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Dietary Saturated Fatty Acids Modulate Host Innate Immunity

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by

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ABSTRACT

Nutrition, cellular metabolism, and inflammatory regulation of the immune system are interconnected, and advancing our understanding of this will greatly improve treatment of metabolic and inflammatory disease. Specifically, dietary saturated fats are becoming increasingly appreciated for their ability to modify innate immune cell function in mammals following absorption into the blood and lymphatic circulation systems. The work herein focuses on the capacity for lipids in the diet to modulate innate immune memory. My thesis first broadly outlines fundamental background, including: the evolution of human nutrition, the discovery of innate immune memory, and key topics that reoccur throughout the text surrounding dietary modulation of innate immunity, and the implications this has for treating disease. Next, I review the trek of dietary fat from digestion to blood and lymph, to the inside of a white blood cell and the inner workings of mitochondrial metabolism—where breakdown occurs, and byproducts travel to the nucleus to unwind or tighten chromatin. The overarching impact of this is an alteration in the way genes that encode inflammatory proteins are made more or less available for transcription. The cellular mechanisms underlying these processes are currently burgeoning in the field of immunometabolism, and the research is important for identifying biomarkers and novel therapeutic targets for treating diseases characterized by systemic inflammation, including obesity, insulin resistance, atherosclerosis, autoimmunity, and sepsis.

A large portion of the experimental work that went into this shows how diet-induced innate immune memory subsequently changes the severity and outcome of

disease using mouse models of acute, systemic inflammation. These experiments reveal how chronic exposure to dietary saturated fats alters inflammation in the blood and worsens systemic inflammation induced by a microbial ligand. The cell culture models used were designed to mimic primary and secondary stimulation of innate immune cells as would occur with diet exposure followed by microbial ligand stimulation. An infection model using a fungal pathogen is utilized to bolster these findings.

Chronic exposure to a diet enriched in saturated fatty acids derived from milkfat is shown here to significantly alter the bone marrow compartment in mice. Specifically, palmitic acid, which is common in many human diets across the globe and consequently found also in the blood, shows a unique capacity for modulating memory responses in macrophages derived from the bone marrow, indicative of the phenomenon known as trained immunity. Exposing mice to palmitic acid alone is enough to alter the outcome of systemic inflammatory disease, and infection with a fungal pathogen. Interestingly, this impact is reversible *in vitro* and *in vivo* using another fatty acid simultaneously that is also common in our diets and blood, the unsaturated oleic acid. This remarkable quality of lipids that regulate inflammation and subsequent disease outcome has great potential to contribute to better treatments for metabolic and inflammatory diseases.

DEDICATION

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An introduction to dietary saturated fatty acids as innate immune modulators

Introduction

Human nutrition has evolved rapidly since the Neolithic Revolution began approximately 10,000 years ago, most notably in a region of the Middle East known as the Fertile Crescent [1]. During this time period humans replaced their hunter-gatherer lifestyles with a way of life that allowed them to settle, and build civilizations that were supported by agriculture, including farming and the domestication of plants and animals [1]. More recently, the Industrial Revolution that began in Europe and the United States in the 1700s lead to a drastic shift in culture and lifestyle for humans in the west due to the industrialization of agriculture. Both of these cultural revolutions stimulated a significant shift in diet toward enhanced saturated fat content [2-4]. These dramatic changes in nutrition comprise only a small fraction of human evolution, and it is important to consider the adaptability of our physiology to these dietary changes that have occurred over a short time frame.

Humans can adapt to a broad range of food intake, and research in evolutionary genetics suggests the selection of "meat-adaptive" genes have allowed for greater fat consumption balanced by a reduced cardiovascular risk [5,6]. Consistent with this hypothesis, another group showed genes involved in lipid and fatty acid (FA) metabolism are enriched in human tissues (liver, heart, and kidneys) compared to other primates, and suggests their regulation evolved under natural selection [7,8].

Dietary FAs have been shown to modulate the inflammatory response of innate immune cells, specifically circulating monocytes and their tissue-resident equivalent, macrophages, and this may impact the outcome of diseases characterized by inflammatory dysregulation [9,10]. Following digestion of lipids, FAs enter the blood as free-floating straight chains of carbon atoms or are packaged inside spherical structures of proteins and lipids called chylomicrons that circulate through the lymphatic system [11]. This voyage through blood and lymph is a direct route for FAs to interact with circulating immune cells while en route to tissues for energy use or storage [12].

A deeper understanding of how lipids affect innate immune cell activity came about through the study of atherosclerotic foam cells in mouse models and *in vitro* model systems using human monocytes [13-15]. These studies led to the discovery that the scavenger receptor, Cluster of differentiation 36 (CD36), transports oxidized low-density lipoprotein (OxLDL) into monocytes and macrophages, driving the formation of foam cells, the cells that make up atherosclerotic plaque [13]. OxLDL is an endogenous lipid synthesized via liver and adipose tissue metabolism in response to fat digestion, and plasma concentrations can be altered by dietary saturated fats [16,17]. Since this study was published, it has been shown endogenous lipids and exogenous (dietary) FAs are immunomodulatory molecules that have significant impact on the metabolism and inflammatory response of innate immune cells [18-22].

It is now appreciated that nutrition influences inflammatory disease outcome by reprogramming innate immune cell metabolism to favor pro- or anti-inflammatory responses to subsequent stimuli. Specifically, pre-treatment of human monocytes with

OxLDL enhances CD36 expression and intracellular ceramide, upregulates glycolysis and oxidative phosphorylation, and induces histone methylation of promoters nearby genes that encode for the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α, hereafter referred to as TNF) and interleukin-6 (IL-6), leading to augmented TNF and IL-6 secretion in response to secondary stimulation with microbial ligands up to 7 days post-OxLDL exposure [23-25]. Likewise, pre-treatment of human monocytes and primary mouse macrophages with saturated fatty acids (SFA) enhances intracellular ceramide and TNF and IL-6 secretion in response to subsequent microbial ligand exposure [12,19-22]. This sensitized response to secondary stimuli has been termed a "memory" response for the innate immune system [26].

Despite these seminal studies that began over 40 years ago, the molecular mechanisms underlying innate immune modulation by SFAs remain enigmatic, and much is left to discover. This gap in mechanistic knowledge is important to fill for personalized nutrition and dietary intervention strategies that could prevent or treat diseases characterized by dysregulated metabolism and inflammation, including infection—both local and systemic (*i.e.*, sepsis), and non-infectious diseases like obesity, type II diabetes (T2D), cardiovascular disease (CVD), and autoimmunity.

My thesis presents an exploration of dietary fatty acids, how they are digested and metabolized by mammalian hosts, circulate throughout the body, interact with, and regulate innate immune cell functions by altering their metabolism and inflammatory response potential to secondary stimuli. I investigate the role of a specific SFA, palmitic acid (PA), in memory phenomena of the innate immune system, a branch of immunity

that has only recently been appreciated for its memory capabilities, and I hypothesize that dietary PA can reprogram innate immune cell metabolism and alter the inflammatory response to secondary stimuli—both sterile and pathogenic.

Innate immune memory

The innate immune system is an ancient branch of immunity that mammals, plants, and invertebrates evolved for protection against invasion by pathogens or exposure to toxins—and innate immune memory is a feature common to these organisms [27,28]. Innate immune cells have long been appreciated for their ability to respond rapidly to intruders, engulf and abolish them via phagocytosis, and alert other immune cells of the breach [29]. However, in mammals, immune memory has mostly been attributed to the more recently evolved adaptive immune branch including T and B lymphocytes and the formation of antigen-specific antibodies, all of which require highly specific and complex mechanisms to build long-term memory of each individual pathogen encountered over prolonged periods of time (14 – 90 days) [29]. Innate immune cells possess non-specific surface receptors called pattern recognition receptors (PRRs) that identify a variety of molecular patterns common to pathogenic microbes [30]. Circulating monocytes and their tissue-specific counterparts, macrophages, are innate immune cells that use PRRs to identify pathogen-associated molecular patterns (PAMPs), or danger-associated molecular patterns (DAMPs) associated with sterile molecules or metabolic byproducts of nutrition [29]. Stimulation of these PRRs initiate a primary signal required to induce memory by regulating metabolites that signal epigenetic enzymes (*e.g.* histone

methyltransferases) to open or tighten chromatin and reveal or conceal inflammatory genes [24,31]. This remodeling can either increase or decrease the inflammatory response of an innate immune cell to secondary stimulation.

When innate immune memory initiates an enhanced inflammatory response to a secondary stimulate it is termed "trained immunity". This term was first coined by Netea *et al*. to describe the underappreciated potential of innate immune cells to harbor long-term resistance to a diversity of pathogens following initial exposure to a single stimulus [26]. The first identified inducer of trained immunity was the Bacillus Calmette-Guerin (BCG) vaccine, a live attenuated vaccine for *Mycobacterium tuberculosis* (Mtb) that lowered regional mortality rates up to 50%, prompting scientists to investigate its capacity to confer resistance to multiple pathogens in addition to Mtb [32,33]. Since this finding, BCG vaccine has been applied to *ex vivo*, *in vitro*, and *in vivo* model systems to show that innate immune cells of humans and mice respond to subsequent pathogenic exposure with enhanced inflammatory prowess even up to 1 year after vaccination [34-36]. Specifically, human monocytes isolated from patients 1 year after receiving the BCG vaccine respond to *ex vivo* infection with Mtb, *Candida albicans* or *Staphylococcus aureus* with significantly enhanced inflammation compared to monocytes from humans not given the BCG vaccine [35].

This long-term feature of trained immune memory befuddled scientists because the lifespan of circulating monocytes is only 1-3 days, however, the mechanism by which BCG vaccine provides long-term memory was shown in mice to expand hematopoietic stem cells in the bone marrow that continually replenish the circulation with

epigenetically modified monocytes and macrophages poised for enhanced pathogenic defense [36]. Together these data highlight the extraordinary capabilities of innate immune cells to provide long-term protection against pathogens.

The discovery that the BCG vaccine induced trained immunity in humans and mice led scientists to study further methods of exploiting the memory capabilities of innate immune cells, including investigations of other attenuated microbial strains. In 1986 Bistoni *et al.* showed that treating mice with a poorly virulent strain of *C. albicans* conferred protection against subsequent infection with pathogenic *C. albicans* or *S. aureus* 2 weeks after initial training. This protection was found to be mediated by monocytes and macrophages [37]. At this point the mechanism underlying trained immunity remained unknown, and scientists began asking questions about innate immune recognition of microbial surface molecules.

The fungal microbe *C. albicans* exists on mucosal surfaces in humans as a commensal organism throughout many regions of the body, and it can also function as an opportunistic pathogen and breach the mucosal barrier of immunocompromised individuals leading to systemic candidiasis [38,39]. Monocytes and macrophages possess PRRs that recognize cell wall features of *C. albicans*, and one that is important for initiating trained immunity is β-glucan [40]. In 2012, Quintin *et al.* recapitulated the training capabilities of heat-killed *C. albicans* in mice and in human monocytes. They showed that this feature was dependent on the recognition of β-glucan by the monocyte PRR, Dectin-1 [41]. β-glucan is now known for its potent induction of trained immunity in

humans and mice, and along with the BCG vaccine it is widely used to study trained immunity [30].

Additionally, β-glucan can inhibit tolerance, the other side of innate immune memory characterized by a suppressed inflammatory response to secondary stimuli [42]. The most well-studied inducer of tolerance is lipopolysaccharide (LPS), an immunogenic molecule on the outer wall of Gram-negative bacteria, including *Escherichia coli*. LPS is recognized by the innate immune cell PRR, toll-like receptor 4 (TLR4), and primarily induces pro-inflammatory cytokine transcription (*Tnf* and *Il-6*) via nuclear translocation of the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [29]. At certain concentrations and lengths of exposure (100 ng/ml; 24 hours) LPS is capable of modifying chromatin in human and mouse macrophages that leads to the suppression of *Tnf* and *Il-6* transcription while maintaining antimicrobial effector function [31]. LPS tolerance prevents excess inflammation that would otherwise induce tissue damage [31].

I have described here the induction of innate immune tolerance and trained immunity with pathogenic molecules. However, current studies have found that sterile stimulants can induce trained immunity, including circulating lipids (*e.g.,* OxLDL) and SFAs (*e.g.,* PA) that are enriched by increasing dietary fat intake [40,43,44]. The influence of diet on innate immune memory is important to investigate in order to understand the mechanisms underlying metabolic and inflammatory diseases. Considering trained immunity alters the innate immune response to subsequent pathogenic exposure, the interplay between diet, trained immunity, and infection must be thoroughly studied.

The impact of diet on innate immune memory

The Western Diet (WD) is enriched in SFAs and sucrose and is fiber deficient. We previously discovered that, independently of diet-induced microbiome changes, wild-type mice fed a WD experience increased disease severity and mortality in a model of LPS-induced sepsis (endotoxemia), which corresponded to altered levels of activated monocytes in the blood compared to mice fed a standard chow (SC) [45]. Further, another group found the WD induced trained immunity in atherosclerotic mice [44]. At this time the exact constituents of the WD that induced trained immunity and were driving the disease phenotypes in atherosclerotic mice or mice with endotoxemia remained unknown.

For the studies I present here we implemented a Ketogenic diet (KD) enriched only in SFAs derived from milkfat—the same fat source used in the WD [43,45]. The KD is devoid of carbohydrates, and these features allowed us to ask if the disease phenotypes we previously showed in WD-fed mice were strictly dependent on the SFA content, rather than the sucrose portion of the WD [45]. Ketogenesis is a metabolic process whereby cells become reliant on the use of ketones as their primary fuel source rather than glucose; it can be induced by a very low carbohydrate diet or fasting, which starves cells of glucose and leads to their use of fatty acid oxidation as their dominant energy source, leading to ketone production as a byproduct of mitochondrial β-oxidation [46]. Although the KD and ketogenesis have proven to be beneficial for the treatment of specific diseases, such as epilepsy and obesity, the impact of excessive fat intake on innate immune cell function in humans has not been fully characterized [47,48].

