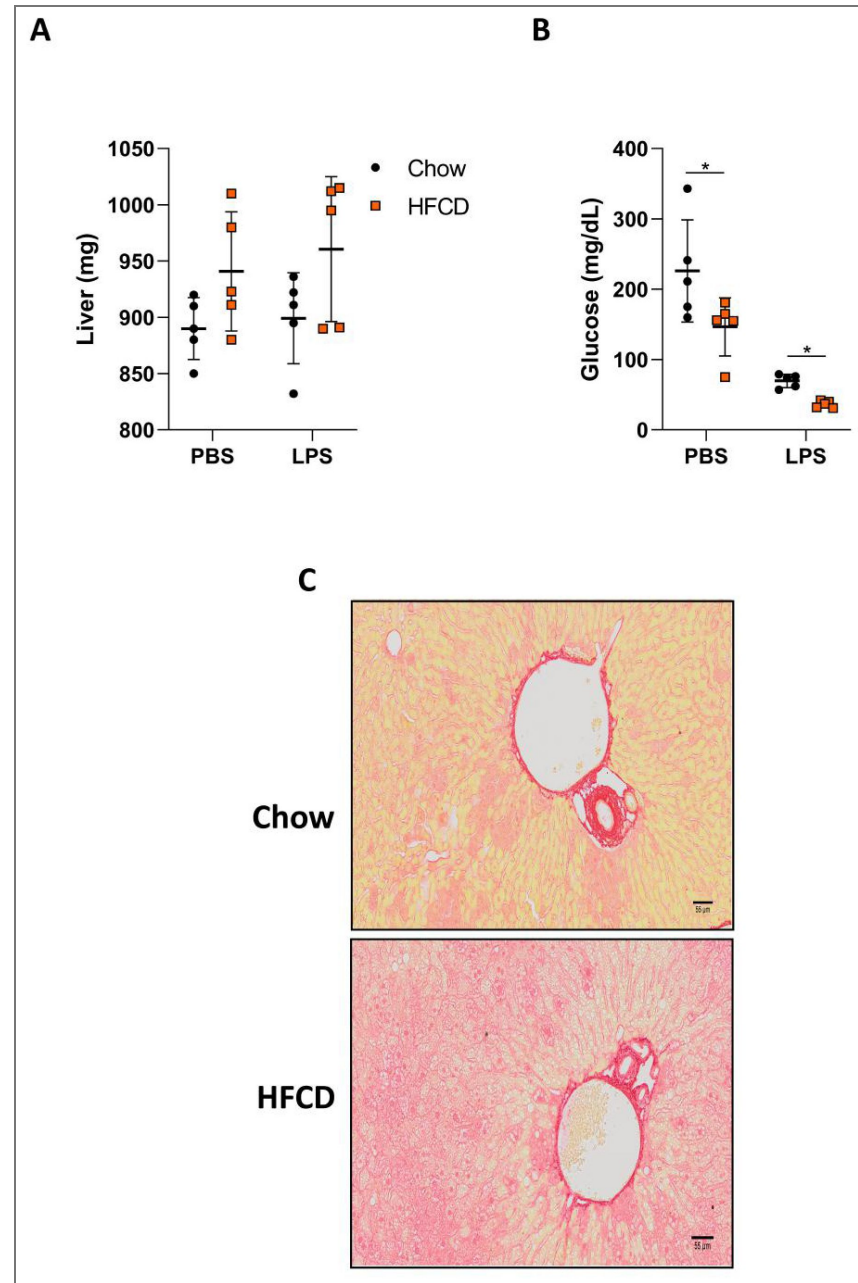


Figure S1. Validation of the HFCD-induced MAFLD experimental model based on liver weight, blood glucose levels, and histological analysis (H&E and Picro Sirius staining). (A) Liver weight, (B) plasma glucose, and (C) representative liver histological sections of lipid accumulation, steatotic morphology and collagen deposition (Picro Sirius Red staining) of mice fed a high-fat, choline-deficient (HFCD) or control chow diet for two weeks and subsequently challenged or not with LPS (10 mg/kg, i.p.) (n=5) (Data are expressed as the mean $\pm$ standard deviation (SD) from one representative experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Each experiment was independently repeated at least twice to ensure reproducibility of the findings

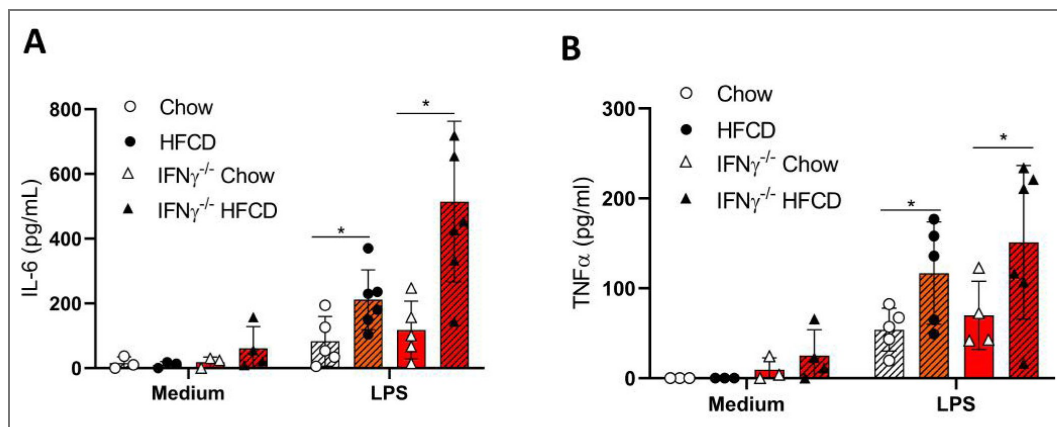


Figure S2. The IFN γ -deficient mice display LPS responses comparable to controls.

(A–B) Splenocytes isolated from C57BL/6 and IFN $\gamma^{-/-}$ mice fed with Chow or HFCD for two weeks were stimulated *in vitro* with LPS (100 ng/mL) for 24 hours. Cytokine levels of (A) IL-6 and (B) TNF- α were quantified in the culture supernatants. Data are expressed as mean $\pm$ standard deviation (SD) from one representative experiment ($n = 5$ per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Each experiment was independently repeated twice to ensure reproducibility.

