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## A medium-chain fatty acid analogue prevents endotoxin liver injury in a murine model

Sarah Z. Wang<sup>1,2,4</sup>✉, Thomas I. Hirsch<sup>1,2</sup>, Scott C. Fligor<sup>1,2</sup>, Savas T. Tsikis<sup>1,2</sup>, Amy Pan<sup>1,2</sup>, Mikayla Quigley<sup>1,2</sup>, Paul D. Mitchell<sup>1,2</sup>, Kathleen M. Gura<sup>1,2</sup>, David A. Fraser<sup>3</sup> & Mark Puder<sup>1,2,4</sup>✉

Parenteral nutrition (PN) is lifesaving for patients with short bowel syndrome and other gastrointestinal disorders, however long-term use may lead to complications including hepatosteatosis and sepsis. We have previously demonstrated the anti-steatotic, -fibrotic, and -inflammatory properties of SEFA-6179, an engineered medium-chain fatty acid analogue. We hypothesized that SEFA-6179 treatment would protect against endotoxin-induced liver injury in a murine model of PN-induced hepatosteatosis. C57Bl/6J mice were administered a high-carbohydrate liquid diet plus intravenous lipid emulsion (Intralipid, 4 g fat/kg/d) or intravenous saline for 19 days to induce hepatosteatosis. SEFA-6179 (100 mg/kg) or vehicle (MCT/medium-chain triglyceride) was administered via oral gavage for four days leading up to intraperitoneal challenge with lipopolysaccharide (15 mg/kg) or saline on day 19. Age-matched, chow-fed controls received the same treatments. The primary outcome was liver biomarkers: alanine aminotransferase and aspartate aminotransferase. Pro-inflammatory cytokines, IL-6, TNF-alpha, and monocyte chemoattractant protein (MCP1), were analyzed. Liver immunofluorescence staining was performed to evaluate macrophage phenotypes. In endotoxin-challenged mice, pre-treatment with SEFA-6179 lowered liver enzymes and pro-inflammatory cytokine levels compared to vehicle. On liver histology, SEFA-6179 pre-treatment led to greater polarization of M1/pro-inflammatory macrophages to an M2/anti-inflammatory phenotype compared to vehicle. SEFA-6179 is currently in Phase II clinical trials. These findings support the potential application of SEFA-6179 in high-risk, PN-dependent patients.

**Keywords** Parenteral nutrition, Liver steatosis, Inflammation, Fatty acid

### Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BSA	Bovine serum albumin
CCL2	Chemokine ligand 2
CLABSI	Central line-associated bloodstream infections
ELISA	Enzyme-linked immunosorbent assay
IFALD	Intestinal failure-associated liver disease
LPS	Lipopolysaccharide
MCFA	Medium-chain fatty acid
MCP1	Monocyte chemoattractant protein 1
MCT	Medium-chain triglyceride
PPAR	Peroxisome proliferator-activated receptor
PN	Parenteral nutrition
PNALD	Parenteral nutrition-associated liver disease
RCF	Relative centrifugal force
SEFA	Structurally-engineered fatty acid
TBS	Tris-buffered saline

<sup>1</sup>Boston Children's Hospital, Boston, MA, USA. <sup>2</sup>Harvard Medical School, Boston, MA, USA. <sup>3</sup>NorthSea Therapeutics, Amsterdam, The Netherlands. <sup>4</sup>Department of Surgery and Vascular Biology Program, Boston Children's Hospital, 300 Longwood Ave, Boston, MA 02115, USA. ✉email: Sarah.Wang@childrens.harvard.edu; Mark.Puder@childrens.harvard.edu

TBST Tris-buffered Saline + Tween20

Short bowel syndrome (SBS) is a malabsorptive state that results from insufficient bowel length and can occur secondary to acquired or congenital conditions. Four million people live with SBS globally, with the number of SBS-related hospitalizations increasing by 55% between 2005 and 2014 in the United States<sup>1,2</sup>. Patients with SBS are unable to absorb adequate nutrition through their gastrointestinal tract and may require intravenous i.e. parenteral nutrition (PN). While PN is lifesaving for SBS patients, long-term use is associated with significant complications including intestinal failure-associated liver disease (IFALD) and central line-associated bloodstream infections (CLABSI). IFALD pathophysiology is multifactorial, involving nutrient imbalance-related steatosis, disruption of the gut-liver axis, and inflammation from a variety of causes including endotoxin-driven sepsis<sup>3</sup>. Limited treatment options exist for patients with IFALD. In patients with IFALD, switching from soybean oil-based to fish oil-based lipid emulsion improved histologic liver injury, improved survival, and reduced rates of organ transplantation<sup>4</sup>. Sepsis is an additional modifiable risk factor associated with liver disease and death in some PN-dependent patients; new treatments that protect the liver can be lifesaving<sup>5</sup>.

Among patients with malabsorptive syndromes, medium-chain fatty acids (MCFAs) are commonly used in nutritional supplementation and intestinal rehabilitation<sup>6</sup>. Unlike long-chain fatty acids which must first be packaged into chylomicrons and transported via the lymphatic system, MCFAs are quickly and passively absorbed from the gastrointestinal tract directly into portal circulation, enabling liver targeting<sup>7</sup>. MCFAs are bioactive molecules that play a role in numerous physiologic mechanisms related to inflammation, fibrogenesis, and metabolism<sup>8–10</sup>. Interestingly, the ten-carbon MCFA decanoic acid has previously been shown to bind peroxisome proliferator-activated receptor (PPAR)-gamma without inducing adipogenesis<sup>11</sup>. However, the therapeutic potential of MCFAs in vivo are likely limited by their rapid utilization as an energy source.

Structurally-engineered fatty acid (SEFA)-6179 is an engineered MCFA analogue that, as for unmodified MCFAs, acts on a variety of receptors in vitro, including PPAR-alpha and -gamma which help to downregulate inflammatory signaling<sup>12</sup>. However, while structurally similar to decanoic acid, SEFA-6179 has moieties that confer resistance to beta-oxidation, with the intention of maximizing availability of the compound for receptor engagement<sup>13</sup>. Our lab has previously demonstrated the anti-fibrotic, anti-steatotic, and anti-inflammatory properties of SEFA-6179 in a preterm piglet model of intestinal failure and murine model of PN-induced hepatosteatosis<sup>13,14</sup>. These animal models resulted in a type of liver injury similar to that of patients with IFALD<sup>15</sup>.

PN-dependent patients universally have indwelling foreign bodies as a means of central venous access for the provision of PN. These patients are not only at risk of developing hepatosteatosis from long-term PN administration, but are additionally susceptible to line-related bacteremia. Murine models of sepsis have long employed the use of intraperitoneally-delivered lipopolysaccharide (LPS), a Gram-negative bacterial cell wall component that drives pro-inflammatory signaling, leading to organ failure and sepsis<sup>16</sup>. Since PN dependence places patients at risk of developing both liver disease and inflammation, we aimed to investigate the potential therapeutic effects of SEFA-6179 in an animal model that encapsulates both of these elements. Recurrent sepsis contributes to the pathophysiology of IFALD<sup>17,18</sup>. We sought to investigate whether SEFA-6179 would be protective against liver injury in a murine model of PN-associated hepatosteatosis and endotoxin-driven inflammation.