Lipids: untapped potential for therapeutic intervention

Lipids are macromolecules essential to all of life and are largely appreciated for their contribution to energy storage and cellular structure, particularly that of cell and organelle membranes [49]. Following digestion by mammals, lipids are transiently upregulated in the blood and lymphatic circulation systems (within minutes to hours) as free-floating fatty acids (FFA) or packaged in chylomicrons [11]. In this way they can be rapidly transported to the tissues that need them most for energy, or are simply stored in the liver for later dispersal. In addition to PA, I focus on the mono-unsaturated fatty acid associated with the Mediterranean Diet, oleic acid (OA), and a sphingolipid species called ceramide. Although PA and OA can be synthesized endogenously, I emphasize exogenous, or dietary, forms throughout. In contrast, ceramide and other sphingolipids are primarily synthesized via metabolic byproducts of SFAs, including PA [50].

Beyond energy storage and membrane assembly, lipids can also act as bioactive molecules and second messengers that stimulate, inhibit, or support intracellular signaling pathways [49]. Specifically, ceramide is known to enhance TLR-mediated signaling in macrophages and contribute to pro-inflammatory cytokine synthesis, and macrophage stimulation with TLR ligands upregulates intracellular ceramide synthesis [51]. Ceramide has been shown to inhibit mitochondrial β-oxidation, while OA restores this function [50,52]. The opposing features of PA and ceramide versus OA on macrophage metabolism and inflammation makes these lipids intriguing candidates, as circulating biomarkers, and in considering personalized nutritional intervention for immune dysfunction.

Nutritional influences on cytokine responses in health and disease

Cytokines are soluble signaling proteins that are fundamental in the host immune response to infection; they facilitate communication between cells, and are analogous to hormones for the endocrine system, or neurotransmitters for the nervous system [53]. All cell types except erythrocytes can produce cytokines, however they are produced primarily by immune cells [54]. Cell metabolism drives the production and secretion of pro-inflammatory and anti-inflammatory cytokines, and when metabolism is perturbed, so is this tightly regulated homeostasis of inflammation [55,56]. Balanced nutrition helps to regulate immunometabolism, and excessive intake of SFAs (an amount that can be difficult to precisely determine due to metabolic variability between individuals) may contribute to dysregulated metabolism and alter the cytokine response of innate immune cells to infectious or non-infectious disease [57-59].

Obesity can also occur in both metabolically healthy and unhealthy individuals depending on the presence of insulin resistance, a pathology that correlates with enhanced serum concentrations of SFAs and ceramide [60,61]. In addition to insulin resistance, metabolically unhealthy individuals may also exhibit metaflammation—chronic inflammation induced by diet [45]. The WD is known to induce metaflammation in humans and mice and this is characterized by chronically enhanced serum concentrations of pro-inflammatory cytokines, including TNF, IL-6, and IL-1β [57]. Considering the potential for diet to dysregulate these cytokines via metaflammation and contribute to non-infectious disease, I have focused on TNF, IL-6, and IL-1β throughout

this work because they also play a central role in the host immune response during sepsis [62,63].

Sepsis is a disease of the immune system characterized by the host response to a microbial infection that has entered the blood, and the host inflammatory response is ultimately responsible for inducing tissue damage, organ failure, and death [64]. There are two distinct phases of inflammation that occur following blood infection: 1) acute and systemic inflammation, and 2) immunosuppression that results as the immune system attempts to alleviate tissue damage induced by the first phase [65]. During the acute response in humans, pro-inflammatory cytokines including TNF, IL-6, and IL-1β are released into the circulation and stimulate the hypothalamus to elevate the body's set temperature, known as fever [29]. Additional characteristics of the acute phase are mediated by systemic TNF, IL-6, and IL-1β, including vasodilation and increased vascular permeability, acute phase protein release from the liver, and infiltration of innate immune cells into the lungs, kidneys, and heart [29,54]. These three cytokines play significant roles in the acute phase of sepsis and that is why we chose to measure them in nearly all of the model systems used here. The data I present provide compelling evidence that SFAs modulate the expression and release of TNF, IL-6, and IL-1β following microbial ligand stimulation [43].

Conclusion

Innate immune memory likely evolved to provide non-specific protection from secondary infections [66]. However, an enhanced immune response to a secondary

stimulus, as shown in this work with primary PA stimulation, can be deleterious in the context of sepsis when the host inflammatory response is severely dysregulated [43,67]. Nutrient intake directly impacts immune cell metabolism and inflammatory responses [68,69]. PA, which is found primarily in animal products, coconut, and palm oil, is the most abundant SFA in human serum, and it is significantly elevated in the blood of those with obesity, metabolic dysfunction, and systemic inflammation—conditions which correlate with enhanced susceptibility to infection and higher sepsis mortality rates [60,70-74]. Currently, feeding tube formulas for critically ill sepsis patients contain various SFAs and are not personalized based on patient need for dampening or enhancing systemic inflammation [75]. Determining the immune modulating capacity of PA may be a useful strategy for therapeutic interventions based on the nutritional status and risk of critically ill sepsis patients.

The models used in this work are primarily oriented toward acute, systemic inflammation (*i.e.*, sepsis) and the impact of excessive dietary SFA on this condition. However, nutritional impact on metabolism and inflammation has far reaching consequences, especially for human populations adapting to diets with swiftly increasing SFA content. Understanding how humans have emphasized dietary fats in the past and present, and how this impacts our long-term innate immune responses during disease, will be critical for the advancement of novel therapeutic strategies needed to prevent and treat metabolic and inflammatory diseases, and infections that lead to sepsis.

REFERENCES:

[1] Alt, K.W.; Al-Ahmad, A.; Woelber, J.P. Nutrition and Health in Human Evolution–Past to Present. Nutrients 2022, 14, 3594.

[2] Carrera-Bastos, Pedro; Fontes-Villalba, Maelan; O'Keefe, James H; Lindeberg, Staffan; Cordain, Loren. The western diet and lifestyle and diseases of civilization. Research Reports in Clinical Cardiology 2011:2 15–35

[3] O'Keefe, James H. and Cordain, Loren. Cardiovascular Disease Resulting From a Diet and Lifestyle at Odds With Our Paleolithic Genome: How to Become a 21st-Century Hunter-Gatherer. Mayo Clin Proc. 2004;79:101-108

[4] Cordain, Loren; Eaton, S Boyd; Sebastian, Anthony; Mann, Neil; Lindeberg, Staffan; Watkins, Bruce A; O'Keefe, James H; Brand-Miller, Janette. Origins and evolution of the Western diet: health implications for the 21st century. Am J Clin Nutr 2005;81:341–54

[5] Leonard WR, Snodgrass JJ, Robertson ML. Evolutionary Perspectives on Fat Ingestion and Metabolism in Humans. In: Montmayeur JP, le Coutre J, editors. Fat Detection: Taste, Texture, and Post Ingestive Effects. Boca Raton (FL): CRC Press/Taylor & Francis; 2010. Chapter 1.

[6] Finch CE, Stanford CB. Meat-adaptive genes and the evolution of slower aging in humans. Q Rev Biol. 2004 Mar;79(1):3-50.

[7] Luca F, Perry GH, Di Rienzo A. Evolutionary adaptations to dietary changes. Annu Rev Nutr. 2010 Aug 21;30:291-314.

[8] Blekhman R, Oshlack A, Chabot AE, Smyth GK, Gilad Y. Gene regulation in primates evolves under tissue-specific selection pressures. PLoS Genet. 2008 Nov;4(11):e1000271.

[9] Palomer X, Pizarro-Delgado J, Barroso E, Vázquez-Carrera M. Palmitic and Oleic Acid: The Yin and Yang of Fatty Acids in Type 2 Diabetes Mellitus. Trends Endocrinol Metab. 2018 Mar;29(3):178-190.

[10] need to find a citation on dietary FAs and how they impact inflammatory disease

[11] Black DD. Development and physiological regulation of intestinal lipid absorption. I. Development of intestinal lipid absorption: cellular events in chylomicron assembly and secretion. Am J Physiol Gastrointest Liver Physiol. 2007;293(3):G519–24.

[12] Seufert AL, Napier BA. A new frontier for fat: dietary palmitic acid induces innate immune memory. Immunometabolism (Cobham). 2023 May 15;5(2):e00021.

[13] Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. J Biol Chem. 1993 Jun 5;268(16):11811-6.

[14] Nicholson AC, Frieda S, Pearce A, Silverstein RL. Oxidized LDL binds to CD36 on human monocyte-derived macrophages and transfected cell lines. Evidence implicating the lipid moiety of the lipoprotein as the binding site. Arterioscler Thromb Vasc Biol. 1995 Feb;15(2):269-75.

[15] Huh HY, Pearce SF, Yesner LM, Schindler JL, Silverstein RL. Regulated expression of CD36 during monocyte-to-macrophage differentiation: potential role of CD36 in foam cell formation. Blood. 1996 Mar 1;87(5):2020-8.

[16] Saladin, KS. Chapter 26/Lipids, in Anatomy & Physiology: The Unity of Form and Function. 8th ed. McGraw Hill; 2017, p. 997-999.

[17] Fitó M, Guxens M, Corella D, et al. Effect of a Traditional Mediterranean Diet on Lipoprotein Oxidation: A Randomized Controlled Trial. *Arch Intern Med.* 2007;167(11):1195–1203.

[18] Bekkering S, Domínguez-Andrés J, Joosten LAB, Riksen NP, Netea MG. Trained Immunity: Reprogramming Innate Immunity in Health and Disease. Annu Rev Immunol. 2021 Apr 26;39:667-693.

[19] Schwartz EA, Zhang W-Y, Karnik SK, et al. Nutrient modification of the innate immune response: a novel mechanism by which saturated fatty acids greatly amplify monocyte inflammation. Arterioscler Thromb Vasc Biol. 2010;30:802–8.

[20] Riera-Borrull M, Cuevas VD, Alonso B, Vega MA, Joven J, Izquierdo E, Corbí ÁL. Palmitate Conditions Macrophages for Enhanced Responses toward Inflammatory Stimuli via JNK Activation. J Immunol. 2017 Dec 1;199(11):3858-3869.

[21] Fang C, Wang L, Qiao J, et al. Differential regulation of lipopolysaccharide-induced IL-1beta and TNF-alpha production in macrophages by palmitate via modulating TLR4 downstream signaling. Int Immunopharmacol. 2022;103:108456.

[22] Caslin HL, Cottam MA, Piñon JM, et al. Weight cycling induces innate immune memory in adipose tissue macrophages. Front Immunol. 2023;13:984859.

[23] Bekkering S, Quintin J, Joosten LA, van der Meer JW, Netea MG, Riksen NP. Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes. Arterioscler Thromb Vasc Biol. 2014 Aug;34(8):1731-8.

[24] Bekkering S, Arts RJW, Novakovic B, Kourtzelis I, van der Heijden CDCC, Li Y, Popa CD, Ter Horst R, van Tuijl J, Netea-Maier RT, van de Veerdonk FL, Chavakis T, Joosten LAB, van der Meer JWM, Stunnenberg H, Riksen NP, Netea MG. Metabolic Induction of Trained Immunity through the Mevalonate Pathway. Cell. 2018 Jan 11;172(1-2):135-146.e9.

[25] Keating ST, Groh L, Thiem K, Bekkering S, Li Y, Matzaraki V, van der Heijden CDCC, van Puffelen JH, Lachmandas E, Jansen T, Oosting M, de Bree LCJ, Koeken VACM, Moorlag SJCFM, Mourits VP, van Diepen J, Strienstra R, Novakovic B, Stunnenberg HG, van Crevel R, Joosten LAB, Netea MG, Riksen NP. Rewiring of glucose metabolism defines trained immunity induced by oxidized low-density lipoprotein. J Mol Med (Berl). 2020 Jun;98(6):819-831.

[26] Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. Cell Host Microbe. 2011 May 19;9(5):355-61.

[27] Melillo D, Marino R, Italiani P, Boraschi D. Innate Immune Memory in Invertebrate Metazoans: A Critical Appraisal. Front Immunol. 2018 Aug 22;9:1915.

[28] Pradeu T, Thomma BPHJ, Girardin SE, Lemaitre B. The conceptual foundations of innate immunity: Taking stock 30 years later. Immunity. 2024 Apr 9;57(4):613-631.

[29] Murphy, Kenneth (Kenneth M.), and Casey Weaver. *Janeway's Immunobiology*. 9th edition. New York, NY: Garland Science/Taylor & Francis Group, LLC, 2017. Print.

[30] Vuscan P, Kischkel B, Joosten LAB, Netea MG. Trained immunity: General and emerging concepts. Immunol Rev. 2024 May;323(1):164-185.

[31] Foster SL, Hargreaves DC, Medzhitov R. Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature. 2007 Jun 21;447(7147):972-8.

[32] Naeslund C. Resultats Des Experiences de Vaccination Par le BCG Poursuivies Dans le Norrbotten (Suède) (Septembre 1927 - Décembre 1931). In: Vaccination Préventive de la Tuberculose de l'homme et des animaux par le B C G Rapports et Documents Provenant Des Divers Pays (la France exceptée) transmis à l'Institut Pasteur en 1932. Paris, France: Mason and Cie; 1932:274–281.

[33] Calmette A. Preventive Vaccination Against Tuberculosis with BCG. Proc R Soc Med. 1931 Sep;24(11):1481-90.

[34] Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, Jacobs C, van Loenhout J, de Jong D, Stunnenberg HG, Xavier RJ, van der Meer JW, van Crevel R, Netea MG. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection

from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A. 2012 Oct 23;109(43):17537-42.

[35] Kleinnijenhuis J, Quintin J, Preijers F, Benn CS, Joosten LA, Jacobs C, van Loenhout J, Xavier RJ, Aaby P, van der Meer JW, van Crevel R, Netea MG. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. J Innate Immun. 2014;6(2):152-8.

[36] Kaufmann E, Sanz J, Dunn JL, Khan N, Mendonça LE, Pacis A, Tzelepis F, Pernet E, Dumaine A, Grenier JC, Mailhot-Léonard F, Ahmed E, Belle J, Besla R, Mazer B, King IL, Nijnik A, Robbins CS, Barreiro LB, Divangahi M. BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. Cell. 2018 Jan 11;172(1-2):176-190.e19.

[37] Bistoni F, Vecchiarelli A, Cenci E, Puccetti P, Marconi P, Cassone A. Evidence for macrophage-mediated protection against lethal Candida albicans infection. Infect Immun. 1986 Feb;51(2):668-74.

[38] Romani L. Immunity to fungal infections. Nat Rev Immunol. 2011 Apr;11(4):275-88.

[39] Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nat Rev Dis Primers. 2018 May 11;4:18026.