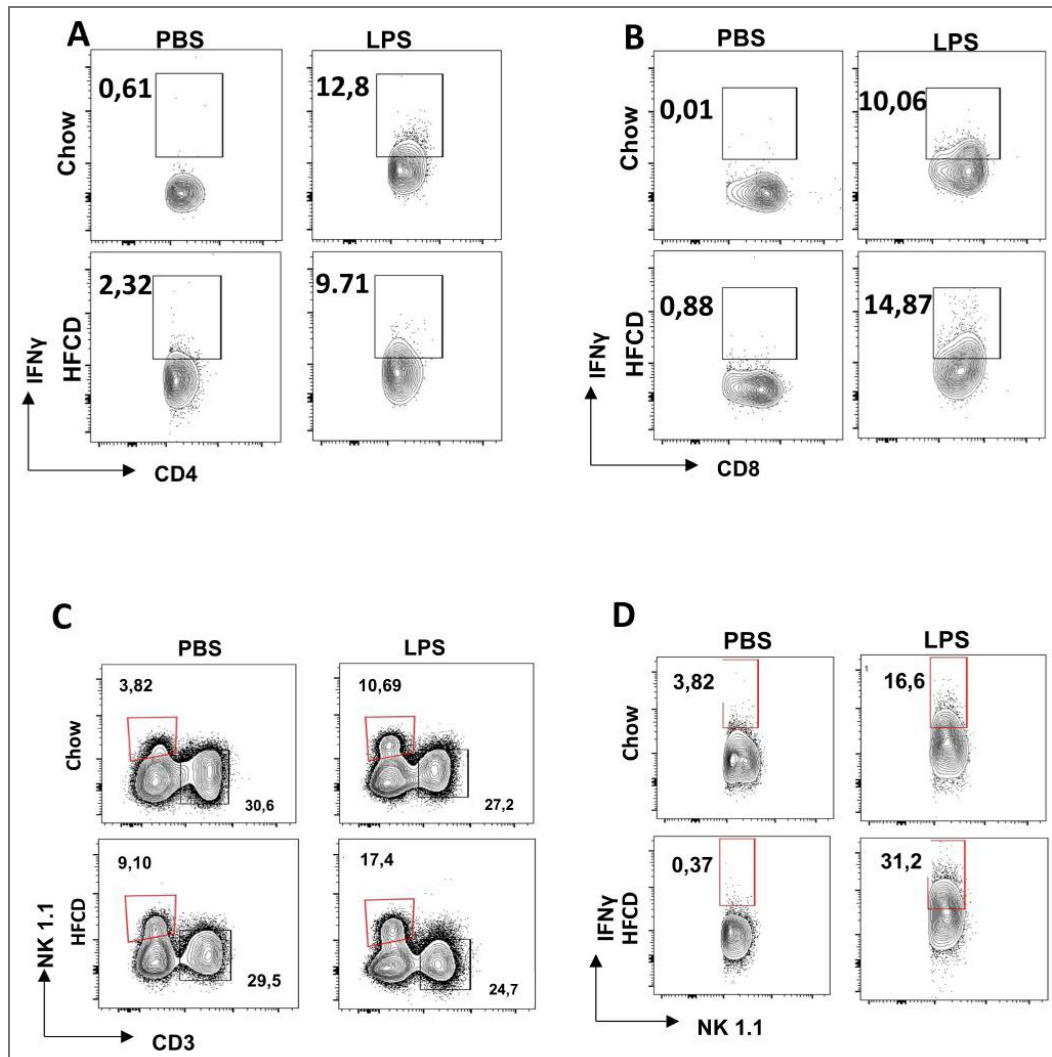


Figure S3. NK cells produce high levels of IFN-γ during endotoxemia in MAFLD

(A) Representative flow cytometry dot plot showing CD4⁺IFN-γ⁺ T cells from the liver of mice fed a high-fat, choline-deficient (HFCD) or chow diet for two weeks and subsequently challenged or not with LPS. (B) Representative dot plot of hepatic CD8⁺IFN-γ⁺ T cells under the same experimental conditions. (C) Representative dot plot showing NK cells (NK1.1⁺CD3⁺) from the liver of HFCD and chow-fed mice challenged or not with LPS. (D) Representative dot plot of IFN-γ expression by NK cells (NK1.1⁺CD3⁺) from the same groups.

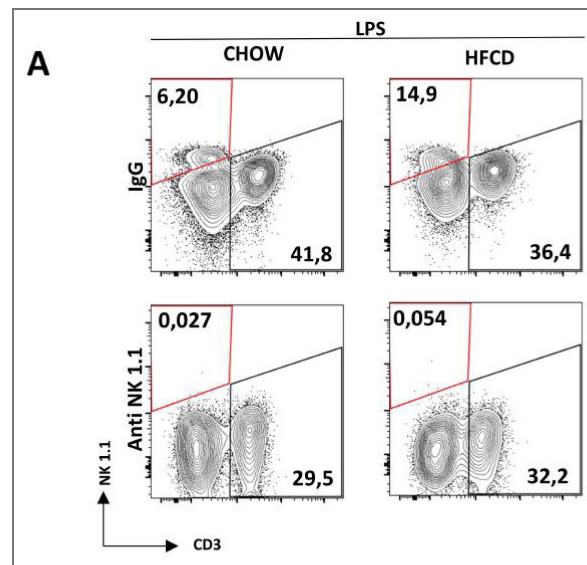


Figure S4. Anti-NK1.1 treatment depletes NK cells in animals with MAFLD during endotoxemia

(A) Representative flow cytometry dot plot showing depletion of NK cells (NK1.1⁺CD3⁻) in the liver of mice fed a high-fat, choline-deficient (HFC) or chow diet for two weeks and challenged with LPS, following treatment with anti-NK1.1 antibody.

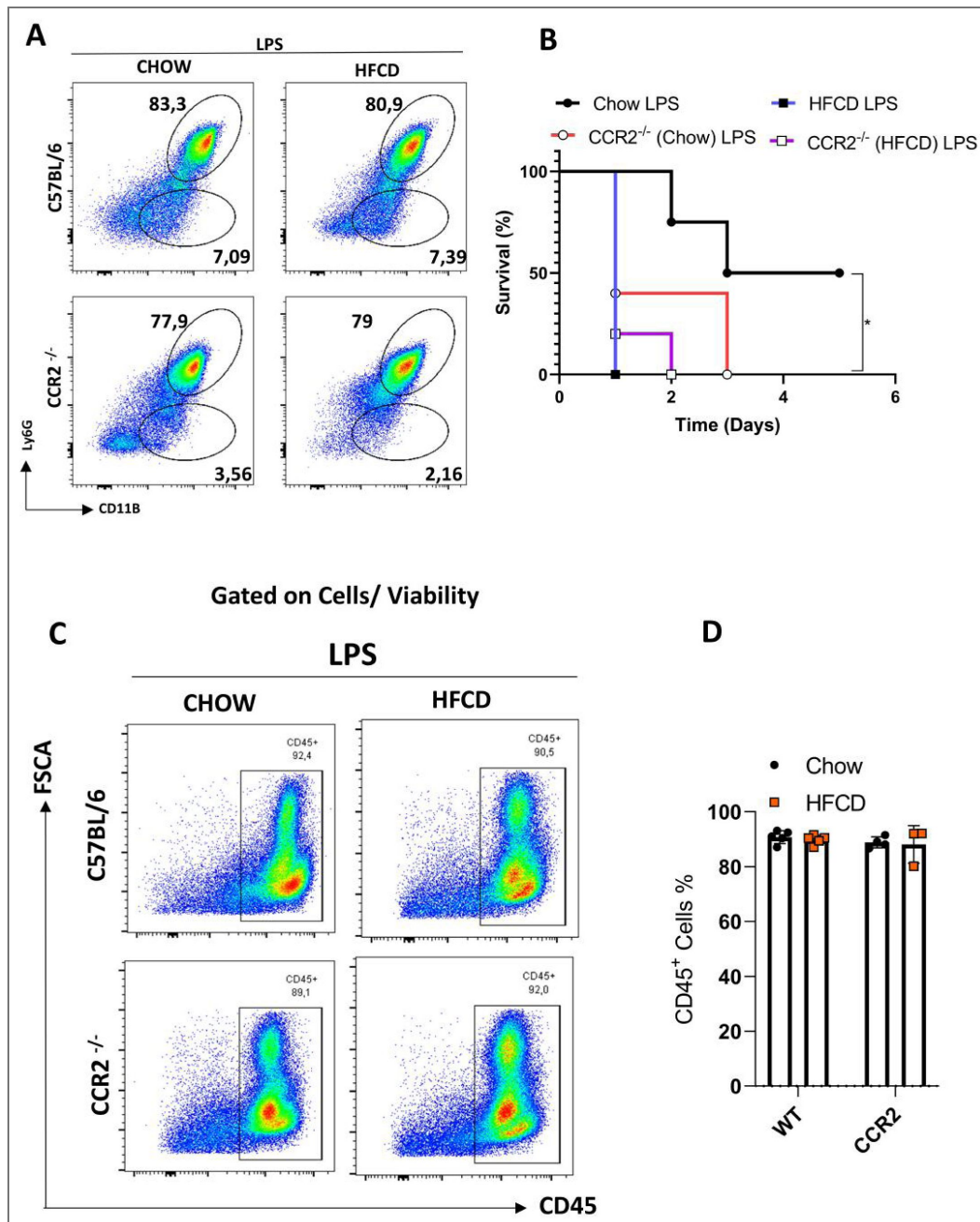


Figure S5. Monocytes do not participate in the susceptibility of mice with MAFLD to endotoxemia

(A) Representative flow cytometry dot plot showing neutrophils (CD45⁺CD11b⁺Ly6G⁺) and monocytes (CD45⁺CD11b⁺Ly6G⁻) in the liver of C57BL/6 and CCR2^{-/-} mice fed a high-fat, choline-deficient (HFCD) or chow diet for two weeks and challenged with LPS. (B) Survival curves of C57BL/6 and CCR2^{-/-} mice under the same experimental conditions. (C) Representative dot plot of total leukocytes (CD45⁺) in the liver of C57BL/6 and CCR2^{-/-} mice after LPS challenge. (D) Frequency of leukocytes (CD45⁺) in the liver of C57BL/6 and CCR2^{-/-} mice fed HFCD or chow diet and challenged with LPS (n=5). Data are representative of one experiment reproduced independently twice with similar results. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined with the following p-values: *p < 0.05, **p < 0.01, ***p < 0.001. Results are expressed as the mean ± standard deviation (SD).