## Results

### Body and organ weights

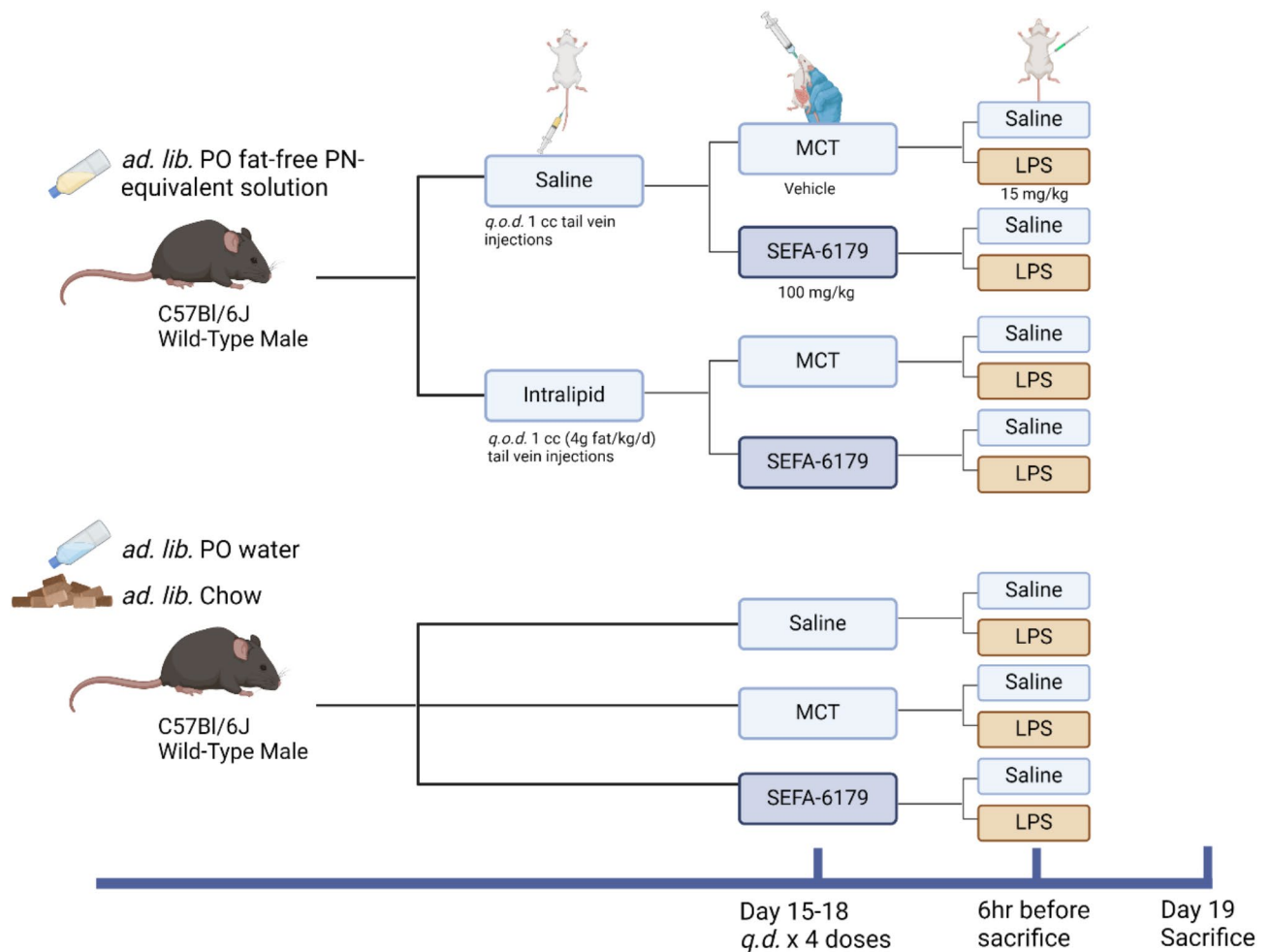
In a murine model of PN-associated hepatosteatosis and endotoxin-induced inflammation (Fig. 1), there were no inter-group differences in overall body weight, amount of PN consumption, or normalized organ weights (liver, spleen, and kidney) (Fig. 2). PN-dependent mice experienced an initial period of mild weight loss ( $1.7 \pm 0.1$ ) prior to steady weight gain until the onset of oral gavage treatments with drug or vehicle on day 15, when PN consumption and weight ( $2.6 \pm 0.04$ ) decreased, followed by recovery to near-baseline weight at euthanasia on day 19. Chow-fed control mice experienced gradual weight gain over time ( $3.2 \pm 0.2$ ) without an initial weight loss period.

### Liver histology

PN-dependent mice developed hepatosteatosis compared to chow-fed controls, as evidenced by macrovascular steatosis on hematoxylin and eosin (H&E) stain and excessive intracellular fat deposition on Oil Red O stain on representative liver histology (Fig. 3). SEFA-6179 treatment led to the reduction of liver steatosis compared to MCT in PN-sustained groups. Chow-fed mice did not develop steatosis in either the MCT or SEFA-6179-treated groups.

### Liver enzymes and pro-inflammatory cytokines

LPS challenge increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations compared to saline controls in all groups, including mice provided with PN solution (ALT  $167.3 \pm 14.6$  vs  $62.3 \pm 7.3$  U/L,  $P=0.001$ ; AST  $233.8 \pm 25.7$  vs  $130.5 \pm 12.9$  U/L,  $P=0.0007$ ) and chow-fed cohorts (ALT  $158.8 \pm 3.0$  vs  $22.8 \pm 5.9$  U/L,  $P<0.0001$ ; AST  $120.8 \pm 8.0$  vs  $44.2 \pm 6.2$  U/L,  $P=0.007$ ) (Fig. 4). In PN-sustained animals challenged with endotoxin, pre-treatment with SEFA-6179 normalized ALT ( $42.5 \pm 16.2$  vs  $160.0 \pm 12.0$  U/L,  $P=0.001$ ) and AST ( $56.3 \pm 10.1$  vs  $159.3 \pm 7.8$  U/L,  $P=0.0002$ ) compared to vehicle-treated controls. Alkaline phosphatase and gamma-glutamyl transferase levels were additionally assessed, however there were no statistically significant differences in these values between MCT and SEFA-6179 treated groups in the present study. SEFA-6179 pretreatment normalized serum levels of pro-inflammatory cytokines IL-6 (interleukin-6) and TNF-alpha (tumor necrosis factor-alpha) (Fig. 5). In endotoxin-challenged chow-fed mice, IL-6 was lower in SEFA-6179-pretreated animals ( $95.7 \pm 3.2$  pg/mL) compared to MCT ( $775.0 \pm 103.7$  pg/mL,  $P=0.003$ ) and