[40] Riksen NP, Netea MG. Immunometabolic control of trained immunity. Mol Aspects Med. 2021 Feb;77:100897.

[41] Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, Jacobs L, Jansen T, Kullberg BJ, Wijmenga C, Joosten LAB, Xavier RJ, van der Meer JWM, Stunnenberg HG, Netea MG. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe. 2012 Aug 16;12(2):223-32.

[42] Domínguez-Andrés J, Novakovic B, Li Y, Scicluna BP, Gresnigt MS, Arts RJW, Oosting M, Moorlag SJCFM, Groh LA, Zwaag J, Koch RM, Ter Horst R, Joosten LAB, Wijmenga C, Michelucci A, van der Poll T, Kox M, Pickkers P, Kumar V, Stunnenberg H, Netea MG. The Itaconate Pathway Is a Central Regulatory Node Linking Innate Immune Tolerance and Trained Immunity. Cell Metab. 2019 Jan 8;29(1):211-220.e5.

[43] Seufert AL, Hickman JW, Traxler SK, Peterson RM, Waugh TA, Lashley SJ, Shulzhenko N, Napier RJ, Napier BA. Enriched dietary saturated fatty acids induce trained immunity via ceramide production that enhances severity of endotoxemia and clearance of infection. Elife. 2022 Oct 20;11:e76744.

[44] Christ A, Günther P, Lauterbach MAR, Duewell P, Biswas D, Pelka K, Scholz CJ, Oosting M, Haendler K, Baßler K, Klee K, Schulte-Schrepping J, Ulas T, Moorlag SJCFM, Kumar V, Park MH, Joosten LAB, Groh LA, Riksen NP, Espevik T, Schlitzer A, Li Y, Fitzgerald ML, Netea MG, Schultze JL, Latz E. Western Diet Triggers NLRP3-Dependent Innate Immune Reprogramming. Cell. 2018 Jan 11;172(1-2):162-175.e14.
[45] Napier BA, Andres-Terre M, Massis LM, Hryckowian AJ, Higginbottom SK, Cumnock K, Casey KM, Haileselassie B, Lugo KA, Schneider DS, Sonnenburg JL, Monack DM. Western diet regulates immune status and the response to LPS-driven sepsis independent of diet-associated microbiome. Proc Natl Acad Sci U S A. 2019 Feb 26;116(9):3688-3694.

[46] Miyamoto J, Ohue-Kitano R, Mukouyama H, Nishida A, Watanabe K, Igarashi M, Irie J, Tsujimoto G, Satoh-Asahara N, Itoh H, Kimura I. Ketone body receptor GPR43 regulates lipid metabolism under ketogenic conditions. Proc Natl Acad Sci U S A. 2019 Nov 19;116(47):23813-23821.

[47] Wilder R.M. High fat diets in epilepsy. The Clinic Bulletin. 1921; 2: 308.

[48] Spigoni V, Cinquegrani G, Iannozzi NT, Frigeri G, Maggiolo G, Maggi M, Parello V, Dei Cas A. Activation of G protein-coupled receptors by ketone bodies: Clinical implication of the ketogenic diet in metabolic disorders. Front Endocrinol (Lausanne). 2022 Oct 20;13:972890.

[49] Gurr MI, Harwood JL, Frayn KN, Murphy DJ, Michell RH. Lipids: Biochemistry, Biotechnology and Health. 6th Edition. Wiley Blackwell, 2016.

[50] Chaurasia B, Summers SA. Ceramides - Lipotoxic Inducers of Metabolic Disorders. Trends Endocrinol Metab. 2015 Oct;26(10):538-550.

[51] Ruysschaert JM, Lonez C. Role of lipid microdomains in TLR-mediated signalling. Biochim Biophys Acta. 2015 Sep;1848(9):1860-7.

[52] Di Paola M, Cocco T, Lorusso M. Ceramide interaction with the respiratory chain of heart mitochondria. Biochemistry. 2000 Jun 6;39(22):6660-8.

[53] Dinarello CA. Historical insights into cytokines. Eur J Immunol. 2007 Nov;37 Suppl 1(Suppl 1):S34-45.

[54] Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. Front Immunol. 2014 Oct 7;5:491.

[55] Ryan DG, O'Neill LAJ. Krebs Cycle Reborn in Macrophage Immunometabolism. Annu Rev Immunol. 2020 Apr 26;38:289-313.

[56] Diskin C, Ryan TAJ, O'Neill LAJ. Modification of Proteins by Metabolites in Immunity. Immunity. 2021 Jan 12;54(1):19-31.

[57] Hotamisligil GS. Inflammation, metaflammation and immunometabolic disorders. Nature. 2017 Feb 8;542(7640):177-185.

[58] Christ A, Latz E. The Western lifestyle has lasting effects on metaflammation. Nat Rev Immunol. 2019 May;19(5):267-268.

[59] Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest. 2007 Jan;117(1):175-84.

[60] Perreault M, Zulyniak MA, Badoud F, Stephenson S, Badawi A, Buchholz A, Mutch DM. A distinct fatty acid profile underlies the reduced inflammatory state of metabolically healthy obese individuals. PLoS One. 2014 Feb 10;9(2):e88539.

[61] Summers SA, Chaurasia B, Holland WL. Metabolic Messengers: ceramides. Nat Metab. 2019 Nov;1(11):1051-1058.

[62] Gogos CA, Drosou E, Bassaris HP, Skoutelis A. Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. J Infect Dis. 2000 Jan;181(1):176-80.

[63] Chousterman BG, Swirski FK, Weber GF. Cytokine storm and sepsis disease pathogenesis. Semin Immunopathol. 2017 Jul;39(5):517-528.

[64] Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, Hotchkiss RS, Levy MM, Marshall JC, Martin GS, Opal SM, Rubenfeld GD, van der Poll T, Vincent JL, Angus DC. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016 Feb 23;315(8):801-10.

[65] Cheng SC, Scicluna BP, Arts RJ, Gresnigt MS, Lachmandas E, Giamarellos-Bourboulis EJ, Kox M, Manjeri GR, Wagenaars JA, Cremer OL, Leentjens J, van der Meer AJ, van de Veerdonk FL, Bonten MJ, Schultz MJ, Willems PH, Pickkers P, Joosten LA, van der Poll T, Netea MG. Broad defects in the energy metabolism of leukocytes underlie immunoparalysis in sepsis. Nat Immunol. 2016 Apr;17(4):406-13.

[66] Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, Joosten LAB, van der Meer JWM, Mhlanga MM, Mulder WJM, Riksen NP, Schlitzer A, Schultze JL, Stabell Benn C, Sun JC, Xavier RJ, Latz E. Defining trained immunity and its role in health and disease. Nat Rev Immunol. 2020 Jun;20(6):375-388.

[67] DiNardo AR, Netea MG, Musher DM. Postinfectious Epigenetic Immune Modifications - A Double-Edged Sword. N Engl J Med. 2021 Jan 21;384(3):261-270.

[68] Håversen L, Danielsson KN, Fogelstrand L, Wiklund O. Induction of proinflammatory cytokines by long-chain saturated fatty acids in human macrophages. Atherosclerosis. 2009 Feb;202(2):382-93.

[69] Korbecki J, Bajdak-Rusinek K. The effect of palmitic acid on inflammatory response in macrophages: an overview of molecular mechanisms. Inflamm Res. 2019 Nov;68(11):915-932.

[70] Richieri GV, Kleinfeld AM. Unbound free fatty acid levels in human serum. J Lipid Res. 1995 Feb;36(2):229-40.

[71] Alsharari ZD, Risérus U, Leander K, Sjögren P, Carlsson AC, Vikström M, Laguzzi F, Gigante B, Cederholm T, De Faire U, Hellénius ML, Marklund M. Serum Fatty Acids, Desaturase Activities and Abdominal Obesity - A Population-Based Study of 60-Year Old Men and Women. PLoS One. 2017 Jan 26;12(1):e0170684.

[72] Vincent JL, Marshall JC, Namendys-Silva SA, François B, Martin-Loeches I, Lipman J, Reinhart K, Antonelli M, Pickkers P, Njimi H, Jimenez E, Sakr Y; ICON investigators. Assessment of the worldwide burden of critical illness: the intensive care over nations (ICON) audit. Lancet Respir Med. 2014 May;2(5):380-6.

[73] Papadimitriou-Olivgeris M, Aretha D, Zotou A, Koutsileou K, Zbouki A, Lefkaditi A, Sklavou C, Marangos M, Fligou F. The Role of Obesity in Sepsis Outcome among Critically Ill Patients: A Retrospective Cohort Analysis. Biomed Res Int. 2016;2016:5941279.

[74] Yaegashi M, Jean R, Zuriqat M, Noack S, Homel P. Outcome of morbid obesity in the intensive care unit. J Intensive Care Med. 2005 May-Jun;20(3):147-54.

[75] Wischmeyer PE. Nutrition Therapy in Sepsis. Crit Care Clin. 2018 Jan;34(1):107-125.

CHAPTER 2:

A new frontier for fat: dietary palmitic acid induces innate immune memory

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Abstract

Dietary saturated fats have recently been appreciated for their ability to modify innate immune cell function, including monocytes, macrophages, and neutrophils. Many dietary saturated fatty acids (SFAs) embark on a unique pathway through the lymphatics following digestion, and this makes them intriguing candidates for inflammatory regulation during homeostasis and disease. Specifically, palmitic acid (PA) and diets enriched in PA have recently been implicated in driving innate immune memory in mice. PA has been shown to induce long-lasting hyper-inflammatory capacity against

secondary microbial stimuli in vitro and in vivo, and PA-enriched diets alter the developmental trajectory of stem cell progenitors in the bone marrow. Perhaps the most relevant finding is the ability of exogenous PA to enhance clearance of fungal and bacterial burdens in mice; however, the same PA treatment enhances endotoxemia severity and mortality. Westernized countries are becoming increasingly dependent on SFA-enriched diets, and a deeper understanding of SFA regulation of innate immune memory is imperative in this pandemic era.

1. Introduction

The innate immune system acts as an efficient non-species-specific shield against infection that thwarts a diverse array of pathogens, and has recently been shown to be highly influenced by the nutritional milieu of the host. Specifically, in humans, the innate immune response has been adapting to constantly changing dietary patterns and, more recently, a variety of diets with enriched saturated fatty acids (SFAs). Decades of research have been dedicated to understanding how SFAs modulate innate immune cell function and inflammatory capacity [1,2]; however, it has only recently been described that enriched dietary SFAs can induce innate immune memory.

Innate immune memory is characterized by epigenetic and metabolic changes within macrophages and monocytes induced by a primary inflammatory stimulus that leads to an enhanced (trained immunity or priming) or decreased (tolerance) response to a secondary inflammatory stimulus [3]. Trained immunity and priming are primitive adaptations of innate host defense that results from exposure to a primary inflammatory stimulus, and

leads to a faster and greater inflammatory response to a secondary homologous or heterologous challenge [4]. Hallmark features of trained immunity specifically include metabolic alterations that induce long-lasting epigenetic changes within innate immune cells, remodeling of the hematopoietic stem cell (HSC) compartment that allows for a sustained augmented response from developing myeloid cells, an amplified inflammatory response to pathogenic infection, and transmission of epigenetic memory across generations [5–9]. In contrast, the effects of priming do not involve long-term epigenetic modifications or HSC remodeling; however, priming does allow a cell to respond more strongly to secondary inflammatory stimuli, but only if the secondary stimulus takes place before the cell returns to basal inflammation and homeostasis [3]. The most studied inducers of trained immunity are the Bacillus Calmette-Guerin (BCG) vaccine and the fungal antigen, β-glucan. BCG and β-glucan induce cross-protection against heterologous pathogens in humans and in mice, respectively, including cross-protection against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [4,10]. Trained immunity is also known for its dual nature, and can be beneficial or detrimental to the host depending on the disease context and inflammatory status.

There is little known about the impact of dietary SFAs on innate immune memory; however, SFA-enriched diets have been shown to induce long-lasting impacts on innate immune inflammation and microbial infection in mice, via remodeling of the HSC compartment in the bone marrow [11,12]. Specifically, enriched dietary palmitic acid ([PA]; C16:0), the most prevalent SFA found in the diet and circulating in human blood, has been shown to induce trained immunity within myeloid cells, and plays an important

role in both homeostasis and infection in vivo [1,12,13]. PA is known to enhance toll-like receptor (TLR)-dependent inflammation by inducing ceramide metabolism, and sensitizes innate immune cells to subsequent TLR stimulation [12,14–16]. During endotoxemia (lipopolysaccharide [LPS]-induced acute systemic shock), mice pre-exposed to PA exhibit enhanced circulating inflammation, disease severity, and mortality compared to vehicle (Veh)-treated mice and showed significantly greater clearance of fungal and bacterial infections compared to Veh-treated mice [12,13]. In an obese mouse model of weight cycling, the adipose tissue (AT) environment skewed resident macrophages toward heightened inflammatory response to microbial stimulation, and this was comparable to a bone marrow-derived macrophage (BMDM) model that used PA pre-treatments to enhance LPS stimulation [17]. The molecular details of SFA-induced and/or PA-induced trained immunity in vitro and in vivo have not been fully defined; however, in light of these exciting results, we will review here the specific relationship between PA and innate immune memory, and discuss the implications of our known data and the need for future investigations that will elucidate the intricate dynamics of this relationship.

2. PA mediates macrophage metabolism and function

Trained immunity in macrophages is accompanied by direct crosstalk between metabolites from the Krebs cycle and histone modifying enzymes necessary for enhanced transcription of pro-inflammatory cytokines via epigenetic mechanisms [18]. It is well known that specific metabolic pathways mediate the transcription and release of pro- and

anti-inflammatory cytokines by macrophages [19,20]. Glycolytic metabolism in the cytosol is upregulated in pro-inflammatory macrophage responses, and is accompanied by breaks in the Krebs Cycle that allow for accumulation of metabolites that also support the Warburg effect (ie, aerobic glycolysis) [21–23]. In contrast, oxidative phosphorylation in the mitochondria is upregulated, and the Krebs cycle remains intact, in order to support anti-inflammatory macrophage responses [19]. The plasticity of macrophage inflammatory polarization is governed by tightly regulated metabolic pathways that can be disrupted by excessive exposure to dietary SFAs, including PA [12,16,24].