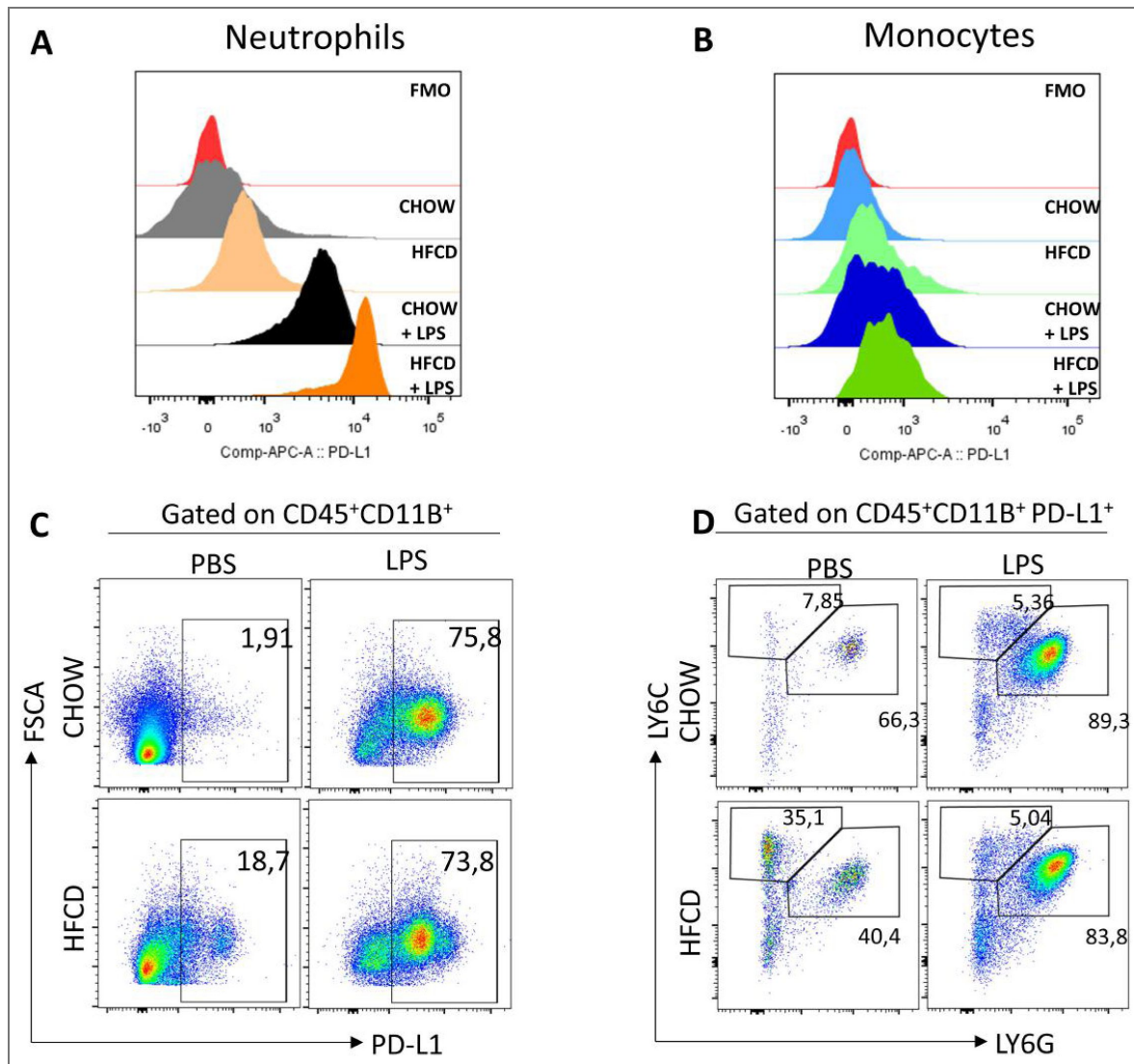


Figure S6. Neutrophils are the main PD-L1-expressing subset among CD45⁺CD11b⁺ leukocytes in MAFLD.

(A and B) Representative flow cytometry histogram showing PD-L1 expression on neutrophils (CD45⁺CD11b⁺Ly6C⁺Ly6G⁺) and monocytes (CD45⁺CD11b⁺Ly6C⁺Ly6G⁻) isolated from the liver of mice fed a high-fat, choline-deficient (HFCD) or control chow diet for two weeks and challenged with LPS (10 mg/kg, i.p.) (n=5) (C) Representative dot plot showing PD-L1 expression within total hepatic CD45⁺CD11b⁺ leukocytes from chow- and HFCD-fed animals for two weeks, either challenged or not with LPS. (D) Representative dot plot depicting the relative proportions of neutrophils (CD45⁺CD11b⁺Ly6C⁺Ly6G⁺) and monocytes (CD45⁺CD11b⁺Ly6C⁺Ly6G⁻) within total CD45⁺CD11b⁺ leukocytes in the liver of chow- and HFCD-fed mice after LPS challenge (n=5). Data are expressed as the mean $\pm$ standard deviation (SD) from one representative experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined as *p < 0.05, **p < 0.01, ***p < 0.001. Each experiment was independently repeated at least twice to ensure reproducibility of the findings.

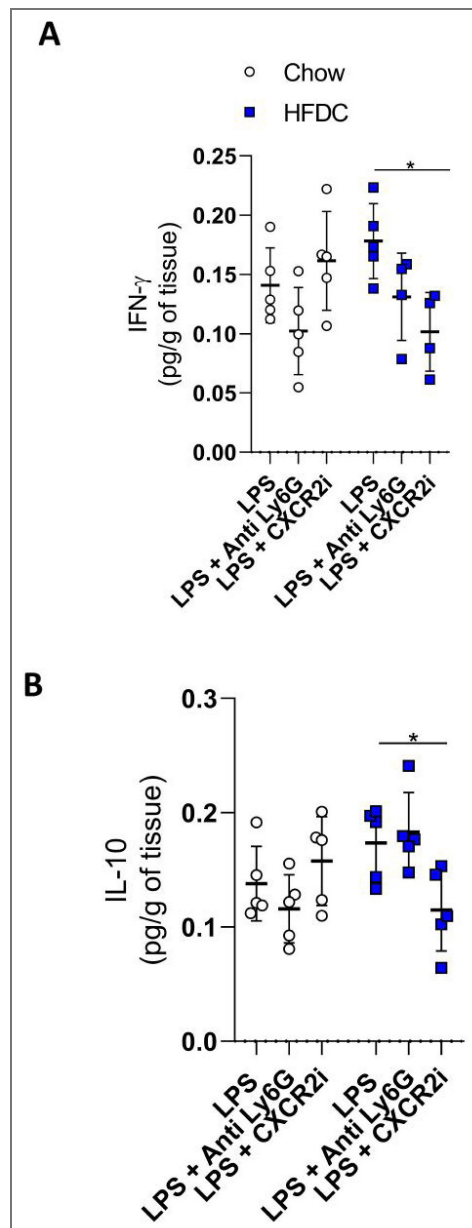


Figure S7. CXCR2i treatment reduces IFN- γ in the liver tissue of animals with MAFLD during endotoxemia

(A) IFN- γ levels in the liver of mice fed a high-fat, choline-deficient (HFCD) or chow diet for two weeks, challenged with LPS, and treated with either anti-Ly6G antibody or a CXCR2 inhibitor (CXCR2i) (n=5).. (B) IL-10 levels in the liver of the same experimental groups (n=5). The data are representative of one experiment reproduced independently twice with similar results. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined with the following p-values: *p < 0.05, **p < 0.01, ***p < 0.001. Results are expressed as the mean $\pm$ standard deviation (SD).