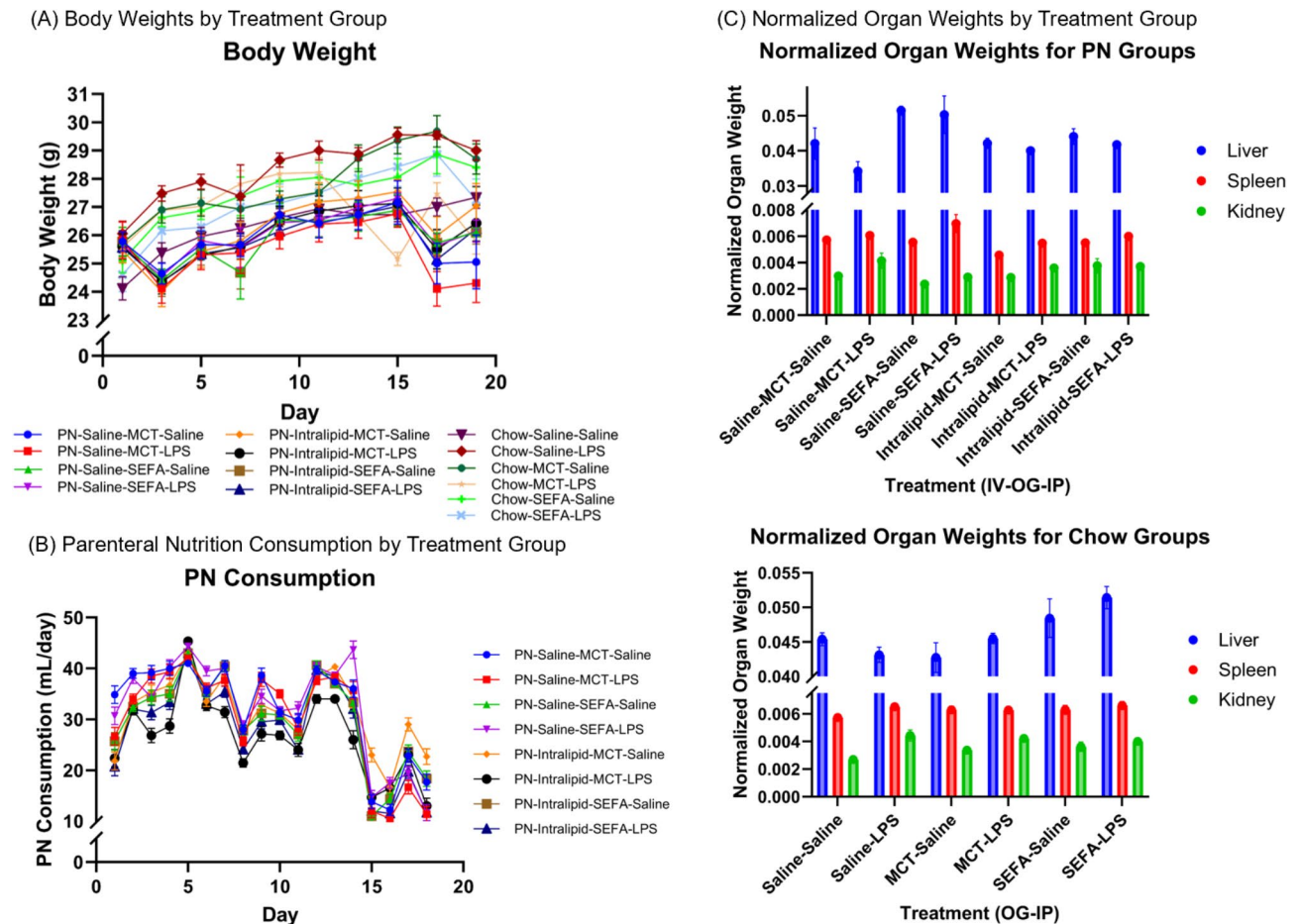


**Fig. 1.** Experimental design investigating an engineered medium-chain fatty acid analogue, SEFA-6179, in a murine model of PN-associated liver disease and endotoxin challenge. Mice were randomized to parenteral nutrition-equivalent intake or standard rodent chow. Mice received various intravenous (saline or soybean oil-based lipid emulsion i.e. Intralipid), oral gavage (MCT or SEFA-6179), and intraperitoneal (saline or LPS) treatments. A total of  $n = 70$  C57Bl/6J mice were utilized ( $n = 5$  per group, total of 14 groups). MCT, medium-chain triglyceride; SEFA-6179, structurally engineered fatty acid-6179; LPS, lipopolysaccharide (*E. coli*).

saline ( $1627 \pm 175$  pg/mL,  $P = 0.0009$ ). TNF- $\alpha$  levels were lower in the SEFA-6179-treated group compared to vehicle-treated controls ( $2556 \pm 65$  vs  $3375 \pm 57$  pg/mL,  $P < 0.0001$ ) among LPS-challenged mice with PN-induced hepatosteatosis. MCP1 (monocyte chemoattractant protein 1) concentration was greater in LPS-exposed animals compared to saline controls ( $5209 \pm 568$  vs  $34.33 \pm 7.89$  pg/mL,  $P < 0.001$ ). Trends in MCP1 generally followed that of IL-6. The mean MCP1 levels trended lower for SEFA-6179-pretreated mice compared to MCT-pretreated mice in the PN + saline ( $3954 \pm 11742$  vs  $7665 \pm 2191$  pg/mL,  $P = 0.210$ ), PN + Intralipid ( $3138 \pm 300$  vs  $4016 \pm 595$  pg/mL,  $P = 0.236$ ), and chow ( $2388 \pm 342$  vs  $3417 \pm 1056$  pg/mL,  $P = 0.381$ ) groups in the setting of LPS exposure, although this did not reach statistical significance. In chow-fed, LPS-exposed animals, SEFA-6179-treated mice had lower MCP1 levels compared to saline-treated mice ( $2388 \pm 342$  vs  $5209 \pm 568$  pg/mL,  $P = 0.003$ ).

#### Macrophage phenotypes on immunofluorescence

Mice that underwent endotoxin challenge had greater hepatic M1/pro-inflammatory macrophage populations compared to animals that received saline control as measured by mean fluorescent signal intensity on immunofluorescence histology (Fig. 6). In LPS-challenged mice, pre-treatment with SEFA-6179 led to a greater relative population of M2/anti-inflammatory to M1/pro-inflammatory macrophages in the liver compared to MCT on immunofluorescence. The ratio of M1 to M2 macrophages was highest for mice administered saline ( $5.411 \pm 1.263$ ), followed by MCT ( $3.676 \pm 1.619$ ), and lowest for SEFA-6179 ( $0.7851 \pm 0.1701$ ). While the difference in M1:M2 macrophage ratios between SEFA-6179 and MCT trended towards but did not reach significance ( $P = 0.13$ ), the difference between SEFA-6179 and saline control was statistically significant ( $P = 0.01$ ).