PA is the most common SFA found in the human body (20%–30% total FAs) and is enriched in meat, dairy products (50%–60% of total fats), and is nearly 30% of total fats in breast milk [2,25]. The western diet (WD) contains high levels of FAs, specifically PA, and sucrose; the ketogenic diet (KD) is exclusively enriched in FAs, and depending on the dietary structure, it may contain excess PA content. In addition to dietary sources, PA can be synthesized endogenously throughout the mammalian body from other FAs, carbohydrates, and amino acids [2]. PA is known as an immunomodulatory molecule, and it has the capacity to regulate inflammatory processes of innate immune cells, including monocytes, macrophages, and neutrophils [12,16,26].

Historically, it was believed that PA was a ligand for TLR4 and induced TLR4-dependent inflammatory cytokine production [27]. However, an elegant study has recently shown that PA is not a ligand for TLR4, but enhances activation of multiple TLR signaling pathways and subsequent NF-κB-dependent transcription of inflammatory cytokines through c-Jun

N-terminal kinase (JNK) activation that is dependent on mitochondrial metabolic regulation via mammalian target of rapamycin (mTOR) [24]. These studies were conclusive in determining PA is not a TLR4 ligand, but much still remains to understand how PA is metabolically enhancing TLR-dependent inflammation in macrophages.

Importantly, when there is an excess of PA in the diet, it is reflected in an increase of free PA systemically [1,28]. When macrophages and monocytes are exposed to excess free PA, it is taken up through the membrane scavenger receptor, CD36. CD36-dependent intake of PA can lead to lipid accumulation and modulation of signaling through metabolic dysfunction, including suppressing AMPK activation [29,30]. It has been found that genetic loss of *Cd36* renders murine macrophages insensitive to some TLR2 ligands and induces hyper-susceptibility of mice to *Staphylococcus aureus* infection [31]. Considering this, inhibition of free PA uptake by genetic deletion of *Cd36* leading to a dampened inflammatory response may contribute to enhanced susceptibility to infection and decreased TLR2-mediated inflammation. Currently, it is unknown if CD36 plays a role in PA-induced innate immune reprogramming, but it is clearly required for PA-dependent inflammatory phenotypes.

After CD36-dependent uptake of PA by the macrophage, PA is converted into phospholipids, diacylglycerol (DAG), and ceramides reviewed here [1]; however, in the presence of excess PA, triglyceride (TAG) synthesis is stalled at the DAG stage causing accumulation in the cell [1]. Excess PA also leads to enhanced macrophage expression of adipose fatty acid binding protein, which acts as a PA chaperone during uptake and

enhances ceramide synthesis [32]. Both DAG and ceramides have been shown to enhance TLR-mediated signaling cascades in macrophages and subsequent activation of NF-κB [1].

Ceramide is a bioactive sphingolipid (SGL) with cell signaling capabilities, and there are three metabolic pathways that can lead to intracellular ceramide synthesis: the de novo pathway, sphingomyelin (SM) hydrolysis, and the endosomal salvage pathway [33]. Stimulating macrophages with LPS induces ceramide synthesis via SM hydrolysis [34]. In contrast, de novo ceramide synthesis in the presence of excess PA is important for increasing TLR4 activation and cytokine production in primary peritoneal macrophages; however, ceramide produced via SM hydrolysis was shown to regulate LPS-induced interleukin 6 (IL-6) secretion in PA-treated RAW macrophages [14,15]. In addition, our laboratory recently showed, using primary BMDMs, that de novo ceramide synthesis is required for PA-induced hyperinflammation in response to LPS [12]. Moreover, inhibiting de novo ceramide synthesis completely abolished the hyper-inflammatory impact with respect to tumor necrosis factor (TNF), however only partially inhibited IL-6-mediated and IL-1β-mediated hyperinflammation [12]. Future studies will benefit from determining precisely which pathway(s) of ceramide synthesis may be responsible for driving innate immune cell reprogramming and which proteins and signaling pathways are targeted by ceramide, as any of these have the potential to be therapeutic targets for inflammatory diseases driven by ceramide.

Together, these data conclude PA is not a TLR ligand, but enters macrophages through CD36, and subsequently mediates metabolism to enhance TLR-dependent cytokine production, which may play a critical role in regulating host response to infection and inflammatory diseases. Importantly, the term excess dietary PA is subjective, and likely depends on disease context, and the ability of the host to regulate homeostasis between endogenous and exogenous PA levels; however, physiologically relevant serum PA concentrations have been mimicked in vitro and in vivo to show a significant impact on metabolic pathways that alter macrophage inflammation [12,14,15,24,35]. To target PA metabolism of macrophages therapeutically, future studies should determine the specific threshold of intracellular PA levels that, when exceeded, can lead to metabolic and inflammatory dysregulation.

3. Exogenous PA and trained immunity

Trained immunity is a feature of innate immune cells that illuminates their capacity for memory via non-specific stimulation [4]. The training phenomenon is unlike memory exhibited by adaptive immune cells because it does not require gene recombination or clonal expansion as lymphocytes do following specific antigen recognition. Instead, innate immune cells acquire memory through metabolic rewiring following encounter with a stimulus, and this induces epigenetic modifications that continue to influence transcription of cytokine genes for weeks to years upon secondary stimulation [4]. Trained immunity has been studied mostly in the context of vaccination with BCG, or microbial ligand stimulation using the fungal molecule β-glucan, but recently dietary products have

been shown to elicit memory capability within innate immune cells [6,8,11,12]. It has recently been proposed that exogenous PA is capable of altering the metabolic and epigenetic landscape of monocytes and macrophages in order to initiate a non-specific, hyper-inflammatory memory response to secondary stimulation with a microbial ligand [12]. Although the precise impact of PA-mediated metabolism on the monocyte/macrophage epigenome has yet to be described, numerous features of innate immune memory, specifically trained immunity, have been revealed by studies involving exogenous PA treatments in vitro and in vivo.

Many studies show the inflammatory impact of combined PA and TLR ligand treatment on monocytes and macrophages; however, few studies show the effect of pre-treating cells with PA followed by TLR agonist or microbial challenge (Table 1). Pre-treatment experiments allow us to understand if the PA-dependent metabolic and epigenetic changes can alter short-term or long-term inflammatory response to secondary stimulation with a microbial challenge (trained immunity). Pre-treatment can be a model for a host that is exposed to excess dietary PA and then challenged by an infection, and these studies can inform how PA may be involved in the induction of innate immune memory.

It was recently shown that immortalized human and mouse macrophage cell lines that were pre-treated with exogenous PA exhibited enhanced LPS-induced TNF secretion [37]. Schwartz et al. showed, in THP-1 monocytes, that the hyper-inflammatory response to LPS following PA-pre-treatment was dependent on ceramide-mediated activation of

protein kinase C and mitogen-activated protein kinase signaling pathways [35]. While the mechanism for augmented TNF secretion was not defined by Fang et al., the hyper-inflammatory effect of PA-pre-treatment was associated with enhanced phosphorylation of p38 and JNK, and enhanced expression of carnitine palmitoyltransferase1A, an enzymatic shuttle on the outer membrane of mitochondria that facilitates uptake of activated fatty acids into the mitochondrial matrix for β-oxidation [37]. These data suggest that mitochondrial metabolism plays an important role in the hyper-inflammatory impact of PA-pre-treatment and subsequent challenge with LPS in macrophages [35,37]. These studies, however, did not distinguish if PA pre-treatment was inducing priming or trained immunity, but they did suggest that PA can alter macrophages to respond more acutely to secondary LPS challenge.

Our team recently showed that PA-pre-treatment of BMDMs subsequently challenged with LPS enhanced TNF, IL-6, and IL-1β release; this was dependent on ceramide synthesis, and reversible when BMDMs were pre-treated simultaneously with both PA and the monounsaturated FA (MUFA) that diverts ceramide synthesis in the presence of PA, oleic acid (OA) [12]. Additionally, the synergistic effect of PA and LPS was shown in primary mouse peritoneal macrophages; however, only simultaneous treatment was used to show significantly enhanced de novo ceramide synthesis, and significantly enhanced TNF and IL-6 secretion [14]. Thus, they could not conclude if this was trained immunity. This effect of combined PA and LPS treatment was recapitulated in RAW murine macrophages to show that ceramide mediates LPS-induced IL-6 secretion via JNK

phosphorylation; interestingly, this process was regulated by fatty acid transporter 1, and the requirement of CD36 was not shown [36].

Although these studies have been immensely important in describing the effect of PA on inflammation, it is still unclear if PA is inducing priming or trained immunity induced in macrophages. Thus, future studies regarding the role of PA in innate immune memory should determine the following: (1) The time point of initial inflammatory release in primary stimulation of macrophages with PA; (2) a return to basal inflammation; and (3) hyperinflammation upon secondary stimulation. Together, these outcomes would bolster the hypothesis that PA induces macrophage-trained immunity in vitro.

In vivo, exogenous PA has been shown to play an important role during infection. Specifically, in *Rag1*$^{-/-}$ mice that lack adaptive immunity, an intraperitoneal (i.p.) injection of a PA solution 12 hours prior to intravenous (i.v.) infection with *Candida albicans* lead to a significant decrease in kidney fungal burden, compared to infected mice only pretreated with a Veh solution [12]. This suggests that PA-induced memory mediates microbial clearance, and this is dependent on innate immune cells. Further, mice injected i.p. with a PA solution 12 hours prior to LPS-induced endotoxemia show significantly enhanced *Tnf* and *Il-6* expression in the blood within 5 hours post-LPS [12]. Importantly, blood draws were taken immediately before LPS injections to show that baseline inflammation was not upregulated in PA-treated mice, suggesting that LPS-induced cytokine expression in the blood was not a priming effect induced by PA, but rather a trained immunity phenomenon [12].

Further, in a study by Reyes et al., defining the role of exogenous PA in *Brucella abortus* infection in mice, oral gavage of PA was shown to significantly reduce splenic bacterial burden when administered for 3 days before, and during 14 days of *B. abortus* infection following an i.p. challenge [13]. This was accompanied by a suppression of serum IL-10; however, the mechanism underlying enhanced bacterial clearance induced by PA remains unknown. It is not entirely clear whether these data represent PA-induced priming or trained immunity, because there was no resting period after 3-day PA exposure, or determination of basal inflammatory status before infection with *B. abortus*. The results are still compelling, and to our knowledge, they are the first to show the impact of dietary PA on the clearance of pathogenic bacteria in vivo. Lastly, as mentioned previously, canonical-trained immunity induced by β-glucans or BCG vaccine leads to long-term metabolic and functional reprogramming of myeloid cells, and relies on epigenetic alterations for sustained inflammatory capacity. Remarkably, PA-induced memory was shown to elicit long-lasting immune reprogramming in vivo using an LPS-induced endotoxemia mouse model [12]. Specifically, 9 daily i.p. injections of PA followed by a 7-day resting period and subsequent LPS challenge led to enhanced endotoxemia severity and mortality compared with mice injected with a Veh solution [12]. PA-injected mice showed significantly increased hypothermia compared with Veh-injected mice, and while mortality was enhanced in PA-injected mice, this survival defect was not significant compared with Veh-injected mice. Thus, PA exacerbates endotoxemia severity, but this is not sufficient to significantly decrease survival in an endotoxemia mouse model [12]. These data suggest that PA regulates epigenetic modifications that persist beyond the

time points of PA exposure, and these alterations adversely impact the ability of the host to regulate body temperature in response to LPS challenge. Thus, PA induces trained immunity in this context, and not priming. Additional experiments will be required to determine whether PA depends solely on innate immune memory mechanisms to exert the long-term adverse effects described here.

Together, these studies build a compelling case that exogenous PA modulates microbial-induced inflammation and clearance in vitro and in vivo. Importantly, the effects of PA exposure in vivo are long-lasting, and PA-exposed mice do not exhibit heightened circulating inflammation before infection, indicating that PA is inducing trained immunity and not priming. The intracellular mechanisms of PA-induced memory and the subsequent augmenting effects on microbial ligand stimulation are not fully characterized. Although intracellular ceramide has been shown to mediate PA-induced memory in macrophages, it is still unclear how the metabolism of PA may lead to epigenetic alterations that ultimately modify the expression of inflammatory response genes.

4. Enriched dietary PA, trained immunity, and obesity

Thus far we have described experimental data involving exogenous PA and its effects on monocyte and macrophage metabolism, inflammation, and memory, in addition to in vivo models of PA injections (i.p.) and oral gavage. Next, we outline the impacts of enriched dietary PA consumption on the development of innate immune cells in the bone marrow,

and how this may contribute to long-term innate immune memory, specifically trained immunity.

Recent studies from our group and others have begun to understand the effect of excess chronic dietary PA on systemic response to microbial challenge and macrophage function ex vivo. Christ et al. found that atherosclerotic mice (*Ldlr*$^{-/-}$ C57BL/6) fed WD for 4 weeks resulted in a hyper-inflammatory response when challenged with LPS ex vivo [11], suggesting exposure to excess dietary PA may be a factor in influencing the inflammatory capacity of myeloid cell populations in vivo. More recently, we have reported wild-type (WT) mice fed WD and KD, both enriched in PA, exhibit increased systemic inflammation in response to endotoxemia, a single-intraperitoneal injection of LPS [12,38]. Importantly, we show that this enhanced systemic inflammation in response to LPS is independent of glycolytic shock and the diet-induced microbiome, further suggesting that enriched dietary PA is leading to an enhanced response to TLR4 agonist LPS [12]. Together, these studies suggest a direct link between enriched dietary PA and trained immunity in vivo.

A hallmark of canonical-trained immunity in vivo is the skewing of the HSC compartment toward increased myeloid cell production and enhanced inflammatory capacity [8,11]. It has been shown that 4 weeks of WD administration was sufficient to alter HSC populations within the bone marrow of atherosclerotic mice; and after reverting back to a standard chow for an additional 4 weeks, stem cell progenitors remained skewed toward developing monocytes with hyper-inflammatory potential [11].

More recently, we published that WT BALB/c mice on WD for 2 weeks do not exhibit altered HSC populations [12]. Importantly, these studies may disagree due to the use of different mouse models (*Ldlr*$^{-/-}$ C57BL/6 vs WT BALB/c) and the length of diet administration; more follow-up studies are required to understand this bifurcation. However, we have additionally shown that exposure to a PA-enriched KD skews the HSC compartment to develop significantly enhanced populations of long-term and short-term HSCs and multipotent progenitors [12]. Importantly, no one has shown whether PA is responsible for the HSC skewing in either of these models.