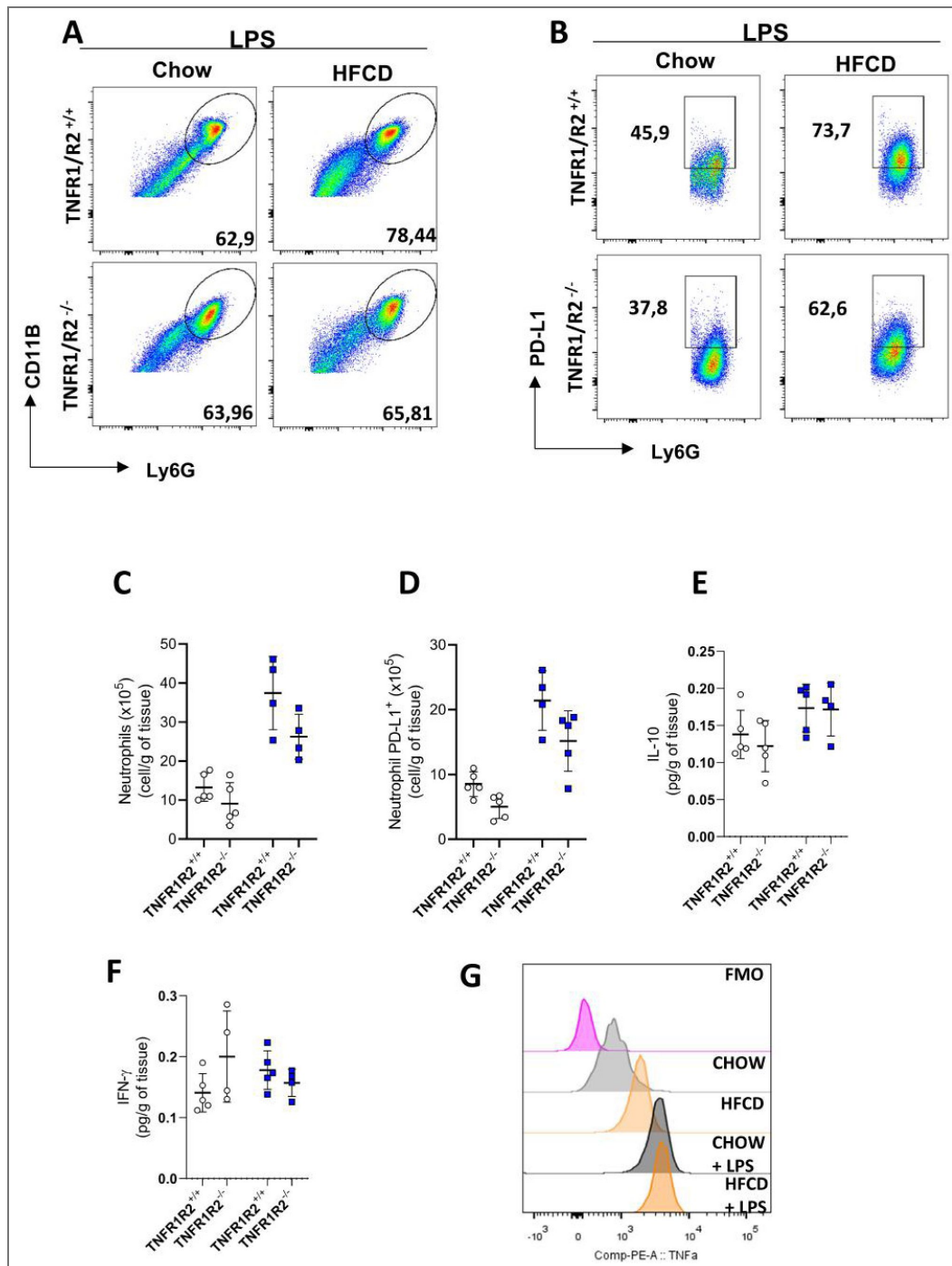


Figure S8. TNFR1/R2^{-/-} animals do not show reduced neutrophil infiltration in the hepatic tissue of mice with MAFLD during endotoxemia.

(A) Representative flow cytometry dot plot showing neutrophils (CD45⁺CD11b⁺Ly6G⁺) in the liver of TNFR1R2^{+/+} and TNFR1R2^{-/-} mice fed a high-fat, choline-deficient (HFCD) or chow diet for two weeks and challenged with LPS. (B) Representative dot plot of PD-L1⁺ neutrophils (CD45⁺CD11b⁺Ly6G⁺PD-L1⁺) under the same experimental conditions. (C) Quantification of neutrophils (CD45⁺CD11b⁺Ly6G⁺) in the liver of TNFR1R2^{+/+} and TNFR1R2^{-/-} mice after LPS challenge. (D) Quantification of PD-L1⁺ neutrophils (CD45⁺CD11b⁺Ly6G⁺PD-L1⁺) in the liver (n=5). (E) IL-10 levels in the liver of TNFR1R2^{+/+} and TNFR1R2^{-/-} mice fed HFCD or chow and challenged with LPS (n=5). (F) IFN-γ levels in the liver of TNFR1R2^{+/+} and TNFR1R2^{-/-} mice under the same conditions. (G) Representative histogram showing TNF-α expression in the liver of C57BL/6 mice fed HFCD or chow for two weeks, challenged or not with LPS. The data are representative of one experiment reproduced independently twice with similar results. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was determined with the following p-values: *p < 0.05, **p < 0.01, ***p < 0.001. Results are expressed as the mean ± standard deviation (SD).

Figure S9. PD-L1 expression in neutrophils occurs in the liver and not in the lung in animals with MAFLD during endotoxemia