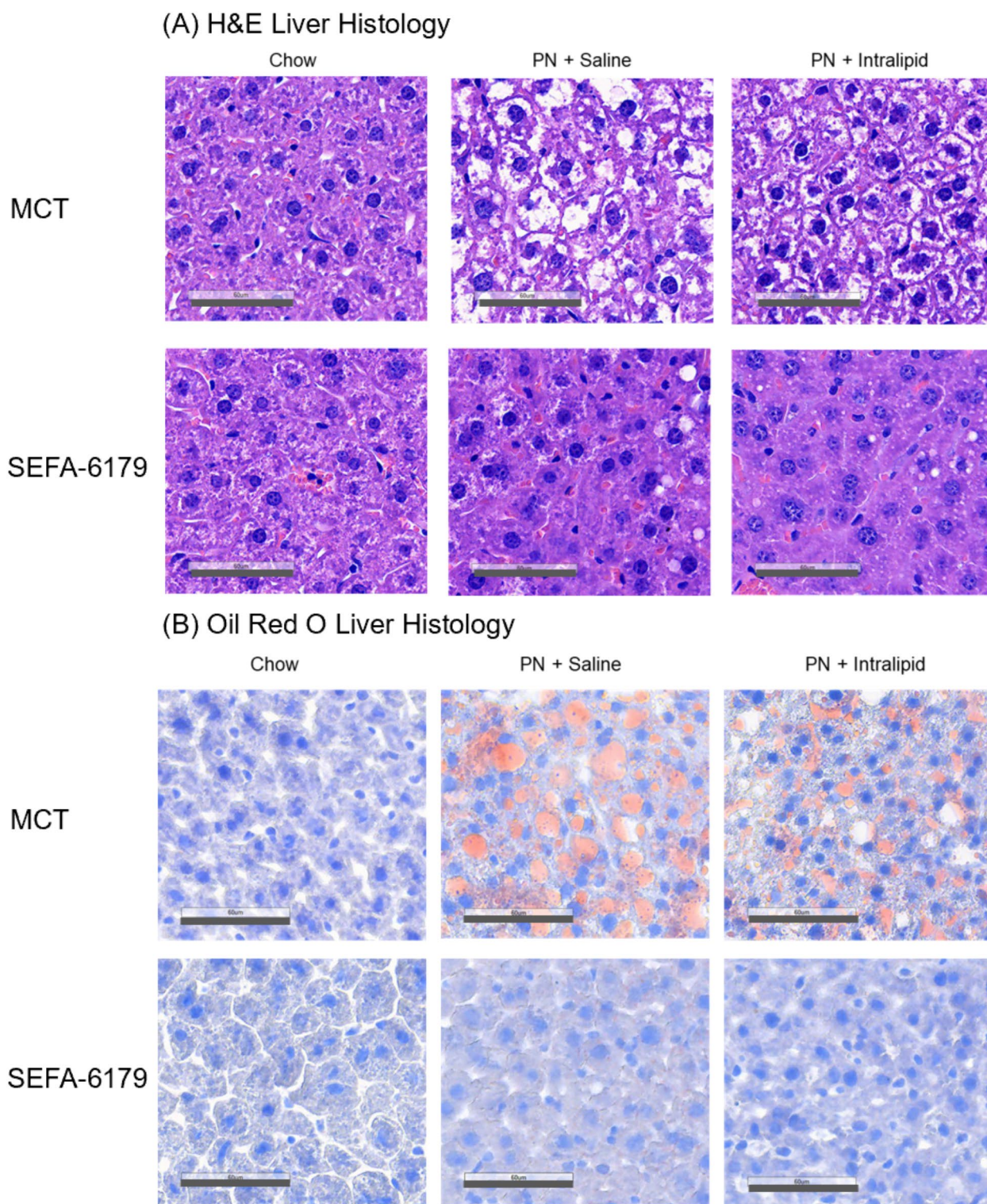
**Fig. 2.** (A) Body weights, (B) Parenteral nutrition consumption, and (C) Normalized organ weights of liver, spleen, and kidney by treatment group over the course of a 19-day experiment involving a murine model of parenteral nutrition-associated liver disease. For (A) and (B), treatment groups are named in the following order: ad libitum oral intake (PN vs. chow), intravenous injection (saline vs. Intralipid), oral gavage (MCT vs. SEFA), and intraperitoneal treatment (saline vs. LPS). Mean  $\pm$  Standard Error of Mean. PN, parenteral nutrition; SEFA, structurally engineered fatty acid (i.e. SEFA-6179); MCT, medium-chain triglyceride; LPS, lipopolysaccharide.

## Discussion

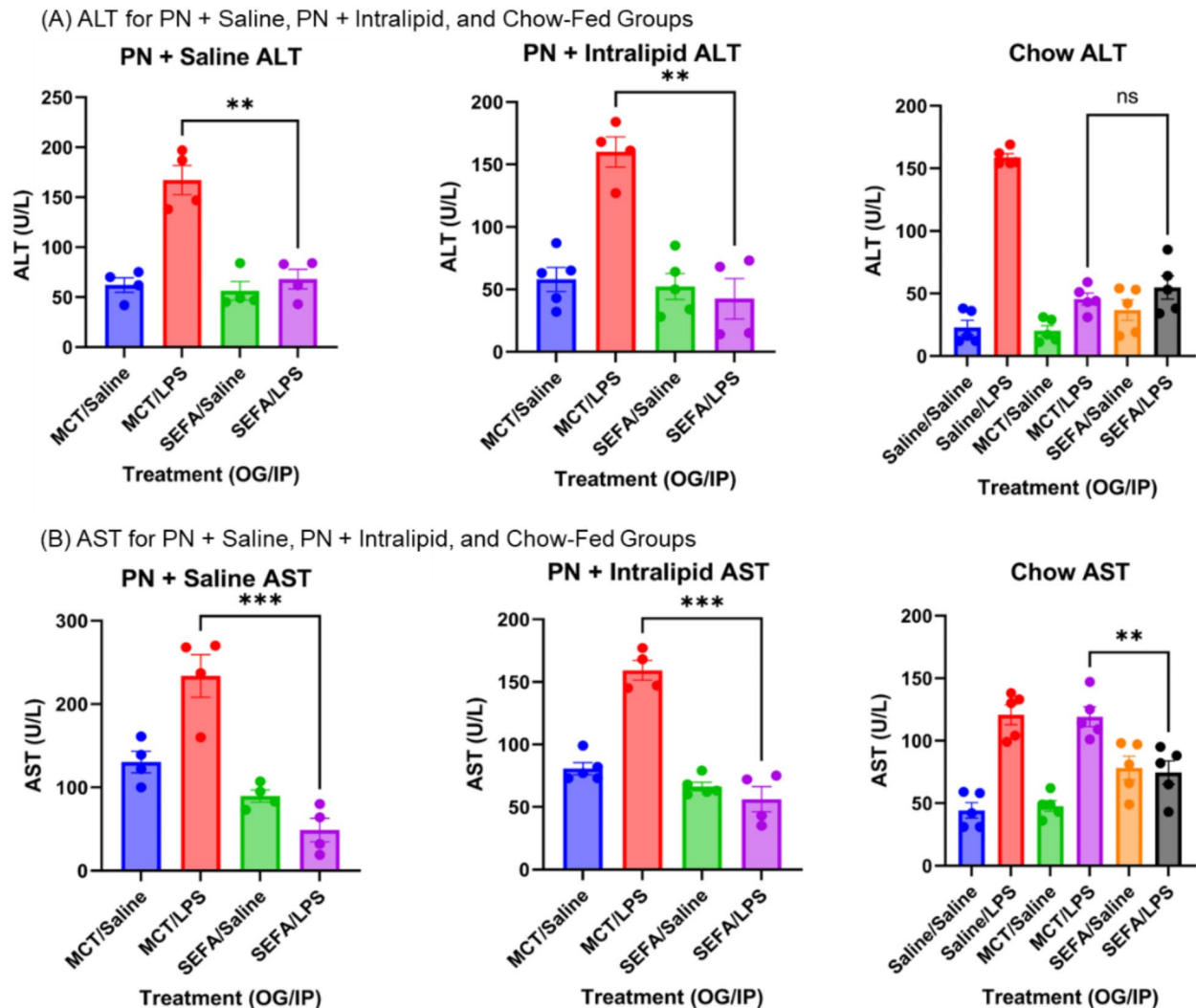
Patients with PN dependence experience a pro-inflammatory state partly due to repeated infections and endotoxin translocation in the setting of a compromised gut barrier<sup>19</sup>. Additionally, the very composition of lipid emulsions administered as part of the PN can lead to the development of hepatosteatosis, which can disrupt physiologic liver-dependent processes<sup>20</sup>. Commercially available soybean oil-based lipid emulsions contain high levels of pro-inflammatory omega-6 fatty acids and phytosterols, which have been linked to IFALD with long-term use<sup>21,22</sup>. Efforts aimed at reducing liver injury resulting from intestinal failure and/or PN administration include changing the lipid composition, restricting the amount of soybean oil-based lipid emulsion administered, increasing enteral feeding, preventing and treating infections, and preserving bowel length during surgical procedures. However, these interventions may not always be clinically feasible. Current therapeutic options for IFALD are limited, leading to the development of SEFA-6179 as a potential treatment.