As mentioned previously, trained immunity can last days to years after initial induction with a primary inflammatory stimulus. While the endurance of dietary PA-induced memory remains unknown, the impacts exhibit hallmark features of trained immunity, including (1) the induction of long-term hyper-inflammatory capacity toward microbial stimuli, (2) the low basal inflammatory status shown in vitro and in vivo following PA treatments before secondary stimulation, (3) the enhanced clearance of microbial infection in vivo, and (4) the induction of HSC remodeling that mimics HSC remodeling in BCG-induced trained immunity [8].

Alongside a growing body of evidence that shows dietary SFAs disrupt metabolic and inflammatory homeostasis, many disorders characterized by immunometabolic dysfunction are associated with excessive WD exposure, including obesity, type II diabetes, cardiovascular disease, neurodegenerative, and autoimmune diseases—all filed under the umbrella term, metabolic syndrome [39]. While obesity is not solely a diet-

driven disease, weight gain is the most observable side effect, and as a result, weight gain is often misrepresented as the driver of enhanced susceptibility to poor outcome during infectious disease. However, our laboratory has shown that WD-induced weight gain does not correlate with endotoxemia severity and mortality in WT mice [38]. This suggests that dietary constituents of the WD, and not the ensuing weight gain, are the drivers of innate immune reprogramming and enhanced susceptibility to secondary microbial exposure. A paradox currently exists in the literature that shows a diversity of severity and mortality outcomes in obese sepsis patients, whereby obesity appears to be protective in some studies, whereas others show that obesity correlates with exacerbated sepsis outcome [40]. Sepsis is characterized by widespread inflammatory dysregulation, brought on by an infection that has entered the bloodstream, and it can be induced by a variety of microbial pathogens [41]. Interestingly, obese populations have been shown to exhibit variability in serum PA levels and also metabolic health—indicated by the presence or absence of insulin resistance [42,43]. For example, a subset of the obese population is considered metabolically healthy if they do not exhibit insulin resistance, and this population exhibits significantly lower levels of PA in their serum compared with the serum of metabolically unhealthy obese individuals [42]. We believe that this correlation between PA levels and metabolic health in obesity, in addition to the obesity paradox in inflammatory disease outcome in humans, align with the variability in disease outcome that PA-induced memory exhibits in mouse studies of trained immunity. Specifically, the widely acknowledged duality of trained immunity is a key feature of this memory phenomenon that explains the ability of SFAs like PA to impose beneficial, or

detrimental, inflammatory disease outcomes that are likely dependent on the type of microbial species acting as the secondary stimuli and disease trigger.

In harmony with numerous obesity studies that highlight the metabolic and inflammatory dysregulation that occurs alongside diet-induced obesity, weight cycling was recently considered in Caslin et al.'s work using a unique BMDM model, whereby adipose tissue (AT) harvested from weight-cycled mice (high fat-diet-induced obesity followed by low fat-diet-induced weight loss within a 9-week period) was used to condition culture media for BMDMs. They showed that, similar to BMDMs given a 24-hour PA pre-treatment, the BMDMs given AT-conditioned media from weight-cycled mice were hyperresponsive to subsequent LPS treatments (TNF and IL-6). They also showed that AT macrophages (ATMs) from weight-cycled mice exhibited enhanced TNF/IL-6 secretion with ex vivo LPS stimulation, compared with ATMs from lean mice [17]. This is important because it suggests that secretions that are put out into the AT environment, not only during obesity, but also—and surprisingly even more so—following subsequent weight loss are capable of reprogramming the immune response of primary macrophages. Moreover, the AT environment likely includes PA as a result of adipocyte lipolysis; however, whether or not PA was the driver of enhanced inflammatory responses shown in their experiments is unknown. How this may impact the host response to subsequent infection is currently of paramount importance, as metabolic syndrome and increased risk of pathogenic exposure are both increasingly evident public health concerns.

Although it was not the main finding of Caslin et al.'s weight cycling study, it is notable that their use of a canonical-trained immunity model using BMDMs showed that PA pre-treatment, followed by a 6-day resting period, not only enhanced TNF/IL-6 secretion in response to subsequent TLR ligand stimulation but also to β-glucan stimulation [17]. Importantly, this hyper-inflammatory response to β-glucan was not present in BMDMs lacking TLR4, suggesting that although PA does not signal through TLR4, PA-induced memory still requires TLR4 in order to enhance the innate immune response to fungal molecules specifically. To our knowledge, this is the first study that shows PA-induced memory requires TLR4 in order to augment the innate immune response to β-glucan. Moreover, our laboratory recently showed that *Rag1/2*$^{-/-}$ mice given i.p. PA injections 12 hours before infection with *Candida albicans*, a fungal pathogen that is recognized by innate immune cells via β-glucan, exhibited enhanced clearance of kidney fungal burden compared with mice only pre-exposed to a Veh solution [12]. Although the studies surrounding PA-induced immunomodulation are still in their infancy, the ability of PA to induce long-lasting, hyper-inflammatory responses to microbial ligands isolated from bacterial and fungal pathogens via innate immune pattern recognition receptors may lend insight into the diversity of diet-induced disease responses currently referred to as paradoxical in the literature. For example, the opposing disease outcomes shown in mice exposed to TLR ligands such as LPS, or Dectin-1 ligands like β-glucan, following the induction of PA-induced innate immune memory, may indicate that not only does the amount and duration of PA exposure play a role in disease outcome following secondary exposure to microbial ligands, but also the specific microbial species themselves that act

as the secondary inflammatory trigger may determine whether or not PA-induced memory will lead to a beneficial or detrimental disease outcome. Future studies should identify the infectious disease contexts that may benefit from prior innate immune modulation via dietary PA, in addition to the infectious agents that may exacerbate the host inflammatory response following dietary PA exposure. Moreover, personalized dietary intervention that uses serum PA measurements may improve inflammatory disease treatments, and prevention strategies for individuals susceptible to infectious disease.

A common co-morbidity in obese patients is the leaky gut that often occurs following a chronic low-fiber diet. Commensal gut microbes metabolize dietary fiber, and during fiber deficiency they will instead feed on the mucus lining the gut, leading to a breach in the epithelial barrier, followed by a release of microbiota-associated LPS into the bloodstream, and potentially life-threatening opportunistic infections [44,45]. Microbial molecules like LPS that breach this barrier will first induce a pro-inflammatory effect known as metabolic inflammation, or metaflammation, characterized by a chronic state of heightened pro-inflammatory cytokine levels in the blood and tissues [46]. Chronic metaflammation can tolerize innate immune cells to microbial ligand exposure, and render them ineffective in response to infection, which can promote immunoparalysis following acute systemic infection during sepsis [38]. Thus, another paradox may arise in considering the induction of trained immunity via dietary PA, whereby exposure to PA may initially cause hyperinflammation in response to secondary ligand exposure; however, if PA exposure if excessive and chronic, innate immune tolerance may play a

role in dampening the host response to infection. We believe that serum lipidomics and personalized nutrition will be necessary in order to balance levels of PA and PA-saturated lipids in the blood of individual patients, to prevent an imbalance in that may skew patients toward hyper-responsive or hypo-responsive inflammatory response during specific disease contexts (local infection vs systemic infection or sepsis).

5. PA from the mouth to the bone marrow

To determine the holistic impact of dietary PA on innate immune memory, we must also consider the pathway that PA follows after digestion, and the byproducts of PA metabolism that may impact innate immune cells enroute to various tissues for energy or storage purposes. PA and its metabolic byproducts encounter innate immune cells before being distributed to tissues, because many fats, depending on their size and diffusion capability, circulate in lipoprotein structures throughout the lymphatics even before entering the bloodstream [47]. Dietary PA digestion, absorption, and mobilization to tissues is complex compared to that of carbohydrates and proteins, which are transported directly to the liver via the superior mesenteric and hepatic portal veins immediately following digestion, and absorption from the small intestine into the blood circulation (Figure 1) [48,49].

The basic structure of PA is a 16-carbon chain saturated with hydrogen, and thus too large to diffuse from the small intestine directly into capillaries leading to the blood [50]. PA requires the emulsification action of bile, and ferrying within bile micelles toward the luminal epithelium of the small intestine [51] (Figure 1). Diffusion can then occur from the

micelles that act as PA carriers, into the enterocytes of the small intestine, where they are broken down, reassembled into triglycerides (TAGs), and packaged into chylomicrons before being taken up by lymphatic lacteals [52]. Chylomicrons are structures with a lipid membrane that contain hydrophobic lipids and proteins internally, and they enter into the lymphatics via lacteals, small ducts at the ends of lymphatic vessels within microvilli of the small intestine. After circulating through the lymph, PA-carrying chylomicrons enter the venous circulation via the thoracic duct and the lymphatic ducts near the subclavian veins. Then, the chylomicrons travel through the heart and lungs and are distributed to the remaining peripheral tissues including AT, skeletal muscle, bone marrow, and finally the liver, where they can be used for energy for various metabolic processes, incorporated into lipid membranes for cells and organelles, or stored as TAGs for later use [52]. The CO_2 waste from PA digestion is excreted via the respiratory system, and the term chylomicron remnants is often used to describe the final remaining TAGs that are taken to the liver after distribution throughout the rest of the body.

There are many opportunities for innate immune cells to encounter PA, which can be in the form of free fatty acids (FAs), TAGs, phosphatidylcholines, and SGLs while circulating in the lymphatics and blood to be distributed to brain, skeletal muscle, adipose, bone marrow, liver, and spleen tissues for energy use or storage [53]. Of particular importance is in the bone marrow, where HSCs may be reprogrammed by dietary PA, and then later differentiate into more specific progenitor cells with enhanced inflammatory capacity [11,12,54]. It is important to consider the likelihood that reprogrammed HSCs may then seed tissues during stress or a localized infection, and

exacerbate inflammation potentially leading to disruption of inflammatory homeostasis within a tissue or whole organ.

This unique pathway that PA embarks on following digestion, and its ability to be transported within chylomicrons to the bone marrow, is especially important for the long-term nature of innate immune memory. Because of the short lifespan of circulating monocytes (1–3 days) before their differentiation into tissue-specific macrophages, it seems unlikely that these cell types would retain long-term, non-specific memory; however, PA-induced HSC reprogramming may have the potential to harbor epigenetically modified progenitor cells within the bone marrow that can be subsequently recruited to infected tissues, and respond with hyper-inflammatory output to quickly clear infection. Moreover, in the context of dysregulated inflammation, such as in a septic response, trained HSCs would likely provide too much inflammation and exacerbate disease via tissue damage. Considering that dietary SFAs can be transported to the bone marrow and alter HSC populations, the role of dietary PA in bone marrow remodeling is crucial to consider when determining therapeutic intervention strategies for infection and inflammatory diseases among populations that consume PA-enriched diets.

6. Plasticity of PA-dependent innate immune memory

There is evidence that PA-dependent innate immune memory induces metabolic and inflammatory changes that are reversible, and in contrast to PA exerting pro-inflammatory effects, certain unsaturated FAs (UFAs) are known to promote an anti-inflammatory outcome. It is now appreciated that PA accumulation can lead to

pathophysiological changes and inflammation when there is an imbalance of dietary PA/MUFAs. OA is the second most prevalent FA in the blood next to PA, and it has been shown to induce low levels of inflammation in macrophages in vitro at certain concentrations [55]. However, OA is considered to be an anti-inflammatory MUFA, due to its ability to counteract or reverse the inflammatory impacts of SFAs in vitro [55]. For example, in mouse peritoneal macrophages, OA reverses PA-induced and stearic acid (SA)-induced IL-1β secretion [56]. In vivo, OA injections have been shown to mitigate the endotoxemia severity and mortality that is exacerbated in KD-fed mice [12]. The polyunsaturated FA (PUFA), docosahexaenoic acid, has also been shown to counteract the inflammatory effects of PA in RAW macrophages and BMDMs, displaying a specific target against de novo synthesis of ceramide [57–59]. Thus, specific ratios of SFAs to MUFAs, or SFAs to PUFAs in the blood, bone marrow, and peripheral tissues, such as liver, spleen, adipose, skeletal muscle, and the central nervous system, will be important to consider when studying the regulatory impact of FAs on innate immune inflammation in vivo.

It is tempting to consider the possibility that a metabolic rheostat exists between trained immunity and inflammatory homeostasis and that can be controlled and balanced with specific ratios of dietary SFAs to UFAs; if inflammation is required to clear microbial burden, then SFAs may work to upregulate it, and if tissue homeostasis requires restoration after a hyper-inflammatory event, then UFAs may serve to downregulate inflammation before it becomes damaging (Figure 2). If innate immune cells must adapt to a changing dietary environment, they may be required to increase or decrease their

uptake of SFAs and UFAs, and perhaps this function is perturbed during excessive and prolonged exposure to SFAs.

7. Conclusion: where to next?

The studies we present here provide compelling evidence that PA is an immunomodulating SFA that reprograms the innate immune cell response to secondary inflammatory stimuli in vitro and in vivo, that is, PA induces innate immune memory. Although some outcomes suggest that PA induces priming, more recent investigations show the long-term impact of PA on innate immune inflammatory regulation. This long-term nature of PA reprogramming, in addition to the ability of PA to alter secondary inflammatory outcomes even after basal inflammation has been reached, strongly supports the hypothesis that PA induces trained immunity.

For future in vivo experiments, more long-term studies need to be done to collectively elucidate the following: (1) The physiologically relevant PA concentrations that induce the initial inflammatory response—are they relevant to consumers of SFA-enriched diets? (2) The initial inflammatory response to dietary PA—is it systemic or localized to specific tissues? (3) The minimum and maximum rest periods from PA exposure required to return to inflammatory homeostasis and still maintain innate immune memory reprogramming—how long does the memory last? (4) The specific metabolites and histone modifications that are required for epigenetic reprogramming of innate immune

cells—what is the mechanism behind the augmented inflammatory response to secondary stimulation? Addressing these questions will advance our knowledge of PA-mediated trained immunity and how it regulates innate immune homeostasis, inflammation, and the response to infection.

PA constitutes 20%–25% of human breast milk, and if PA induces trained immunity, it is interesting to postulate that enhanced maternal dietary intake of PA may induce trained immunity in nursing neonates [60]. Enhanced PA within maternal milk may influence the capacity of a neonate to induce inflammation and or enhance protection against microbial challenge. Interestingly, a study from Du et al. shows that maternal WD consumption in mice causes the production of milk that contains excessive PA, ceramide accumulation, and inflammation in nursing neonates [61]. Additionally, this ceramide accumulation and inflammation in nursing neonates was TLR4/2 dependent; however, they did not look into how enhanced PA milk effected neonatal (1) protection against microbes, (2) neonate HSC composition, or (3) neonatal macrophage response to LPS challenge.