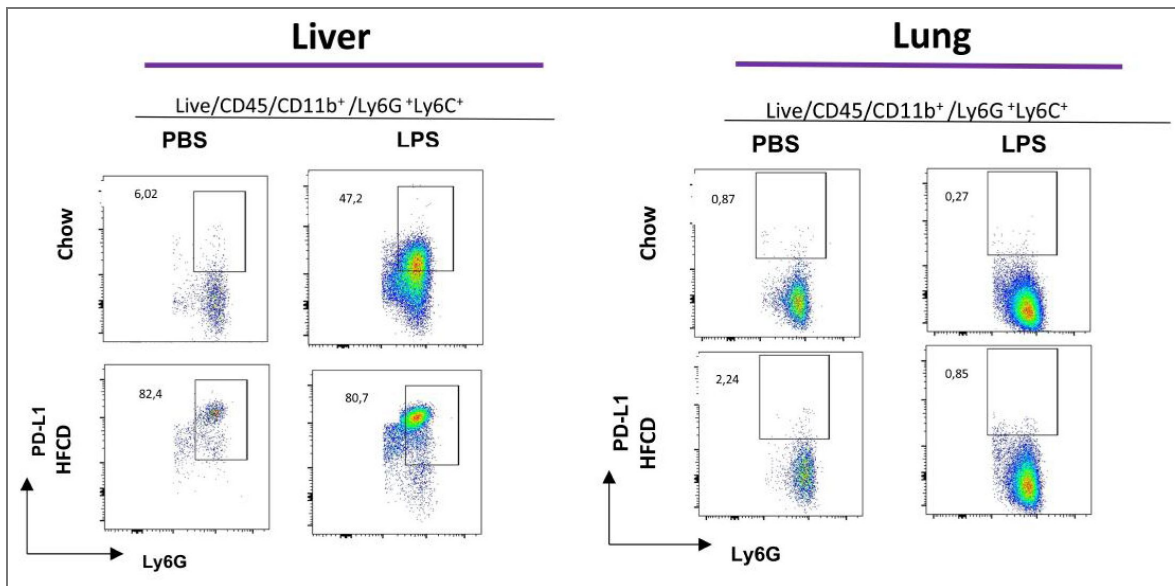
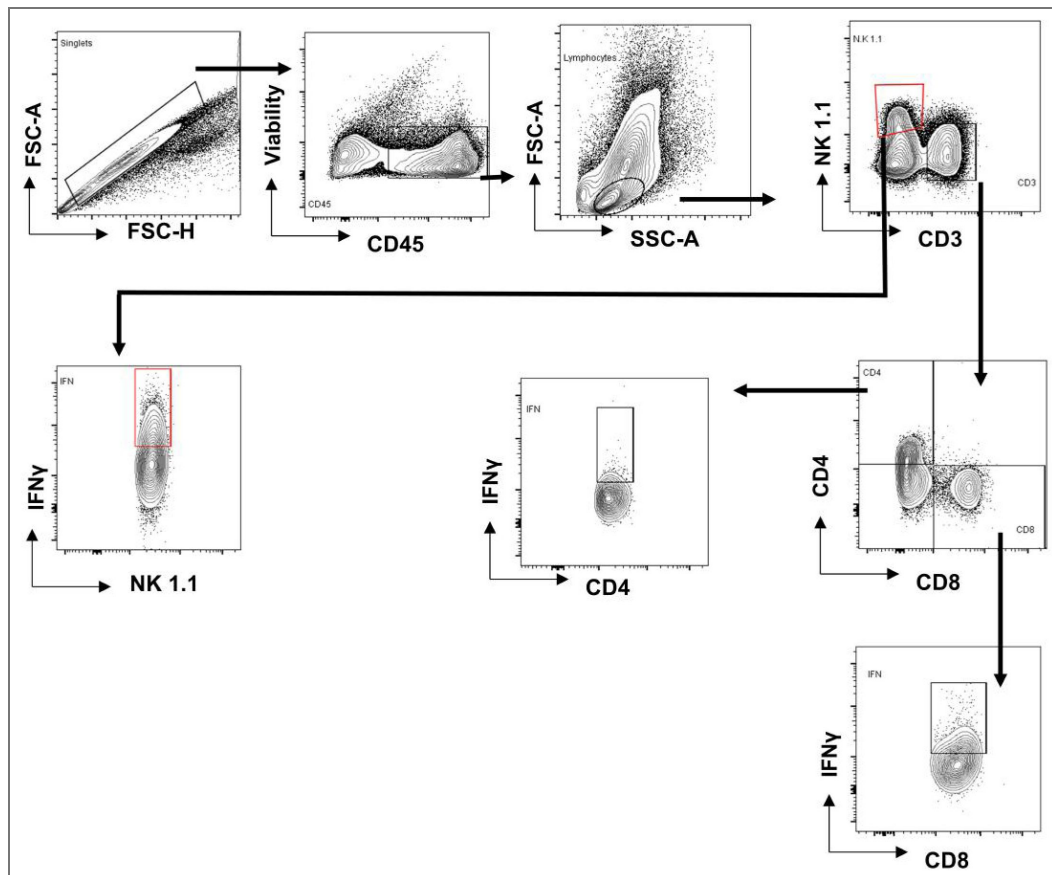


Figure S10. Gating strategy for identification of NK, CD4 and CD8 T cells



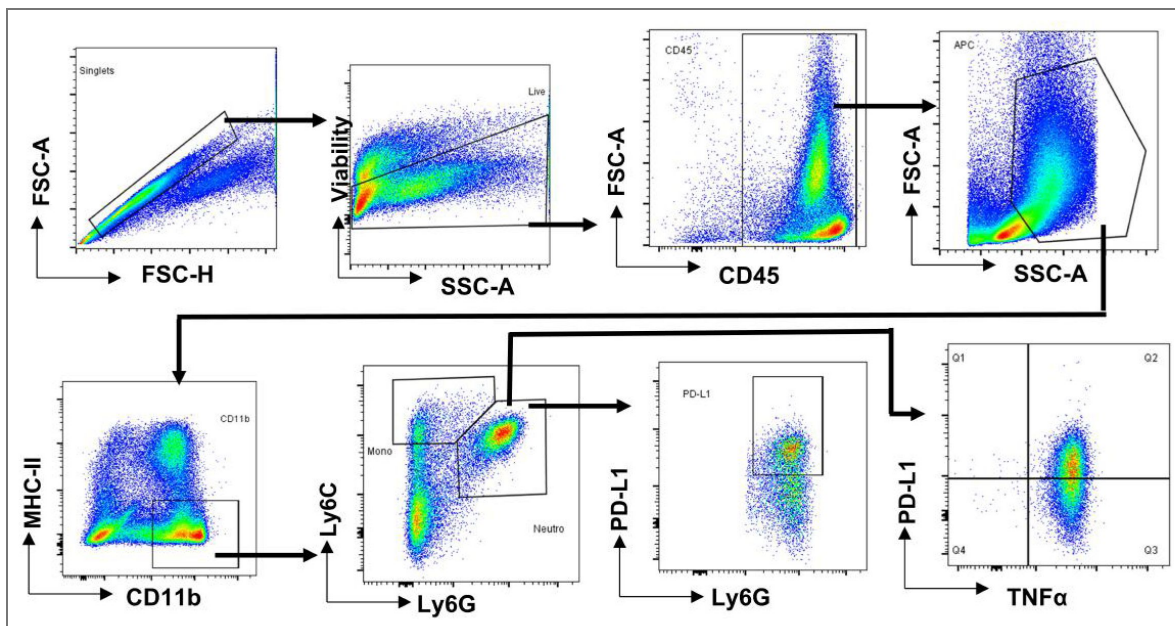


Figure S11. Gating strategy for identification of PD-L1+ neutrophils

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Additional information

Data availability

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Author contributions

CCOB, AK, and FQC designed the study. CCOB, AK, PSAA and GCMC performed the mouse experiments. CCOB, AK, and FQC wrote the manuscript. GVLS performed the bioinformatic analysis. CCOB, AK, LOL, TMC, JCFAF, and FQC reviewed the manuscript.

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Peer reviews

Reviewer #1 (Public review):

Summary:

By using an established NAFLD model, choline-deficient high-fat diet, Barros et al show that LPS challenge causes excessive IFN- γ production by hepatic NK cells which further induces recruitment and polarization of a PD-L1 positive neutrophil subset leading to massive TNF α production and increased host mortality. Genetic inhibition of IFN- γ or pharmacological blockade of PD-L1 decreases recruitment of these neutrophils and TNF α release, consequently preventing liver damage and decreasing host death.

Since NAFLD is often accompanied by chronic, low-grade inflammation, it can lead to an overactive but dysfunctional immune response and increase the body's overall susceptibility to infections, therefore this is very important research question.

Strengths:

The biggest strength of the manuscript is vast number of mouse strains used.

Weaknesses:

After the review, there are still some open questions from my side:

(1) I would like the authors to defend their choice of diet type since this has not been done in the review/response to authors. In case they cannot, we need additional proof (HFD or WD model).

(2) Since the authors used same control groups (chow and HFCD), as required by the animal ethics committee, they must have power analysis test to show that the number of controls (but also in other groups) they used is enough to see the effect. Please provide it.

<https://doi.org/10.7554/eLife.103843.2.sa2>

Reviewer #2 (Public review):

Summary:

This is an extremely interesting mouse study, trying to understand how sepsis is tolerated during obesity/NAFLD. The researchers combine a well-established model of NASH (Choline-deficiency with High Fat Diet) with a sepsis model (IP injection of 10mg/kg LPS), leading to dramatic mortality in mice. Using this model, they characterize the complex contributions of immune cells. Specifically, they find that NK-cells and Neutrophils contribute the most to mortality in this model due to IFNG and PD-L1+ Neutrophils.

Strengths:

The biggest strength of the manuscript is how clear the primary phenotypes/endpoints of their model are. Within 6 hours of LPS injection, there is a stark elevation of liver inflammation and damage, which is exacerbated by a High Fat/CholineDeficient diet (HFCD). And after 1 day, almost all of the mice die. Using these endpoints, the authors were able to identify which cells were critical for mortality in the model and the specific mediators involved.

Comments on revisions:

I have no further comments.

<https://doi.org/10.7554/eLife.103843.2.sa1>

Author response:

The following is the authors' response to the original reviews.