Our lab has previously demonstrated the anti-inflammatory, -steatotic, and -fibrotic effects of SEFA-6179, a fully synthetic medium-chain fatty acid (MCFA) analogue, in a piglet model of intestinal failure and a murine model of PN-induced hepatosteatosis<sup>13,14</sup>. Based on this knowledge, the present study aimed to evaluate SEFA-6179 in a murine model that combines the hepatotoxic effects from PN provision and bacterial endotoxin. SEFA-6179 did not produce statistically significant differences in body or organ weights relative to MCT vehicle. SEFA-6179 had demonstrable safety and tolerability in Phase I clinical trials, and is currently under investigation in Phase II in adult patients<sup>23</sup>. The efficacy of SEFA-6179 is likely associated with structural modifications that, in contrast to unmodified MCFAs, prevent its rapid oxidation as an energy source, permitting activation of key receptors in the liver and elsewhere regulating inflammation and metabolism<sup>23,24</sup>. Pharmacokinetic studies in a piglet model of SBS with intestinal failure demonstrated that SEFA-6179 has superior gastrointestinal absorption when dissolved in MCT compared to saline<sup>25</sup>. For the present study, MCT was chosen as the vehicle not only due to this enhanced absorption, but also to isolate the effect of SEFA-6179 from that of MCT which





**Fig. 3.** Representative liver histology in a murine model of PN-associated liver disease. **(A)** Standard H&E stain demonstrates evidence of steatosis such as hepatocyte ballooning. **(B)** Oil Red O stain demonstrates areas of fat and triglyceride deposition as orange-red in color. 40X magnification, Bar = 60µm. MCT, medium-chain triglyceride; PN, parenteral nutrition; SEFA, structurally engineered fatty acid (i.e. SEFA-6179).



**Fig. 4.** Plasma liver enzyme concentrations including (A) alanine aminotransferase and (B) aspartate aminotransferase in a murine model of parenteral nutrition-associated liver disease for PN + Saline, PN + Intralipid, and Chow-Fed Groups. Values expressed are as the mean  $\pm$  standard error of the mean. ALT, alanine aminotransferase; AST, aspartate aminotransferase; PN, parenteral nutrition; MCT, medium-chain triglyceride; LPS, lipopolysaccharide; SEFA, structurally engineered fatty acid (i.e. SEFA-6179); OG, oral gavage; IP, intraperitoneal; whereby \* indicates  $P < 0.05$ ; \*\* indicates  $P < 0.01$ , \*\*\* indicates  $P < 0.001$  on unpaired Student t-test.

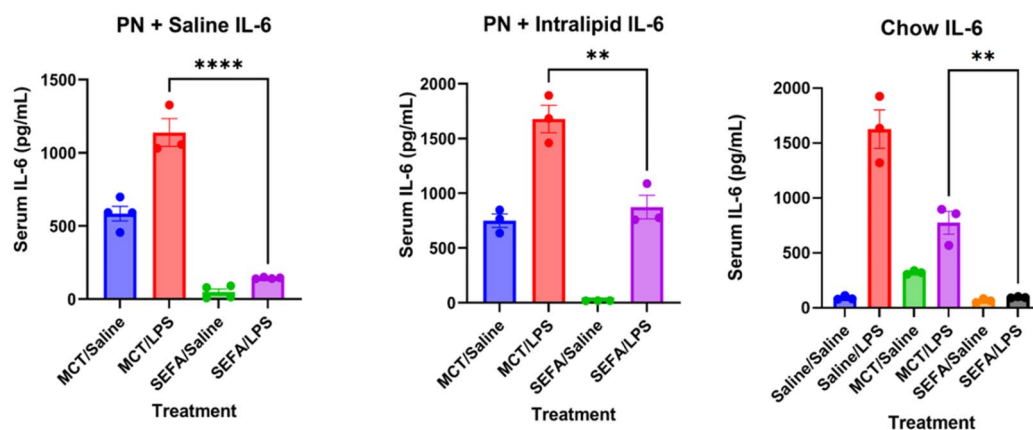
also has anti-inflammatory effects<sup>26,27</sup>. We induced steatotic liver injury in previously healthy, wild-type mice by providing a high-carbohydrate liquid diet and injecting a pro-inflammatory, soybean oil-based lipid emulsion used in clinical practice. The provision of a high-carbohydrate diet promotes de novo lipogenesis with resultant liver injury. This experiment corroborates previous work which demonstrated that treatment with SEFA-6179 maintained normal liver histology in a murine model of PN-induced hepatosteatosis<sup>14</sup>.

In the current study, mice were additionally challenged with bacterial endotoxin to mimic the acute inflammation and sepsis that may affect PN-dependent patients in the clinical setting. Commonly used blood biomarkers including ALT and AST increase in response to liver injury<sup>28</sup>. In the PNALD group that received endotoxin challenge, mice pre-treated with SEFA-6179 had significant reductions in liver enzymes compared to those treated with MCT. The liver enzyme levels of SEFA-6179-treated, LPS-challenged mice were comparable to those of non-LPS challenged mice. These data suggest that SEFA-6179, when delivered as a pre-treatment before acute inflammatory injury, may confer broadly anti-inflammatory effects in other sepsis models. Absolute values of AST were greater for the PN plus intravenous saline group compared to the lipid emulsion group, possibly due to the absence of an exogenous fat source which can lead to essential fatty acid deficiency and related liver dysfunction<sup>29</sup>.