Although most immunological studies highlight the impact of PA on monocytes and macrophages, neutrophils may also play a role in SFA-induced inflammation and innate immune memory. For example, neutrophil recruitment and inflammation are enhanced by PA-treated BMDMs through a chemotactic response mechanism. Specifically, PA induces pannexin channel formation and nucleotide release, which then attracts neutrophils to the high-SFA region that macrophages were exposed to [62]. Other dietary SFAs are also known to stimulate the release of neutrophil precursors from the bone

marrow of mice into the bloodstream [63]. Importantly, the direct impact that PA has on neutrophil metabolism and subsequent inflammation has not been fully studied. Future studies should focus on illustrating the impact that PA-enhanced neutrophil recruitment may have on inflammatory homeostasis, and the host response to microbial triggers.

The discovery that PA induces innate immune memory, and modulates inflammatory and infection outcomes in mice, is just the beginning of a much greater appreciation for the nuances of dietary-regulated immune function. A deeper understanding of the crosstalk between dietary SFAs and innate immune inflammation is imperative for understanding the long-term impact of diets enriched in SFAs on the host response to infection and inflammatory disease. This will contribute to effective prevention and treatment of diseases exacerbated by excessive SFA intake, advanced personalized nutrition, and a greater sense of agency over our immune health while living in societies heavily dependent on dietary SFAs.

Table 1. The impact of PA on innate immune inflammatory responses and memory.

Conditions	Responses	Biological effects	Models used	References
Simultaneous PA + LPS (*in vitro)*	Priming	Enhanced ceramide; enhanced TNF/IL-6 secretion; JNK dependent	Primary & immortalized mouse macrophages	Schilling *et al.*, 2013; Jin *et al.*, 2013; Nishiyama *et al.*, 2018
PA pre-treatment + LPS (*in vitro)*	Trained immunity	Enhanced TNF/IL-6/IL-1β secretion, all ceramide dependent; role of mitochondria and MAPK signaling	Primary mouse macrophages; immortalized human & mouse macrophages	Seufert *et al.*, 2022; Fang *et al.*, 2022; Caslin *et al.*, 2023; Schwartz *et al.*, 2010
PA pre-treatment + LPS (*in vivo)*	Trained immunity	Enhanced circulating inflammation; decreased survival	Wild type female BALB/c mice	Seufert *et al.*, 2022
PA pre-treatment + infection (*in vivo)*	Trained immunity	Enhanced microbial clearance	*Rag*$^{-/-}$ mice + *Candida albicans* infection; wild type female BALB/c mice + *Brucella abortus* infection	Seufert *et al.*, 2022; Reyes *et al.*, 2021

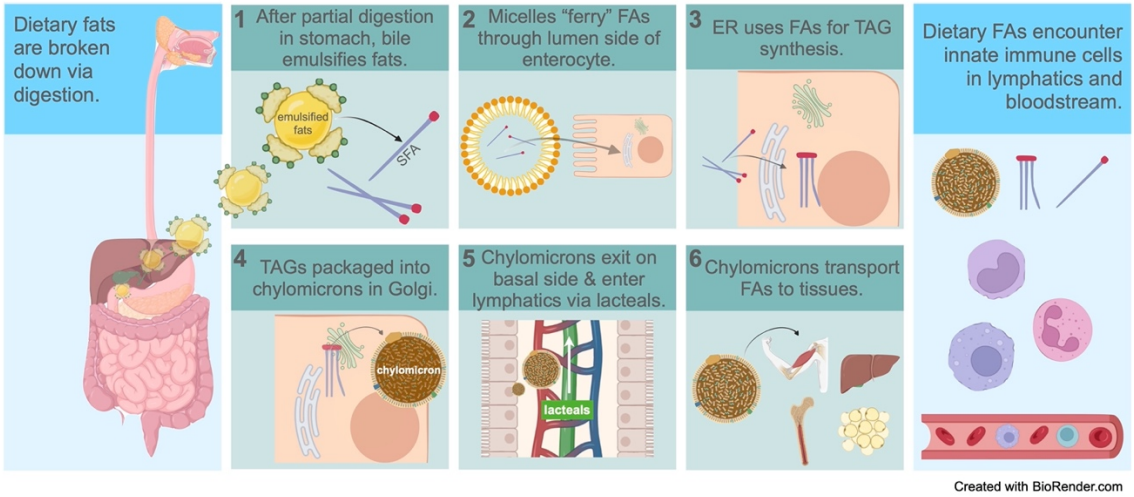

Figure 1. Pathway of dietary fatty acids following digestion. Following enzymatic digestion in stomach, (1) bile salts from the gall bladder emulsify FAs and MAGs during entry into the duodenum. (2) FAs greater than 14-carbons in length are packaged into micelles that ferry them into enterocytes from the lumen. (3) FAs are resynthesized into TAGs in the ER, followed by (4) repackaging into chylomicrons within the Golgi. (5) Chylomicrons are carried in vesicles through the lymphatics within chylomicrons, and may encounter monocytes, macrophages, and neutrophils. ER, endoplasmic reticulum; FA, fatty acid; MAG, monoacylglyceride; TAG, triacylglyceride.

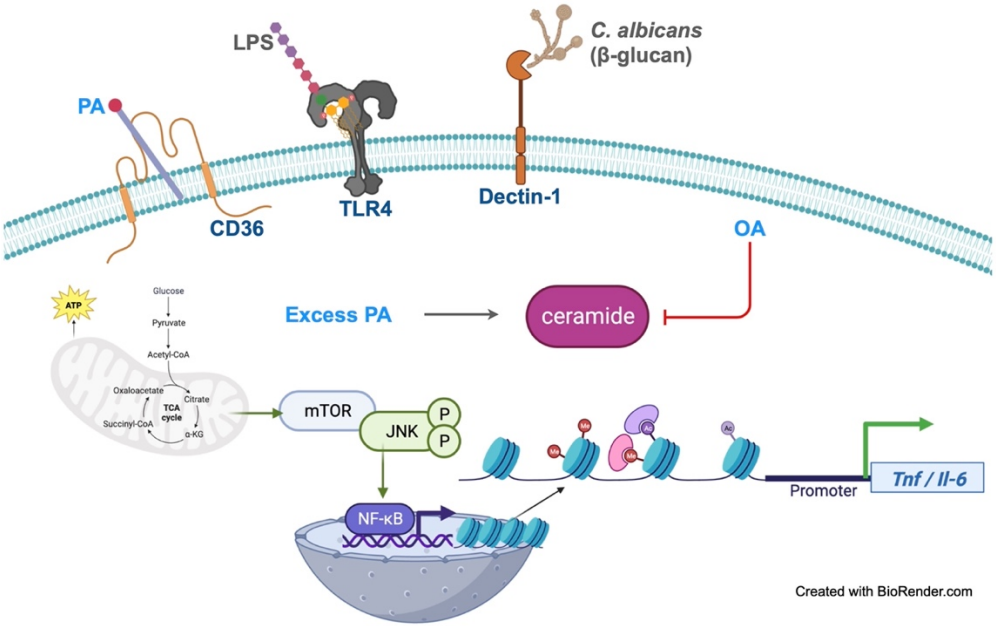

Figure 2. A dietary rheostat for macrophage inflammatory homeostasis and trained immunity. PA enters macrophage through the scavenger receptor, CD36; excess PA increases de novo ceramide; subsequent TLR4 stimulation by LPS enhances intracellular ceramide and activates NF-κB; PA metabolic byproducts induce histone modifications at *Tnf/Il-6* promoters; fungal β-glucans signal through Dectin-1 and activate NF-κB; mTOR regulates JNK activation and NF-κB transcription; OA inhibits ceramide and reverses inflammatory polarization. JNK, c-Jun *N*-terminal kinase; LPS, lipopolysaccharide; mTOR, mammalian target of rapamycin; NF-κB, Nuclear factor kappa B; OA, oleic acid; PA, palmitic acid; TLR4, toll-like receptor 4.

REFERENCES:

[1]. Korbecki J, Bajdak-Rusinek K. The effect of palmitic acid on inflammatory response in macrophages: an overview of molecular mechanisms. Inflamm Res. 2019;68:915–32.

[2]. Carta G, Murru E, Banni S, et al. Palmitic acid: physiological role, metabolism and nutritional implications. Front Physiol. 2017;8:902.

[3]. Divangahi M, Aaby P, Khader SA, et al. Trained immunity, tolerance, priming and differentiation: distinct immunological processes. Nat Immunol. 2021;22:2–6.

[4]. Netea MG, Domínguez-Andrés J, Barreiro LB, et al. Defining trained immunity and its role in health and disease. Nat Rev Immunol. 2020;20:375–88.

[5]. Arts RJW, Novakovic B, Ter Horst R, et al. Glutaminolysis and fumarate accumulation integrate immunometabolic and epigenetic programs in trained immunity. Cell Metab. 2016;24:807–19.

[6]. Saeed S, Quintin J, Kerstens HHD, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science. 2014;345:1251086.

[7]. Mitroulis I, Kalafati L, Hajishengallis G, et al. Myelopoiesis in the context of innate immunity. J Innate Immun. 2018;10:365–72.

[8]. Kaufmann E, Sanz J, Dunn JL, et al. BCG educates hematopoietic stem cells to generate protective innate immunity against tuberculosis. Cell. 2018;172:176–190.e19.

[9]. Katzmarski N, Domínguez-Andrés J, Cirovic B, et al. Transmission of trained immunity and heterologous resistance to infections across generations. Nat Immunol. 2021;22:1382–90.

[10]. Covian C, Retamal-Diaz A, Bueno SM, et al. Could BCG vaccination induce protective trained immunity for SARS-CoV-2? Front Immunol. 2020;11:970.

[11]. Christ A, Günther P, Lauterbach MAR, et al. Western diet triggers NLRP3-dependent innate immune reprogramming. Cell. 2018;172:162–175.e14.

[12]. Seufert AL, Hickman JW, Traxler ST, et al. Enriched dietary saturated fatty acids induce trained immunity via ceramide production that enhances severity of endotoxemia and clearance of infection. Elife. 2022;11:e76744. doi: 10.7554/eLife.76744.

[13]. Reyes AWB, Huy TXN, Vu SH, et al. Protection of palmitic acid treatment in RAW264.7 cells and BALB/c mice during Brucella abortus 544 infection. J Vet Sci. 2021;22(2):e18.

[14]. Schilling JD, Machkovech HM, He L, et al. Palmitate and lipopolysaccharide trigger synergistic ceramide production in primary macrophages. J Biol Chem. 2013;288(5):2923–32. doi: 10.1074/jbc.M112.419978.

[15]. Jin J, Zhang X, Lu Z, et al. Acid sphingomyelinase plays a key role in palmitic acid-amplified inflammatory signaling triggered by lipopolysaccharide at low concentrations in macrophages. Am J Physiol Endocrinol Metab. 2013;305(7):E853–67.

[16]. Zhang X, Li X, Xiong G, et al. Palmitic acid promotes lung metastasis of melanomas via the TLR4/TRIF-Peli1-pNF-κB pathway. Metabolites. 2022;12(11):1132.

[17]. Caslin HL, Cottam MA, Piñon JM, et al. Weight cycling induces innate immune memory in adipose tissue macrophages. Front Immunol. 2023;13:984859.

[18]. Ryan DG, O'Neill LAJ. Krebs cycle reborn in macrophage immunometabolism. Annu Rev Immunol. 2020;38:289–313.

[19]. Viola A, Munari F, Sanchez-Rodriguez R, et al. The metabolic signature of macrophage responses. Front Immunol. 2019;10:1462.

[20]. Curi R, de Siqueira Mendes R, de Campos LA, et al. Crispin A past and present overview of macrophage metabolism and functional outcomes. Clin Sci. 2017;131(12):1329–42.

[21]. Jha AK, Huang SC-C, Sergushichev A, et al. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. Immunity. 2015;42(3):419–30.

[22]. O'Neill L. A broken krebs cycle in macrophages. Immunity. 2015;42(3):393–4.

[23]. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. J Gen Physiol. 1927;8(6):519–30.

[24]. Lancaster GI, Langley KG, Berglund NA, et al. Evidence that TLR4 is not a receptor for saturated fatty acids but mediates lipid-induced inflammation by reprogramming macrophage metabolism. Cell Metab. 2018;27:1096–110.e1095.

[25]. Innis SM. Palmitic acid in early human development. Crit Rev Food Sci Nutr. 2016;56(12):1952–9.

[26]. Hidalgo MA, Caretta MD, Burgos RA. Long chain fatty acids as modulators of immune cells function: contribution of FFA1 and FFA4 Receptors. Front Physiol. 2021;12:668330.

[27]. Morris A. Palmitic acid is not a TLR4 agonist. Nat Rev Endocrinol. 2018;14:382.

[28]. Abdelmagid SA, Clarke SE, Nielsen DE, et al. Comprehensive profiling of plasma fatty acid concentrations in young healthy canadian adults. PLoS One. 2015;10(2):e0116195.

[29]. Pepino MY, Kuda O, Samovski D, et al. Structure-function of CD36 and importance of fatty acid signal transduction in fat metabolism. Annu Rev Nutr. 2014;34:281–303.

[30]. Samovski D, Sun J, Pietka T, et al. Regulation of AMPK activation by CD36 links fatty acid uptake to β-oxidation. Diabetes. 2015;64(2):353–9.

[31]. Hoebe K, Georgel P, Rutschmann S, et al. CD36 is a sensor of diacylglycerides. Nature. 2005;433(7025):523–7.

[32]. Zhang Y, Rao E, Zeng J, et al. Adipose fatty acid binding protein promotes saturated fatty acid-induced macrophage cell death through enhancing ceramide production. J Immunol. 2017;198:798–807.

[33]. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signaling lipid. Nat Rev Mol Cell Biol. 2003;4(5):397–407.

[34]. Ruysschaert JM, Lonez C. Role of lipid microdomains in TLR-mediated signaling. Biochim Biophys Acta. 2015;1848(9):1860–7.

[35]. Schwartz EA, Zhang W-Y, Karnik SK, et al. Nutrient modification of the innate immune response: a novel mechanism by which saturated fatty acids greatly amplify monocyte inflammation. Arterioscler Thromb Vasc Biol. 2010;30:802–8.