We thank the editor and reviewers for their constructive questions, valuable feedback, and for approving our manuscript. We truly appreciate the opportunity to improve our work based on their insightful comments. Before addressing the editor's and each referee's remarks individually, we provide below a point-by-point response summarizing the revisions made.

| *Duplication of control groups across experiments*

We appreciate the reviewers' concern regarding the potential duplication of control groups. In the revised manuscript, we have explicitly clarified that independent groups of control mice were used for each experiment. These details are now clearly indicated in the Materials and Methods section to avoid any ambiguity and to reinforce the rigor of our experimental design (Page 15, Line 453-455): "Furthermore, knockout animals and those treated with pharmacological inhibitors or neutralizing antibodies shared the same control groups (chow and HFCD), as required by the animal ethics committee."

Validation of the MASLD model

To strengthen the metabolic characterization of our MASLD model, we have now included additional parameters, including liver weight, Picrosirius staining and blood glucose measurements. These data are presented as new graphs in the revised manuscript and support the metabolic relevance of the HFCD diet model (Figure Supplementary S1). The corresponding description has been added to the Results section (Page 5, Lines 116–117) as follows: “Mice fed HFCD showed no increase in liver weight and collagen deposition as evidenced by Picrosirius staining (Fig. S1A and Fig. S1C)”

Assessment of liver injury in RagKO and anti-NK1.1 mice

We fully agree that assessment of liver injury is essential for these models. For mice treated with antiNK1.1, ALT levels are shown in Figure 4G, confirming increased liver injury after treatment. Regarding Rag^{-/-} mice, the animals exhibit exacerbation of liver injury when fed a HFCD diet and challenged with LPS (Page 7, Lines 183–184). The corresponding description has been added to the Results section (Page 7, Lines 175–176) as follows: “Interestingly, Rag1-deficient animals under the HFCD remained susceptible to the LPS challenge (Fig. 4C) with exacerbation of liver injury (Fig. 4D) ”

Discussion of limitations

We have expanded the Discussion section to provide a more comprehensive and balanced perspective on the limitations of our model and experimental approach (Page 13–14, Lines 401–414) “Our study presents several limitations that should be acknowledged and discussed. First, we cannot entirely rule out the possibility that our mice deficient in pro-inflammatory components exhibit reduced responsiveness to LPS. However, our ex vivo analyses using splenocytes from these animals revealed a preserved cytokine production following LPS stimulation. These results suggest that the in vivo differences observed are primarily driven by the MAFLD condition rather than by intrinsic defects in LPS sensitivity. Second, the absence of publicly available single-cell RNA-seq datasets from MAFLD subjects under endotoxemic or septic conditions limited our ability to perform direct translational comparisons. To overcome this, we analyzed existing MAFLD patients and experimental MAFLD datasets, which consistently demonstrated upregulation of IFN- γ and TNF- α inflammatory pathways in MAFLD. In line with these findings, our murine model revealed TNF- α ⁺ myeloid and IFN- γ ⁺ NK cell populations, thereby reinforcing the validity and translational relevance of our results.”. This revision highlights the constraints of the MASLD model, the inherent variability among in vivo experiments, and the interpretative limitations related to immunodeficient mouse strains.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

(1) In Figure 4 the authors are showing the number of IFN γ positive CD4, CD8, and NK 1.1⁺ cells. Could they show from total IFN γ production, how much it goes specifically on NK cells and how much on other cell populations since NK1.1 is NK but also NKT and gamma delta T cell marker? Also, in Figure 2E the authors see a substantial increase in IFN γ signal in T cells.

While we did not specifically assess IFN γ production in NKT cells or other minor populations, our data indicate that the NK1.1⁺CD3⁺ cells (NKT cells) cited in Page 7, Lines 188–192 were essentially absent in the liver tissue of LPS-challenged animals, as shown in Supplementary Figures 3C and S10. The corresponding description has been added to the Results section (Page 7, Lines 188–192) as follows: “We observed that the number of NK cells increased in the liver tissue of PBS-treated MAFLD mice compared with mice fed a control diet (Fig. 4E). LPS

challenge increased the accumulation of NK1.1+CD3⁻ NK cells in the liver tissue of MAFLD mice and the absence of NK1.1+CD3⁺ NKT cells (Fig. S3C and 4E)”.

This absence was consistent across all experimental conditions, corroborating our focus on NK1.1+CD3⁻ cells as the primary source of IFN γ production. Furthermore, data demonstrated in Figure 2E illustrate the presence of IFN γ primarily in NK cells. Therefore, the observed IFN γ signal, attributed to NK1.1⁺ cells, predominantly reflects conventional NK cells, with minimal contribution from NKT or $\gamma\delta$ T cells.

(2) In Figure 4C, the authors state that the results suggest that T and B cells do not contribute to susceptibility to LPS challenge. However, they observe a drop in survival compared to chow+LPS. Are the authors certain there is no statistical significance there?

The observed decrease in survival is consistent with our expectations, as T and B cells are not the primary source of interferon-gamma (IFN γ) in this context. Even in their absence, animals remain susceptible to LPS challenge due to the presence of other IFN γ -producing cells that drive the observed lethality. We have carefully re-examined the statistical analysis and confirm that it was correctly performed.

(3) Since the survival curve and rate are exactly the same (60%) in Figures 3F, 3G, 4C, 4F, 5G, and 5H I would just like to double-check that the authors used different controls for each experiment.

The number of mice used in each experiment was carefully determined to ensure sufficient statistical power while fully complying with the limits established by our institutional Animal Ethics Committee. To minimize animal use, the same control group was shared across multiple survival experiments. Despite using shared controls, the total number of animals per experimental group was adequate to produce robust and reproducible survival outcomes. All groups were properly randomized, and the shared control data were rigorously incorporated into statistical analyses. This strategy allowed us to maintain both ethical standards and the scientific rigor of our findings.

(4) In Figure 5 the authors are saying that it is neutrophils but not monocytes mediate susceptibility of animals with NAFLD to endotoxemia. However, CXCR2i depletion and CCR2 knock out mice affect both monocytes/macrophages and neutrophils. And in Figures 5E, 5G, and 5H they see that a) LPS+CXCR2i decreases liver damage more than LPS+anti Ly6G, b) HFCD mice challenged with LPS and treated with anti-LY6G do not rescue survival to levels of CHOW LPS and c) anti Ly6G treatment helps less than CXCR2i. Therefore, from both knock out mice and depletion experiments the authors can conclude that most likely monocytes (but potentially also other cells) together with neutrophils are substantial for the development of endotoxemic shock in choline-deficient high-fat diet model.

While neutrophils express CCR2, our data clearly show that CCR2 deficiency does not impair neutrophil migration, as demonstrated in Supplemental Figures 5A and 5B (added to the manuscript, page 8, lines 213–217). The corresponding description has been added to the Results section (Page 8, Lines 213217) as follows: Interestingly, animals deficient in monocyte migration (CCR2^{-/-}) showed a high mortality rate compared to wild type after LPS challenge and neutrophil migration is not altered (Fig. 5SA and Fig. 5SB). In contrast, CCR2 deficiency primarily affects monocyte recruitment, yet in our experimental conditions, monocyte depletion or CCR2 knockout did not significantly alter the severity of endotoxemic shock, indicating that monocytes play a minimal role in mediating susceptibility in HFCD-fed mice.

To specifically investigate neutrophils, we used pharmacological blockade of CXCR2 to inhibit migration and antibody-mediated neutrophil depletion. Both approaches have consistently demonstrated that neutrophils are critical factors in endotoxemic shock.

These findings support our conclusion that neutrophils are the primary cellular contributors to susceptibility in HFCD-fed mice during endotoxemia, with monocytes making a negligible contribution under the tested conditions.

(6) In Figure 6A (but also others with PD-L1) did the authors do isotype control? And can they show how much of PD1+ population goes on neutrophils, and how much on all the other populations?

To address this issue, we performed additional analyses to assess the distribution of PD-L1 expression on CD45+CD11B+ leukocytes. These new results, detailed on Page 9, lines 245-250, and now presented in Supplemental Figure 6, demonstrate that PD-L1 expression is predominantly enriched in neutrophils compared to other immune subsets. This observation further reinforces our conclusion that neutrophils represent a major source of PD-L1 in our experimental model.