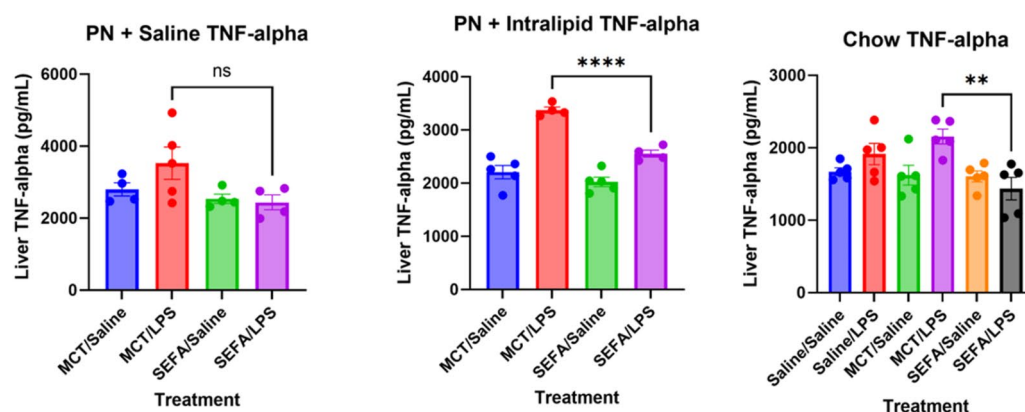
A murine intestinal injury model demonstrated that bacterial endotoxin-mediated activation of toll-like receptor 4 (TLR-4) signaling led to hepatocyte damage<sup>30</sup>. The same group of investigators demonstrated that mice with aberrant TLR-4 expression had reduced macrophage activation and improved plasma chemistry,



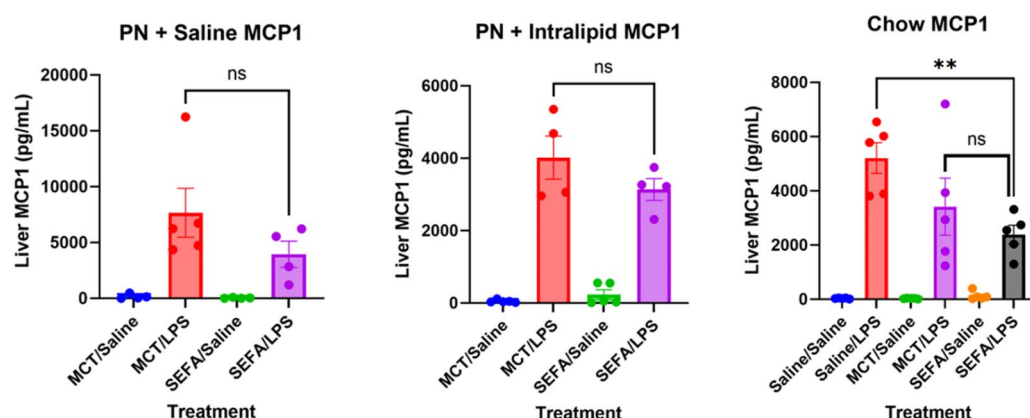
## (A) IL-6 Levels for PN + Saline, PN + Intralipid, and Chow-Fed Groups



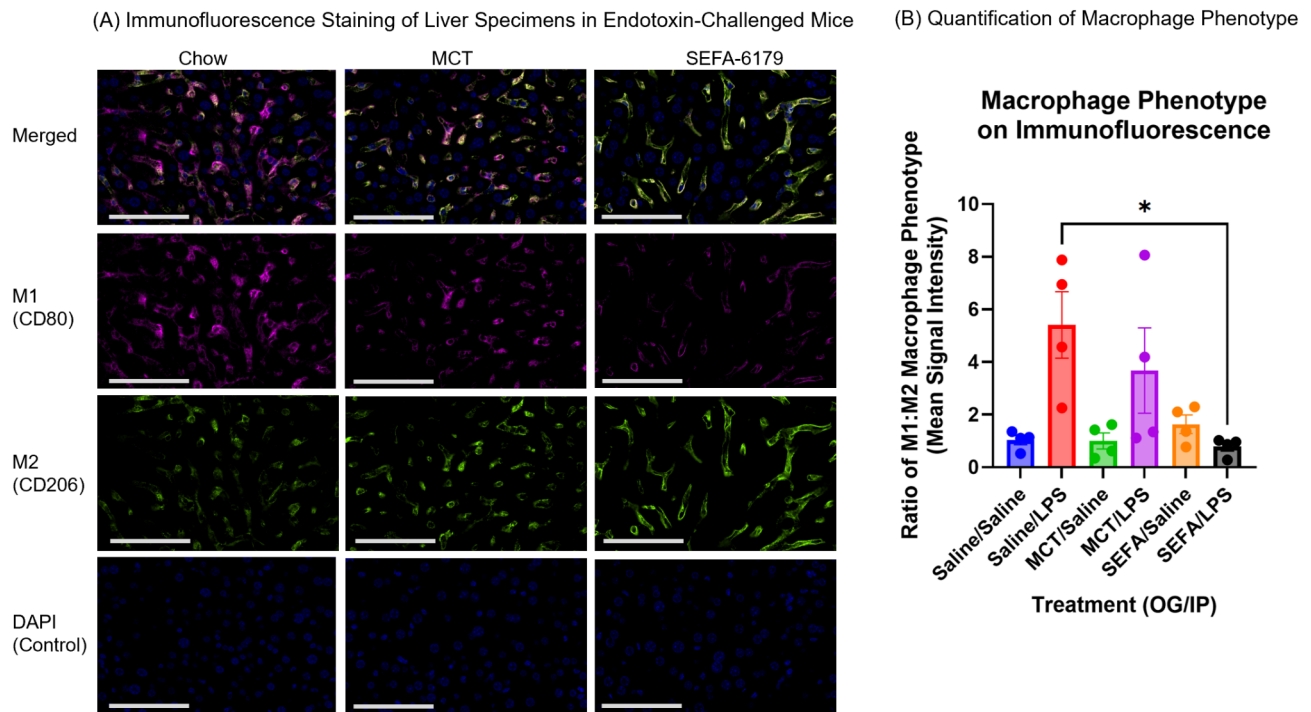
## (B) TNF-alpha Levels for PN + Saline, PN + Intralipid, and Chow-Fed Groups



## (C) MCP1 Levels for PN + Saline, PN + Intralipid, and Chow-Fed Groups



**Fig. 5.** Pro-inflammatory cytokine levels in a murine model of parenteral nutrition-associated liver disease for PN + Saline, PN + Intralipid, and Chow-Fed Groups. (A) Serum IL-6 Levels, (B) Liver Lysate TNF-alpha Levels, (C) Liver Lysate MCP1 Levels. Mean  $\pm$  Standard Error of Mean. PN, parenteral nutrition; ALT, alanine aminotransferase; AST, aspartate aminotransferase; MCT, medium-chain triglyceride; LPS, lipopolysaccharide; SEFA, structurally engineered fatty acid (i.e. SEFA-6179); OG, oral gavage; IP, intraperitoneal; IL-6, interleukin-6; TNF-alpha, tumor necrosis factor-alpha; MCP1, monocyte chemoattractant protein-1, whereby \* indicates  $P < 0.05$ ; \*\* indicates  $P < 0.01$ , \*\*\* indicates  $P < 0.001$  on unpaired Student t-test.