[36]. Nishiyama K, Fujita T, Fujimoto Y, et al. Fatty acid transport protein 1 enhances the macrophage inflammatory response by coupling with ceramide and c-Jun N-terminal kinase signaling. Int Immunopharmacol. 2018;55:205–15.

[37]. Fang C, Wang L, Qiao J, et al. Differential regulation of lipopolysaccharide-induced IL-1beta and TNF-alpha production in macrophages by palmitate via modulating TLR4 downstream signaling. Int Immunopharmacol. 2022;103:108456.

[38]. Napier BA, Andres-Terre M, Massis LM, et al. Western diet regulates immune status and the response to LPS-driven sepsis independent of diet-associated microbiome. Proc Natl Acad Sci U S A. 2019;116:3688–94.

[39]. Christ A, Latz E. The Western lifestyle has lasting effects on metaflammation. Nat Rev Immunol. 2019;19:267–8.

[40]. Ng PY, Eikermann M. The obesity conundrum in sepsis. BMC Anesthesiol. 2017;17:147.

[41]. Singer M, Deutschman CS, Seymour CW, et al. The third international consensus definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016;315:801–10.

[42]. Perreault M, Zulyniak MA, Badoud F, et al. A distinct fatty acid profile underlies the reduced inflammatory state of metabolically healthy obese individuals. PLoS One. 2014;9:e88539.

[43]. Fernandez F, Hinton M, Van Gils B. Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to Salmonella Enteritidis colonization. Avian Pathol. 2002;31:49–58.

[44]. Kumar NG, Contaifer D, Madurantakam P, et al. Dietary bioactive fatty acids as modulators of immune function: implications on human health. Nutrients. 2019;11(12):2974.

[45]. Sun Z, Song ZG, Liu C, et al. Gut microbiome alterations and gut barrier dysfunction are associated with host immune homeostasis in COVID-19 patients. BMC Med. 2022;20(1):24.

[46]. Hotamisligil GS. Foundations of Immunometabolism and Implications for Metabolic Health and Disease. Immunity. 2017;47:406–20.

[47]. Black DD. Development and physiological regulation of intestinal lipid absorption. I. Development of intestinal lipid absorption: cellular events in chylomicron assembly and secretion. Am J Physiol Gastrointest Liver Physiol. 2007;293(3):G519–24.

[48]. Mansbach CM II, Gorelick F. Development and physiological regulation of intestinal lipid absorption. II. Dietary lipid absorption, complex lipid synthesis, and the intracellular packaging and secretion of chylomicrons. Am J Physiol Gastrointest Liver Physiol. 2007;293(4):G645–50.

[49]. Dixon BJ. Mechanisms of chylomicron uptake into lacteals. Ann N Y Acad Sci. 2010;1207(Suppl 1):E52–7.

[50]. Tso P, Nauli A, Lo C-M. Enterocyte fatty acid uptake and intestinal fatty acid-binding protein. Biochem Soc Trans. 2004;32(Pt 1):75–8.

[51]. Hall JE. Textbook of Medical Physiology. 12th ed. W B Saunders CoSaunders; 2010.

[52]. Hussain MM, Kancha RK, Zhou Z, et al. Chylomicron assembly and catabolism: role of apolipoproteins and receptors. Biochim Biophys Acta. 1996;1300(3):151–70.

[53]. Hubler MJ, Kennedy AJ. Role of lipids in the metabolism and activation of immune cells. J Nutr Biochem. 2016;34:1–7.

[54]. Mitroulis I, Ruppova K, Wang B, et al. Modulation of myelopoiesis progenitors is an integral component of trained immunity. Cell. 2018;172:147–161.e12.

[55]. Palomer X, Pizarro-Delgado J, Barroso E, et al. Palmitic and oleic acid: the Yin and Yang of fatty acids in type 2 diabetes mellitus. Trends Endocrinol Metab. 2018;29(3):178–90.

[56]. Karasawa T, Kawashima A, Usui-Kawanishi F, et al. Saturated fatty acids undergo intracellular crystallization and activate the NLRP3 inflammasome in macrophages. Arterioscler Thromb Vasc Biol. 2018;38(4):744–56.

[57]. Lee JY, Sohn KH, Rhee SH, et al. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through toll-like receptor 4. J Biol Chem. 2001;276(20):16683–9.

[58]. Lee JY, Zhao L, Youn HS, et al. Saturated fatty acid activates but polyunsaturated fatty acid inhibits toll-like receptor 2 dimerized with toll-like receptor 6 or 1. J Biol Chem. 2004;279(17):16971–9.

[59]. Jin J, Lu Z, Li Y, et al. Docosahexaenoic acid antagonizes the boosting effect of palmitic acid on LPS inflammatory signaling by inhibiting gene transcription and ceramide synthesis. PLoS One. 2018;13:e0193343.

[60]. Havlicekova Z, Jesenak M, Banovcin P, et al. Beta-palmitate - a natural component of human milk in supplemental milk formulas. Nutr J. 2016;15:28.

[61]. Du Y, Yang M, Lee S, et al. Maternal western diet causes inflammatory milk and TLR2/4-dependent neonatal toxicity. Genes Dev. 2012;26(12):1306–11.

[62]. Tam TH, Chan KL, Boroumand P, et al. Nucleotides released from palmitate-activated murine macrophages attract neutrophils. J Biol Chem. 2020;295(15):4902–11.

[63]. Ortega-Gomez A, Lopez S, Varela LM, et al. New evidence for dietary fatty acids in the neutrophil traffic between the bone marrow and the peripheral blood. Food Chem (Oxf). 2022;5:100133.

CHAPTER 3:

Enriched dietary saturated fatty acids induce trained immunity via ceramide production that enhances severity of endotoxemia and clearance of infection

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Abstract

Trained immunity is an innate immune memory response that is induced by a primary inflammatory stimulus that sensitizes monocytes and macrophages to a secondary pathogenic challenge, reprogramming the host response to infection and inflammatory disease. Dietary fatty acids can act as inflammatory stimuli, but it is unknown if they can act as the primary stimuli to induce trained immunity. Here we find mice fed a diet enriched exclusively in saturated fatty acids (ketogenic diet; KD) confer a hyper-inflammatory response to systemic lipopolysaccharide (LPS) and increased mortality, independent of diet-induced microbiome and hyperglycemia. We find KD alters the composition of the hematopoietic stem cell compartment and enhances the response of

bone marrow macrophages, monocytes, and splenocytes to secondary LPS challenge. Lipidomics identified enhanced free palmitic acid (PA) and PA-associated lipids in KD-fed mice serum. We found pre-treatment with physiologically relevant concentrations of PA induces a hyper-inflammatory response to LPS in macrophages, and this was dependent on the synthesis of ceramide. In vivo, we found systemic PA confers enhanced inflammation and mortality in response to systemic LPS, and this phenotype was not reversible for up to 7 days post-PA-exposure. Conversely, we find PA exposure enhanced clearance of *Candida albicans* in *Rag1*$^{-/-}$ mice. Lastly, we show that oleic acid, which depletes intracellular ceramide, reverses PA-induced hyper-inflammation in macrophages and enhanced mortality in response to LPS. These implicate enriched dietary SFAs, and specifically PA, in the induction of long-lived innate immune memory and highlight the plasticity of this innate immune reprogramming by dietary constituents.

Introduction

Historically, immune memory has been defined as a trait limited to the adaptive immune system; however, it is now well established that innate immune cells have the capacity for metabolic, epigenetic, and functional reprogramming that leads to long-lasting increases in host resistance to infection [1-4]. Specifically, trained immunity is an adaptation of innate host defense in vertebrates and invertebrates that results from exposure to a primary inflammatory stimulus and leads to a faster and greater response to a secondary challenge. Unlike adaptive memory responses, trained immunity does not

require genome rearrangements, B and T lymphocytes, and receptors that recognize specific antigens [1-4]. Furthermore, trained immunity has been documented in organisms that lack canonical adaptive immune responses, such as plants and invertebrates, suggesting this is a primitive immune memory system that is conserved throughout vertebrates and invertebrates [5].

The Bacillus Calmette-Guérin (BCG) vaccine and yeast β-glucans are canonical inducers of trained immunity in humans and stimulate long-lasting metabolic and epigenetic reprogramming of myeloid-lineage cells resulting in hyper-responsiveness upon restimulation with heterologous or homologous inflammatory stimuli. This innate immune memory has been shown to be heritable and can last up to months in humans and mice and, thus, likely evolved to provide non-specific protection from secondary infections. Most recently, it was described that countries with higher rates of BCG vaccine at birth had fewer coronavirus disease 2019 (COVID-19) cases making this immunological phenomenon extremely relevant [6-8]. Importantly, it is easily ascertained that inflammatory hyper-responsiveness could be deleterious in the context of diseases where more inflammation can lead to greater pathology (e.g. acute septic shock, autoimmune disorders, and allergies). Thus, trained immunity can be regarded as a double-edged sword – providing increased resistance to tissue-specific infection but exacerbating diseases exacerbated by systemic inflammation. Consequently, identifying novel inducers of trained immunity will provide clinically relevant insight into harnessing innate immune cells to attain long-term therapeutic benefits in a range of infections and inflammatory diseases.

Typically, the primary inflammatory stimulus that initiates trained immunity is danger- or pathogen-associated molecular patterns (PAMPs); however, recent publications have shown that β-glucan found in mushrooms, baker's and brewer's yeast, wheat and oats, and unknown components of bovine milk can induce trained innate immune memory in monocytes in vitro [9,10]. Our data reported here contribute to the growing evidence supporting the multifaceted immunoregulatory role of certain dietary constituents.

Currently, Westernized nations are increasingly dependent on diets enriched in saturated fatty acids (SFA), which have been shown to mimic PAMP effects on inflammatory cells, regulate innate immune cell function, and alter outcomes of inflammatory disease and infection [11-17]. Specifically, we have shown the Western diet (WD), a diet enriched in sucrose and SFAs, correlates with increased disease severity and mortality in response to systemic LPS, independent of the diet-dependent microbiota, demonstrating the possibility that the dietary components of this diet may be driving the hyper-responsiveness to LPS [18]. Currently, it is unknown if enriched dietary SFAs alone mediate trained immunity.

Our work presented herein identifies a ketogenic diet (KD) enriched exclusively in SFAs, and not sucrose, confers an increased systemic response to LPS independent of diet-associated microbiome, ketosis, or glycolytic regulation during disease, and alters inflammatory capacity and composition of the hematopoietic compartment. While others have shown that the WD induces trained immunity in atherosclerotic mice ($Ldrl^{-/-}$), we are the first to show that trained immunity, including its hallmark long-term persistence,

can be induced in wild-type (WT) mice with exposure to enriched SFAs alone [19]. A lipidomic analysis of blood fat composition after KD exposure revealed a significant increase of free palmitic acid (PA; C16:0) and fatty acid complexes containing PA. PA is known to act synergistically with LPS to enhance intracellular ceramide levels and proinflammatory cytokine expression in macrophages; however, it is currently unknown if ceramide, a bioactive sphingolipid (SG), specifically mediates a heightened inflammatory response to LPS following pre-exposure to PA [20,21]. Here we find macrophages pre-treated with physiologically relevant concentrations of PA followed by a secondary exposure to LPS lead to enhanced proinflammatory cytokine expression and release, which were reversible with the inhibition of ceramide.

We find that both short- and long-term exposure to PA, the predominant SFA found in high-fat diets, enhance systemic response to microbial ligands in mice even after a 7-day rest period from PA exposure. Thus, our data suggest exposure to PA leads to a long-lasting innate immune memory response in vivo [7]. Importantly, trained immunity is induced when a primary inflammatory stimulus changes transcription of inflammatory genes, the immune status returns to basal levels, and challenge with a secondary stimulus enhances transcription of inflammatory cytokines at much higher levels than those observed during the primary challenge [22]. While the dynamics of an initial inflammatory event induced by PA in vivo are not defined in this paper, we show that basal levels of *Tnf*, *Il6*, *Il1b,* and *Il10* in the blood of mice pre-exposed to PA were comparable to control mice immediately prior to endotoxin challenge, indicating that mice were not in a primed state prior to disease. This suggests that the hyper-inflammation and poor disease

outcome we show in PA-exposed mice are not due to priming but a trained immune response.

The dual nature of trained immunity is also a hallmark feature of the phenomenon, in that non-specific innate immune memory can be either beneficial or detrimental depending on the disease context. The majority of research has demonstrated the protective role of trained immunity against a variety of infections, such as with BCG vaccination and β-glucan stimulation [3,23]. Our work is unique because we focus on the detrimental role that trained immunity has on disease characterized by inflammatory dysregulation; however, we also highlight the beneficial nature of this novel phenotype by showing that when mice lacking adaptive immunity (*Rag1*$^{-/-}$) are pre-exposed to systemic PA, they exhibit enhanced clearance of kidney fungal burden compared to control mice.

We further identify a novel role of SFA-dependent intracellular ceramide required for the enhanced systemic response to microbial ligands, and show intervention with OA, a mono-unsaturated fatty acid that depletes PA-dependent ceramide, can reverse these phenotypes in macrophages and in vivo. Our data presented here highlight the dynamic plasticity of dietary intervention on inflammatory disease outcomes. These data are consistent with the current knowledge that SFAs and ceramide are immunomodulatory molecules, and build on these by highlighting a previously unidentified role of PA in driving long-lived trained immunity.

Results

Diets enriched in SFAs increase endotoxemia severity and mortality

To examine the immune effects of chronic exposure to diets enriched in SFAs on lipopolysaccharide (LPS)-induced endotoxemia, we fed age matched (6–8 weeks), female BALB/c mice either with a WD (enriched in SFAs and sucrose), a KD (enriched in SFAs and low-carbohydrate), or standard chow (SC; low in SFAs and sucrose), for 2 weeks (Table 1). We defined 2 weeks of feeding as chronic exposure, because this is correlated with WD- or KD-dependent microbiome changes and confers metaflammation in WD mice [18], sustained altered blood glucose levels in WD mice (Figure 1—figure supplement 1A), and elevated levels of ketones in the urine and blood in KD mice (Figure 1—figure supplement 1B-C). We then induced endotoxemia by a single intraperitoneal (i.p.) injection of LPS. We measured hypothermia as a measure of disease severity and survival to determine outcome [18,24,25]. WD- and KD-fed mice showed significant and prolonged hypothermia, starting at 10 hr post-injection (p.i.), compared to the SC-fed mice (Figure 1A). In accordance with these findings, WD- and KD-fed mice displayed 100% mortality by 26 hr p.i. compared to 100% survival of SC-fed mice (Figure 1B). Hypoglycemia is a known driver of endotoxemia, and each of these diets has varying levels of sugars and carbohydrates (Table 1) [26,27]. However, mice in all diet groups displayed similar levels of LPS-induced hypoglycemia during disease (Figure 1—figure supplement 1D), indicating that potential effects of diet on blood glucose were not a driver of enhanced disease severity.