To ensure the robustness of these findings, we also included FMO controls for PD-L1 staining in the newly added Supplemental Figure S6. These controls validate the specificity of our gating strategy and confirm the reliability of the detected PD-L1 signal. The corresponding description has been added to the Results section (Page 9, Lines 245-250) as follows: First, we observed that only the MAFLD diet caused a significant increase in PD-L1 expression in CD45+CD11b+ leukocytes after LPS challenge (Fig. S6C). We observed that within this population, neutrophils predominate in their expression when compared to monocytes (Fig. 6SA, Fig. 6SB, and Fig. 6SD). Furthermore, PD-L1+ neutrophils showed an exacerbated migration of PD-L1+ neutrophils towards the liver (Fig. 6A and 6B)”

(7) In Figure 6D it is interesting that there is not an increase in PD-L1+ neutrophils in LPS HFCD IFNg+/+ mice in comparison to LPS chow IFNg+/+ mice, since those should be like WT mice (Figure 6A going from 50% to 97%) and so an increase should be seen?

The apparent difference between Figures 6A and 6D likely reflects inter-experimental variability rather than a biological discrepancy. Although the absolute percentages of PD-L1+ neutrophils varied slightly among independent experiments, the overall phenotype and trend were consistently maintained namely, that PD-L1 expression on neutrophils is enhanced in response to LPS stimulation and modulated by IFN γ signaling. Thus, the data shown in Figure 6D are representative of this consistent phenotype despite minor quantitative variation.

(8) In Figure 7 do the authors have isotype control for TNFa because gating seems a bit random so an isotype control graph would help a lot as supplementary information, in order to make the figure more persuasive

To address the concern regarding gating in Figure 7, we have included the FMO showing TNF α as a histogram Supplemental Figure 8gG. These control reaffirm the accuracy and reliability of our gating strategy for TNF α , further supporting the robustness of our data. The corresponding description has been added to the Results section (Page 9, Lines 272-274) as follows: We observed an exacerbated TNF- α expression by PD-L1+ neutrophils from MAFLD when compared to control chow animals (Fig. 7A, Fig. 7B, Fig. 7D, and Fig8SG).

(9) Figure 6C IFNg+/+ mice on CHOW +LPS is same as Figure 8E mice chow +LPS but just with different numbers. Can the authors explain this?

Although the data points in Figures 6C and 8E may appear similar, we confirm that they originate from entirely independent experiments and represent distinct datasets. To enhance clarity and avoid any potential confusion, we have adjusted the figure presentation and sizing in the revised manuscript. These changes make it clear that the datasets, while comparable, are derived from separate experimental replicates.

(10) Figure 1E chow B6+LPS is the same as Figure 5D B6+LPS but should they be different since those should be two different experiments?

We confirm that Figures 1E and 5D correspond to data obtained from independent experiments. Although the experimental conditions were similar, each dataset was generated and analyzed separately to ensure the reproducibility and robustness of our results.

Reviewer #2 (Recommendations for the authors):

(1) Why did you look at kidney injury in Figure 1D? I think this should be explained a little.

We assessed kidney injury alongside ALT, a marker of liver damage, because both the liver and kidneys are among the primary organs affected during sepsis and endotoxemia. This rationale has been added to the manuscript (page 5, lines 129–131): “Remarkably, compared to the Chow group, HFCD mice exposed to LPS did not show greater changes in other organs commonly affected by endotoxemia, such as the kidneys (Figure 1D).” By evaluating markers of injury in both organs, we aimed to determine whether our physiopathological condition was liver-specific or indicative of broader systemic injury.

(2) I know Figure 2C isn't your data, but why are there so few NK cells, considering NK cells are a resident liver cell type? Doesn't that also bring into question some of your data if there are so few NK cells? And the IFN γ expression (2E) looks to mostly come from T-cells (CD8?).

The data shown in Figure 2C were reanalyzed from a separate NAFLD model based on a 60% high-fat diet. Although this model differs from ours, the observed low number of NK cells is consistent with expectations for animals subjected solely to a hyperlipidic diet, which primarily provides an inflammatory stimulus that promotes recruitment rather than maintaining high baseline NK cell numbers.

In our experimental model, these observations align with published data. Specifically, liver tissue from NAFLD animals typically exhibits low baseline NK cell numbers, but upon LPS challenge, there is a marked increase in NK cell recruitment to the liver. This dynamic illustrates the interplay between diet-induced inflammation and immune cell recruitment in our experimental context and supports the interpretation of our IFN γ data.

(3) In your methods, I think you didn't explain something. You said LPS was administered to 56 week old mice, but that HFCD diet was started in 5-6 week old mice and lasted 2 weeks, then LPS was administered. So LPS administration happened when the mice were 7-8 weeks old, right?

We thank the reviewer for pointing out this inconsistency in our Methods section. The reviewer is correct: the HFCD diet was initiated in 5–6-week-old mice, and LPS was administered after 2 weeks on the diet, such that LPS challenge occurred when the mice were 7–8 weeks old.

We have revised the Methods section (add page 15-16, lines 474–480). to clarify this timeline and ensure it is accurately described in the manuscript. The corresponding description has been added to the Materials and Methods section (Page 14, Lines 436-442) as follows: “Lipopolysaccharide (LPS; Escherichia coli (O111:B4), L2630, Sigma-Aldrich, St. Louis, MO, USA) was administered intraperitoneally (i.p.; 10 mg/kg) in C57BL/6, CCR2 $^{-/-}$, IFN $^{-/-}$, and TNFR1R2 $^{-/-}$ mice. The HFCD was initiated in 5–6 week-old mice, and LPS was administered after 2 weeks on the diet, meaning that LPS administration occurred when the mice were 7–8 weeks old, with body weights ranging from 22 to 26 g. LPS was previously solubilized in sterile saline and frozen at -70°C . The animals were euthanized 6 hours after LPS administration”.

(4) Throughout the manuscript, I would consider changing the term NAFLD to something else. I think HFCD diet is a closer model to NASH, so there needs to be some discussion on that. And the field is changing these terms, so NAFLD is now MASLD and NASH is now MASH.

We appreciate the reviewer's comment regarding the terminology and disease classification. In our experimental conditions, the animals were subjected to a high-fat, choline-deficient (HFCD) diet for only two weeks, a period considered very early in the progression of diet-induced liver disease. At this stage, histological analysis revealed lipid accumulation in hepatocytes without evidence of hepatocellular injury, inflammation, or fibrosis. Therefore, our model more closely resembles the metabolic-associated fatty liver disease (MAFLD, formerly NAFLD) stage rather than the more advanced metabolic-associated steatohepatitis (MASH, formerly NASH).

Indeed, prolonged exposure to HFCD diets, typically 8 to 16 weeks, is required to induce the inflammatory and fibrotic features characteristic of MASH. Since our objective was to study the initial metabolic and immune alterations preceding overt liver injury, we believe that using the term MAFLD more accurately reflects the pathological stage represented in our model. Accordingly, we have revised the text to align with the updated nomenclature and disease context.

(6) I am concerned about over interpretation of the publicly available RNA-seq data in Figure 2. This data comes from human NAFLD patients with unknown endotoxemia and mouse models using a traditional high-fat diet model. So it is hard to compare these very disparate datasets to yours. Also, if these datasets have elevated IFNG, why does your model require LPS injection?

We thank the reviewer for their thoughtful comments regarding the interpretation of the RNA-seq data presented in Figure 2. We would like to clarify that the human NAFLD datasets referenced in our study do not specifically include patients with endotoxemia; rather, they focus on individuals with NAFLD alone.

Comparing data from human and murine MAFLD models, we observed that NK cells, T cells, and neutrophils are present and contribute to the hepatic inflammatory environment. Our reanalysis indicates that the elevations of IFN γ and TNF in NAFLD are primarily derived from NK cells, T cells, and myeloid cells, respectively.

In our experimental model, LPS administration was used to evaluate whether these immune populations particularly NK cells are further potentiated under a hyperinflammatory state, leading to exacerbated IFN γ production. This approach allows us to determine whether increased IFN γ contributes to worsening outcomes in NAFLD, providing mechanistic insights that cannot be obtained from static human or traditional mouse datasets alone.