**Fig. 6.** (A) Representative immunofluorescence stains of liver specimens in a murine model of PN-induced hepatosteatosis and endotoxin-driven inflammation. M1/pro-inflammatory macrophages were stained with anti-CD80 (pink, 1:100), M2/anti-inflammatory macrophages were stained with anti-CD206 (green, 1:100), nuclear material was stained with DAPI as a control (blue). Bright field microscopy at 40X magnification, Bar = 100  $\mu$ M. (B) Ratio of M1 to M2 macrophages quantified using ImageJ software according to published protocol. MCT, medium-chain triglyceride; LPS, lipopolysaccharide; SEFA, structurally engineered fatty acid (i.e. SEFA-6179); OG, oral gavage; IP, intraperitoneal, whereby \* indicates  $P < 0.05$  on unpaired Student t-test.

thus elucidating a potential mechanism by which liver injury may be attenuated. SEFA-6179 acts on a variety of receptors in vitro, including PPAR- $\alpha$  and PPAR- $\gamma$ , which belong to a class of nuclear receptors involved in the regulation of inflammation and glucose and lipid metabolism<sup>13,31</sup>. With respect to liver disease, PPAR-targeted therapies are currently under development for patients with metabolic dysfunction-associated steatohepatitis and are approved for the treatment of primary biliary cholangitis<sup>32,33</sup>. In the present study, we demonstrated that SEFA-6179 treatment not only prevented the development of steatosis in a well-established murine model of PN-associated hepatosteatosis, but additionally had hepatoprotective effects in the presence of endotoxin administration. LPS classically activates pro-inflammatory M1 macrophages via a TLR4-dependent pathway that leads to the production of acute phase cytokines, such as IL-6 and TNF- $\alpha$ , that recruit downstream mediators of inflammation<sup>34</sup>. MCP1, also called chemokine ligand 2 (CCL2), is a chemoattractant factor that recruits monocytes; MCP1 levels correlate with the degree of liver injury and in fact, MCP1 deficiency has been found to protect mice against alcoholic liver disease<sup>35,36</sup>. Our results demonstrate that liver MCP1 levels are elevated in the presence of LPS exposure, compared to saline control. Additionally, mean MCP1 levels were lower in SEFA-6179-treated mice compared to MCT-treated mice in all treatment groups, though this did not reach statistical significance and may be a testament to the known anti-inflammatory and liver-protective properties of MCT alone<sup>37,38</sup>. PPAR- $\gamma$  activation aids in the polarization of pro-inflammatory M1 macrophages to an anti-inflammatory M2 phenotype<sup>39,40</sup>. The liver is the primary organ responsible for the activation of the initial cytokine storm, thus the early anti-inflammatory effects of SEFA-6179 may have led to the attenuated liver injury as measured by transaminases.

This study has several limitations. IFALD is a complex, multifactorial disease that is difficult to replicate in the laboratory setting. Unlike in human subjects, mice in this investigation did not have central venous catheters for the provision of PN. Although other investigators have conducted total parenteral nutrition experiments in mice, our 19-day PN-induced hepatosteatosis model has the benefit of avoiding catheter-related animal stress, which often result in animal welfare concerns and early death<sup>14,41</sup>. The present study involved mice with intact gastrointestinal tracts capable of normal absorption, thus it is not a short bowel syndrome model. Previous work from our lab has demonstrated that orally-administered SEFA-6179 is readily absorbed in a SBS model of Yucatan minipigs, albeit with reduced absorption compared to intestinally-intact animals<sup>25</sup>. The current findings may have limited translatability in human patients with malabsorption related to altered gut anatomy. At the time of LPS injection, mice did not have histologic evidence of steatosis which is attributable to pre-treatment with SEFA-6179. However, our study was designed to approximate the pathophysiology of patients with IFALD who experience acute inflammatory episodes such as infection. In chow-fed, LPS-challenged mice, SEFA-6179



conferred a reduction in AST levels compared to MCT. No statistically significant difference was observed in ALT between MCT and SEFA-6179 groups, although this may be related to limited power within our sample size. Stool was not collected from experimental animals for analysis, although the gut microbiome has proven to have an increasingly important role in modulating nutrient imbalance-related liver disease<sup>42</sup>. As in any small animal disease model, further dose–response studies involving large animals with greater physiological similarities to human subjects should be conducted.

## Conclusions

Parenteral nutrition is lifesaving for patients with intestinal malabsorption. However, long-term administration of PN may cause liver injury due to the inflammatory nature of frequently used commercially available lipid emulsions, as well as patients' increased susceptibility to infection. Treatment options in this vulnerable patient population are limited. In the current murine model of PN-induced hepatosteatosis and endotoxin-driven inflammation, treatment with SEFA-6179 normalized liver enzymes and reduced levels of pro-inflammatory cytokines, IL-6 and TNF-alpha, compared to MCT vehicle. The anti-inflammatory properties of SEFA-6179 are partly attributable to its ability to polarize pro-inflammatory M1 macrophages to an anti-inflammatory M2 phenotype. Few treatment options exist for patients with IFALD. SEFA-6179 is currently in a multi-institutional Phase II clinical trial in adults with IFALD. Prophylactic treatment with SEFA-6179 may be valuable in high risk PN-dependent patients, such as those with anticipated long-term PN use and history of recurrent infections. In light of current findings, future studies should also examine the potential effects of SEFA-6179 in non-cholestatic inflammatory/fibrosing liver disease models.