(7) The zoom-ins for the histology (for example, Figure 1E) don't look right compared to the dotted square. The shape and area expanded don't match. And the cells in the zoom-in don't look exactly the same either.

We have thoroughly re-examined the histological sections and the corresponding zoom-ins, including the example in Figure 1E. Upon verification, we confirm that the zoom-ins accurately represent the highlighted areas indicated by the dotted squares. The apparent discrepancies in shape or cellular appearance are likely due to minor differences in orientation or cropping during figure preparation. Nevertheless, the content and regions depicted are consistent with the original sections.

(8) Did the authors measure myeloid infiltration in the CCR2^{-/-} mice? Did you measure Neutrophil infiltration in the TNF-Receptor KO mice?

Analysis of CD45+ cell migration in CCR2 knockout mice, as shown in Supplemental Figure 5C and 5D, demonstrates that the absence of CCR2 does not impair overall leukocyte migration. Similarly, assessment of neutrophil migration in TNF receptor (TNFR1/2) knockout mice, presented in Supplemental Figure 8A, shows that neutrophil trafficking is not affected in these animals. These results indicate that the respective knockouts do not compromise the migration of the analyzed immune populations, supporting the interpretations presented in our study.

(9) Regarding Methods for RNA-seq Analysis. Was the Mitochondrial percentage cutoff 0.8%, because that seems low. And was there not a Padj or FDR cutoff for the differential expression?

The mitochondrial percentage in our scRNA-seq analysis reflects the proportion of mitochondrial gene expression per cell, which serves as a quality control metric. A low mitochondrial gene expression percentage, such as the 0.8% cutoff used here, is indicative of highly viable cells.

For differential gene expression analysis, we employed the FindMarkers function in Seurat with standard parameters: adjusted p-value (Padj) < 0.05 and log2 fold change > 0.25 for upregulated genes, and adjusted p-value < 0.05 with log2 fold change < -0.25 for downregulated genes. These thresholds ensure robust identification of differentially expressed genes while balancing sensitivity and specificity.

(10) Regarding Methods for Flow Cytometry. How were IFNG and TNF staining performed? Was this an intracellular stain? Did you need to block secretion? TNF and IFNG antibodies have the same fluorophore (PE), so were these stainings and analyses performed separately?

Six hours after LPS challenge, non-parenchymal liver cells were isolated using Percoll gradient centrifugation. Because the animals were in a hyperinflammatory state induced by LPS, no in vitro stimulation was performed; all staining was carried out immediately after cell isolation. Detection of IFN γ and TNF was performed via intracellular staining using the Foxp3 staining kit (eBioscience). Due to both antibodies being conjugated to PE, IFN- γ and TNF- α staining and analyses were conducted in separate experiments. These distinct staining protocols and analyses are detailed in Supplemental Figures 10 and 11. The corresponding description has been added to the Materials and Methods section (Page 16, Lines 490-493) as follows: As animals were already in a hyperinflammatory state, no additional in vitro stimulation was required. Intracellular detection of IFN- γ and TNF- α was conducted using the Foxp3 staining kit (eBioscience). Since both antibodies were conjugated to PE, staining and analyses were performed in separate experiments`

Reviewer #3 (Recommendations for the authors):

(1) Achieving an NAFLD model/disease is the starting point of this study. I understand that a two-week HFCD diet period was applied due to the decrease in lymphocyte numbers. Was it enough to initiate NAFLD then? Or is it a milder metabolic disease? Which parameters have been evaluated to accept this model as a NAFLD model?

Indeed, the two-week HFCD diet induces an early-stage form of NAFLD, characterized by initial fat accumulation in the liver without significant hepatic injury. While this represents a milder metabolic phenotype, it is sufficient to study the inflammatory and immune responses associated with NAFLD. To validate this model, we assessed multiple parameters: liver weight, blood glucose levels, and collagen deposition. These measurements confirmed the presence of early-stage NAFLD features in the animals, providing a relevant and reliable context for investigating susceptibility to endotoxemia and immune cell dynamics. They are shown in Figure Supplementary 1 and the text was included in the manuscript (Page 5, Lines

116-117): “Mice fed HFCD showed no increase in liver weight and collagen deposition as evidenced by Picrosirius staining (Fig. S1A and Fig. S1C) ”.

(2) It is true that the CD274 gene (encoding PD-L1) and the IFNGR2 gene, corresponding to the IFN γ receptor, are among the upregulated genes when authors analyzed the publicly available RNAseq data but they are not the most significantly elevated genes. What is the reasoning behind this cherry-picking? Why are other high DEGs not analyzed but these two are analyzed?

We highlighted the expression of the IFN- γ receptor (IFNGR2) and CD274 (encoding PD-L1) in the publicly available RNA-seq data to align and corroborate these findings with the key results observed later in our study. To avoid redundancy, we chose to present these genes in the initial figures as they are directly relevant to the subsequent analyses. Regarding the broader analysis of human RNA-seq data, our primary objective was to identify enriched biological processes and pathways, which served as a foundation for the focus and direction of this study.

(3) Figures 3C-3G: I understand that IFN γ -/- and TNFR1R2a-/- mice are not showing elevated liver damage but it may simply be because of the non-responsiveness to the LPS challenge. I suggest using a different challenge or recovery experiments with the cytokines to show that the challenge is successful and results are caused by NAFLD, truly. The same goes for Figure 6: Looking at Figure 6D one may think that IFN γ deficiency alters the LPS response independent of the diet condition (or NAFLD condition).

We appreciate the reviewer’s insightful comment and fully understand the concern regarding the potential non-responsiveness of IFN- γ ^{-/-} and TNFR1R2a^{-/-} mice to the LPS challenge. To address this point and confirm that these knockout animals are indeed responsive to LPS stimulation, we conducted an additional set of ex vivo experiments.

Specifically, WT and cytokine-deficient (IFN- γ ^{-/-}) mice were fed either Chow or HFCD for two weeks, after which spleens were collected, and splenocytes were challenged in vitro with LPS. We then quantified TNF, IFN, and IL-6 production to confirm that these mice are capable of mounting cytokine responses upon LPS stimulation.

Due to current breeding limitations and a temporary issue in colony maintenance of TNF-deficient mice, we were unable to include TNFR1R2a^{-/-} animals in this additional experiment. Nevertheless, we prioritized performing the analysis with the available knockout line to avoid leaving this important point unaddressed.

These additional data demonstrate that IFN- γ -deficient mice remain responsive to LPS, reinforcing that the differences observed in vivo are related to the NAFLD condition rather than a lack of LPS responsiveness.

(4) Figure 1 vs Figure 4: Rag-/- mice seem more susceptible to LPS-derived death even after normal conditions. But If I compare the survival data between Figure 1 and Figure 4, Rag-/- HFCD diet mice seem to be doing better than wt mice after LPS treatment. (1 day survival vs 2 days survival). How do you explain these different outcomes?

We thank the reviewer for this insightful question regarding the survival data in Figures 1 and 4. Although there is a one-day difference in survival outcomes, Rag-/- mice consistently exhibit increased susceptibility to LPS-induced mortality can influence the exact survival timing. Nonetheless, across all experiments, Rag-/- mice display a reproducible phenotype of heightened sensitivity to LPS challenge, which is supported by multiple independent observations in our study.

(5) How do you explain Figure 4J in connection to the observation presented with Figure 7: TNFa tissue levels, even though significant, seem very similar between the conditions?

We would like to clarify that the animals in this study are in a metabolic syndrome state, with early-stage NAFLD characterized by hepatic fat accumulation without significant tissue injury, as shown in Figure 1C.

Under these conditions, the LPS challenge triggers an exacerbated inflammatory response, leading to increased secretion of IFN- γ and TNF- α , primarily from NK cells and neutrophils. While TNF α levels may appear visually similar across conditions, the HFCD mice exhibit a heightened predisposition for an amplified immune response compared to chow-fed mice. This difference is consistent with the functional outcomes observed in our study and highlights the diet-specific sensitization of the immune system.

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