## Methods

### Animal model

All protocols were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee (IACUC) per National Institutes of Health (NIH) Animal Research Advisory Committee guidelines. All methods were carried out in accordance with relevant guidelines and regulations, and reported in accordance with ARRIVE guidelines. Hepatosteatosis was induced in otherwise healthy eight-week-old male C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) using a well-established murine model<sup>15</sup>. Experimental design is summarized in Fig. 1. Briefly, mice received an ad libitum fat-free, high-carbohydrate parenteral nutrition (PN)-equivalent oral solution for 19 days, with every-other-day tail vein injection of a commercial soybean oil-based lipid emulsion (Intralipid, 4 g fat/kg body weight/day, Fresenius Kabi, Sweden) or isovolumetric saline control. The composition of the PN solution is identical to that of PN administered to patients at Boston Children's Hospital: 20% dextrose, 2% amino acid, 30 mEq/L sodium, 20 mEq/L potassium, 15 mEq/L calcium, 10 mEq/L magnesium, 10 mMol/L phosphate, 36.67 mEq/L chloride, 19.4 mEq/L acetate, multivitamins, and essential trace elements. PN consumption was measured twice daily. Body weights were measured every other day. PN-dependent mice did not receive any other source of nutrition or hydration. Mice were randomized to one of two oral treatments: SEFA-6179 drug (100 mg/kg) (NorthSea Therapeutics, Netherlands) dissolved in medium-chain triglyceride (MCT) vehicle (Nestle, Switzerland) or isovolumetric MCT. Treatments were administered via oral gavage daily on days 15–18. On day 19, a single dose of *Escherichia coli* O111:B4-derived lipopolysaccharide (LPS, 15 mg/kg body weight, Sigma-Aldrich, St. Louis, MO) or isovolumetric saline vehicle was administered intraperitoneally six hours before planned euthanasia. The dose of SEFA-6179, 100 mg/kg, was based on previous work from our lab that demonstrated its safety and tolerability<sup>14</sup>. The dose of LPS, 15 mg/kg, was based on published rodent sepsis models which most frequently utilized *E. coli*-derived LPS (ranging from 10–25 mg/kg) and subsequent lot-specific pilot testing in the lab, as toxicity may vary in a batch-dependent manner<sup>16,43,44</sup>. Animals were anesthetized using inhaled isoflurane (3% induction and 2% maintenance, VetOne, Boise, ID) prior to euthanasia via terminal blood draw followed by cervical dislocation. Age-matched, chow-fed mice received either SEFA-6179 drug (100 mg/kg), isovolumetric MCT (vehicle control), or normal saline (negative control) daily via oral gavage on days 15–18. Chow-fed controls were similarly treated with intraperitoneal endotoxin (15 mg/kg) or isovolumetric saline and sacrificed on day 19. Mice were housed under the same conditions (12-h light/dark cycle, 21 °C ambient temperature) and underwent concurrent treatment in order to minimize the potential for confounding. Fourteen experimental groups were utilized (n = 5 per group) for a total of 70 animals.

### Biochemical assay

The primary outcome was biochemical assessment of liver injury through plasma concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Whole blood was collected from live animals and centrifuged at 1,500 relative centrifugal force (RCF) for 15 min at 4 °C in lithium heparin plasma separator tubes (BD, Franklin Lakes, NJ). Plasma was flash frozen and stored at –80 °C, then assayed for chemistry using veterinary rotors on the VetScan VS2 device (Zoetis, Parsippany, NJ). Enzyme-linked immunosorbent assays (ELISAs) were used to measure levels of pro-inflammatory cytokines, including mouse-specific plasma IL-6 (Abcam, Waltham, MA), liver TNF-alpha (RayBiotech, Norcross, GA), and liver MCP1/CCL2 (R&D Systems, Minneapolis, MN). For the liver TNF-alpha and MCP1 assays, the same mass of liver tissue (30.0 µg/sample) was digested in identical amounts of lysis buffer (300 µL/sample for TNF-alpha and 250 µL/sample for MCP1) prior to completing the kits according to the manufacturer's instructions.

### Histopathology

The largest (i.e. left) lobe of the liver, weighing approximately 0.4–0.5 g underwent 24 h of formalin fixation, paraffin-embedding, and subsequent hematoxylin and eosin (H&E) staining to assess hepatic architecture. Frozen

optimal cutting temperature (OCT) compound-embedded caudate lobes of the liver, weighing approximately 0.2–0.3 g, were additionally stained using Oil Red O to evaluate fatty deposition.

### Immunofluorescence

Immunofluorescence staining of liver specimens, specifically the largest (i.e. left) lobe, was performed to assess for M1 and M2 macrophage phenotypes. Slides were deparaffinized and rehydrated as follows: 15 min xylene, 15 min xylene, 5 min 100% alcohol, 5 min 100% alcohol, 5 min 70% alcohol, and water rinse. Slides were transferred to antigen retrieval buffer (Antigen Unmasking Solution, citric acid-based pH=6, Vector Laboratories, Newark, CA). Antigen retrieval was completed in a pressure cooker at 110 °C for 15 min. Slides were allowed to cool for 20 min before they were brought to room temperature through the addition of water to the retrieval container. Once slides reached room temperature, they were washed three times for 5 min each in TBS (Tris-buffered saline), TBS again, and TBST (Tris-buffered Saline + Tween20). Sections were blocked with a 3% bovine serum albumin (BSA) and incubated at room temperature for 20 min. Following blocking, sections were incubated with the primary antibodies diluted in 3% BSA overnight at 4 °C: anti-CD80 (1:100, Invitrogen, Waltham, MA) for M1 macrophages and CD206 (1:100, R&D Systems, Minneapolis, MN) for M2 macrophages. The following morning, slides were washed as before. Donkey anti-Goat IgG diluted in TBS (1:100, Alexa Fluor Plus 647, Thermo Fisher Scientific, Waltham, MA) was applied to sections and allowed to incubate for 1 h at room temperature. Slides were washed as before. Goat anti-Rabbit IgG diluted in TBS (1:100, Alexa Fluor Plus 488, Thermo Fisher Scientific, Waltham, MA) was applied to sections and allowed to incubate for 1 h at room temperature. Slides were washed as before. Slides were stained with Sudan Black for 20 min to quench background and autofluorescence, then washed under running water for 15 min. Slides were washed three times for 5 min each in TBS, TBS again, and TBST. Slides were counterstained with DAPI diluted in PBS (2 µg/mL, ServiceBio, Wuhan, China) and cover-slipped using Fluoroshield (Sigma-Aldrich, St. Louis, MO). Whole slide-scanning was performed (bright field 40x, MoticEasyScan Infinity, Hong Kong) for analysis. ImageJ software (ImageJ) 1.54 for Windows, National Institutes of Health, USA) was used to quantify immunofluorescence signal intensity of M1 and M2 macrophages, normalized against DAPI, according to published protocol<sup>45</sup>.

### Statistical analysis

The Shapiro–Wilk test was used to assess distribution of data. Continuous outcomes were assessed using analysis of variance (ANOVA) and described as the mean plus or minus standard error of the mean (SEM). We chose not to adjust for multiple comparisons due to limited sample sizes. All tests of significance were two-sided with  $P < 0.05$  deemed statistically significant. Analysis was performed in SAS version 9.4 (SAS, Cary, NC). Plots were generated with GraphPad Prism version 10.3.1 (GraphPad Software, Boston, MA).

### Data availability

Datasets from the present study are available from the corresponding author upon reasonable request.

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## Author contributions

Conceptualization: SZW, SCF, MP; Methodology: SZW, SCF, TIH, MQ, AP; Analysis: SZW, PDM; Writing—Original Draft: SZW; Writing—Review & Editing: All Authors; Supervision: KMG, MP; Funding Acquisition: SZW, TIH, SCF, MP. All authors reviewed and approved the final manuscript.

## Declarations

## Competing interests

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### Additional information

**Correspondence** and requests for materials should be addressed to S.Z.W. or M.P.

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