Considering mice fed KD experience a shift toward nutritional ketosis, we wanted to understand if our phenotype was dependent on nutritional ketosis. 1,3-butanediol (BD) is

a compound that induces ketosis by enhancing levels of the ketone β-hydroxybutyrate in the blood [28]. Age matched (6–8 weeks), female BALB/c mice were fed for 2 weeks with KD, SC supplemented with saccharine and 1,3-butanediol (SC + BD), or SC-fed with the saccharine vehicle solution (SC + Veh). BD supplementation was sufficient to increase blood ketones (Figure 1—figure supplement 1C). We next injected LPS i.p. and found KD-fed mice showed significantly greater hypothermia, and increased mortality, compared to SC + BD and SC + Veh (Figure 1—figure supplement 1E, F). Though short-lived, when compared to SC + Veh, the SC + BD mice did confer an increase in hypothermia, suggesting that nutritional ketosis may play a minor role in KD-dependent susceptibility to endotoxemia (Figure 1—figure supplement 1E, F). Together these data suggest that diets enriched in SFAs promote enhanced acute endotoxemia severity, and this is independent of diet-dependent hypoglycemic shock or nutritional ketosis.

Diets enriched in SFAs induce a hyper-inflammatory response to LPS and increased immunoparalysis

Endotoxemia mortality results exclusively from a systemic inflammatory response characterized by an acute increase in circulating inflammatory cytokine levels (e.g. TNF, IL-6, and IL-1β) from splenocytes and myeloid-derived innate immune cells [29-32]. Additionally, pre-treatment of myeloid-derived cells with dietary SFAs has been shown to enhance inflammatory pathways in response to microbial ligands [33,34]. Considering this, we hypothesized that exposure to enriched systemic dietary SFAs in WD- and KD-fed mice would enhance the inflammatory response to systemic LPS during the acute

inflammatory response. 5 hr p.i., age matched (6–8 weeks), female BALB/c mice fed all diets showed induction of *Tnf, Il6*, and *Il1b* expression in the blood (Figure 1C–E). However, at 5 hr p.i., WD- and KD-fed mice experienced significantly higher expression of *Tnf* and *Il6* in the blood, compared with SC-fed mice, and WD-fed mice also showed significantly higher *Il1b* expression (Figure 1C–E), indicating that diets enriched in SFAs are associated with a hyper-inflammatory response to LPS.

Importantly, septic patients often present with two immune phases: an initial amplification of inflammation, followed by or concurrent with an induction of immune suppression (immunoparalysis), that can be measured by a systemic increase in the anti-inflammatory cytokine IL-10 [35,36]. Furthermore, in septic patients, a high IL-10:TNF ratio equates with the clinical immunoparalytic phase and correlates with poorer sepsis outcomes [37,38]. Interestingly, we found there was significantly increased *Il10* expression in WD- and KD-fed mice, compared to SC-fed mice (Figure 1F), and WD- and KD-fed mice had significantly higher *Il10:Tnf* ratios at 10–20 hr and 15–20 hr, respectively, compared to SC-fed mice (Figure 1G). These data conclude that mice exposed to diets enriched in SFAs show an initial hyper-inflammatory response to LPS, followed by an increased immunoparalytic phenotype, which correlates with enhanced disease severity, similar to what is seen in the clinic.

Diets enriched in SFAs drive enhanced responses to systemic LPS independent of diet-associated microbiome

We have previously shown that WD-fed mice experience increased endotoxemia severity

and mortality, independent of diet-associated microbiome [18]. In order to confirm the increases in disease severity that correlated with KD were also independent of KD-associated microbiome changes, we used a germ-free (GF) mouse model. 19–23-week-old female and 14–23-week-old male and female GF C57BL/6 mice were fed SC, WD, and KD for 2 weeks followed by injection with 50 mg/kg of LPS, our previously established LD_{50} in GF C57BL/6 mice [18]. As we saw in the conventional mice, at 10 hr p.i. WD- and KD-fed GF mice showed enhanced hypothermia and mortality, compared to SC-fed GF mice (Figure 1H, I). These data show that, similar to WD-fed mice, the KD-associated increase in endotoxemia severity and mortality is independent of diet-associated microbiome.

Our previous studies (Figure 1A–G) in conventional mice were carried out in 6–8-week female mice on a BALB/c background. Importantly, genetic background and age differences can have large effects on LPS treatment outcome. The GF mice used in this study (Figure 1H–N) were on a C57BL/6 background, between the ages of 14 and 23 weeks. Thus, we confirmed WD- and KD-fed conventional C57BL/6 mice aged 20–21 weeks old show enhanced disease severity and mortality in an LPS-induced endotoxemia model (4.5 mg/kg), compared to mice fed SC, similar to what is seen in younger BALB/c mice (Figure 1—figure supplement 1G, H).

Additionally, to confirm that the hyper-inflammatory response to systemic LPS was independent of the WD- and KD-dependent microbiome, we measured systemic inflammation during endotoxemia via the expression of *Tnf*, *Il6*, and *Il1b* in the blood at

0–10 hr p.i. We found, WD- and KD-fed GF mice displayed enhanced expression of *Tnf* and *Il1b* at 5–10 hr, and significantly enhanced expression of *il-6* at 5 hr, compared to SC-fed GF mice (Figure 1J–L). Interestingly, *Il10* expression and the *Il10:Tnf* ratio were not significantly different throughout all diets, suggesting the SFA-dependent enhanced immunoparalytic phenotype is dependent on the diet-associated microbiomes in WD- and KD-fed mice (Figure 1M–N). These data demonstrate that the early hyper-inflammatory response, but not the late immunoparalytic response, to LPS associated with enriched dietary SFAs is independent of the diet-dependent microbiota.

A diet enriched exclusively in SFAs induces trained immunity

Thus far we find feeding diets enriched in SFAs (WD and KD) leads to enhanced expression of inflammatory cytokines in the blood after treatment with systemic LPS, suggesting that the SFAs may be inducing an innate immune memory response that leads to a hyper-inflammatory response to secondary challenge. Specifically, trained immunity is an innate immune memory response characterized by reprogramming of myeloid cells by a primary inflammatory stimulus, that then responds more robustly to secondary inflammatory challenge. Trained immunity has been shown to mediate cell sub-types within the HSC compartment that gives rise to "trained" myeloid progeny for weeks to years [39]. A previous study in *Ldlr*$^{-/-}$ mice has shown 4 weeks of WD feeding significantly enhances multipotent progenitors (MPPs) and granulocyte and monocyte precursors (GMPs) and skews development of GMPs toward a monocyte lineage that is primed to respond with a hyper-inflammatory response to LPS [19]. Currently, it is

unknown if diets enriched in SFAs fed to WT mice can induce changes within the HSC compartment or long-lasting trained immunity.

In order to determine the impact of dietary SFAs on bone marrow reprogramming in vivo, we next evaluated HSCs and progenitor cells via flow cytometry from age-matched (6–8 weeks) female WT BALB/c mice fed SC, WD, and KD for 2 weeks. Using previously published panels for analyzing HSC populations in the bone marrow, we collected bone marrow and measured relative proportions of long-term HSCs (LT-HSCs; $CD201^{+}CD27^{+}CD150^{+}CD48^{-}$), short-term HSCs (ST-HSCs; $CD201^{+}CD27^{+}CD150^{+}CD48^{+}$), and MPPs ($CD201^{+}CD27^{+}CD150^{-}CD48^{+}$) (Figure 2A and B) [23,40,41]. Strikingly, we find that KD-fed mice showed significantly enhanced ST- and LT-HSCs, and MPPs compared to SC-fed mice (Figure 2C). Unlike previously reported in *Ldlr*$^{-/-}$ mice, there was no significant change in ST-HSCs, LT-HSCs, or MPPs within WD-fed WT mice (Figure 2C). Furthermore, we did not see a significant increase in MPP3s for WD-fed mice, as previously published for $Ldlr^{-/-}$ mice, or KD-fed mice; however, this may be due to the difference in genetic backgrounds, or length of diet administration (Figure 2—figure supplement 1A) [19]. These data are the first to show that the KD, a diet solely enriched in SFAs, alters hematopoiesis by enhancing expansion and differentiation of HSCs, similar to previously described inducers of trained immunity.

Furthermore, it is unknown if enriched dietary SFAs lead to long-lasting functional reprogramming associated with trained immunity that leads to a hyper-inflammatory response. Thus, we fed age-matched (6–8 weeks) female BALB/c mice SC, WD, or KD

for 2 weeks, isolated bone marrow, differentiated into BMDMs for 7 days, and analyzed baseline inflammation and response to LPS. We found that untreated BMDMs isolated from mice fed SC and WD showed no significant differences in TNF or IL-6, and those from KD-fed mice showed a modest increase only in IL-6 compared to BMDMs from SC-fed mice (Figure 2—figure supplement 1B). However, when BMDMs were stimulated with LPS for 24 hr ex vivo, BMDMs from WD- and KD-fed mice showed significantly higher secretion of TNF, and only those from KD-fed mice showed significantly enhanced IL-6 secretion (Figure 2D–E). These data show that diets enriched in SFAs are inducing long-lasting inflammatory reprogramming of myeloid cells in vivo, and that reprogramming takes place within the bone marrow.

Importantly, monocytes and splenocytes are necessary for induction of systemic inflammatory cytokines during endotoxemia [31,32]. Thus, we wanted to assess if enriched dietary SFA induces in vivo reprogramming of monocytes and splenocytes, leading to an enhanced response to LPS ex vivo. First, we fed age-matched (6–8 weeks) female BALB/c mice SC, WD, or KD for 2 weeks, isolated bone marrow monocytes (BMMs) via magnetic negative selection using bone marrow extracted from femurs and tibias, and determined baseline expression of inflammatory cytokines. We found that prior to ex vivo LPS stimulation, BMMs isolated from mice fed SC, WD, or KD showed no significant difference in *Tnf* expression, and *Il6* expression was significantly decreased in BMMs from KD-fed mice (Figure 2—figure supplement 1C). However, when BMMs were stimulated with LPS for 2 hr ex vivo, those from KD-fed mice showed significantly higher expression of *Tnf* and *Il6*, while those from WD-fed mice exhibited no

significance in expression compared to SC-fed mice (Figure 2—figure supplement 1D). Similarly, we isolated splenocytes from SC-, WD-, and KD-fed mice and found no difference between homeostatic inflammation of splenocytes between diets, but a significantly enhanced expression of *Tnf* in the splenocytes of KD-fed mice, and not WD-fed mice, challenged with LPS (2 hr) compared to splenocytes from SC-fed mice (Figure 2—figure supplement 1E, F).

These data show the KD stimulates expansion of HSC populations and skew differentiation of myeloid progenitors that then give rise to macrophages with enhanced inflammatory potency (Figure 2A–E; Figure 2—figure supplement 2). Furthermore, these data suggest that BMDMs, BMMs, and splenocytes from WD- and KD-fed mice are not more inflammatory at homeostasis; however, when challenged with LPS, KD feeding confers a hyper-inflammatory response. Together, our results suggest the KD, a diet that comprised 90.5% SFAs, leads to reprogramming of the HSC compartment and long-lasting trained immunity.

PA and PA-associated fatty acids are enriched in the blood of KD-fed mice

It is known that the SFAs consumed in the diet determine the SFA profiles in the blood, and that these SFAs have the potential to be immunomodulatory [42-44]. Thus, we next wanted to identify target SFAs enriched in the blood of mice fed a diet exclusively enriched in SFAs that may be altering the systemic inflammatory response to LPS. Considering that the KD is enriched in SFAs and not sucrose, and that KD-fed mice showed distinct HSC alterations and LPS-induced hyper-inflammation in BMDMs,

BMMs, and splenocytes treated ex vivo, the subsequent studies were performed exclusively on KD-fed mice. We used mass spectrometry lipidomics to create diet-dependent profiles of circulating fatty acids in SC- and KD-fed mice [45]. Age matched (6–8 weeks), female BALB/c mice were fed SC or KD for 2 weeks, then serum samples were collected and analyzed using qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry. We used principal component analysis (PCA) to visualize how samples within each data set clustered together according to diet, and how those clusters varied relative to one another in abundance levels of free fatty acids (FFA), triacylglycerols (TAG), and phosphatidylcholines (PC). For all three groups of FAs, individual mice grouped with members of the same diet represented by a 95% confidence ellipse with no overlap between SC- and KD-fed groups (Figure 3A–C). These data indicate that 2 weeks of KD feeding are sufficient to alter circulating FFAs, TAGs, and PCs, and that SC- and KD-fed mice display unique lipid blood profiles. Similarly, the relative abundance of SGs in SC- and KD-fed mice displayed unique diet-dependent profiles with no overlapping clusters, and abundance of specific SGs was significantly higher in the serum of KD-fed mice compared to SC-fed mice (Figure 3—figure supplement 1A, B). Though the independent role of each FFA, TAG, PC, and SG species has not been clinically defined, each are classes of lipids that when accumulated is associated with metabolic diseases, which have been shown to enhance susceptibility to sepsis and exacerbate inflammatory disease [16,46-48].

Importantly, we identified a significant increase in multiple circulating FFAs within the KD-fed mice, compared to the SC-fed mice, many of which were SFAs (Figure 3D).

Interestingly, in KD-fed mice we found a significant increase in free PA (C16:0), an immunomodulatory SFA that is found naturally in animal fats, vegetable oils, and human breast milk and is eightfold enriched in KD (Figure 3D, Table 1) [49]. Additionally, PA-containing TAGs and PCs were significantly elevated in KD-fed mice serum, compared to SC-fed mice (Figure 3—figure supplement 1C, D). These data indicate that KD feeding not only enhances levels of freely circulating PA, but also enhances the frequency PA is incorporated into other lipid species in the blood.

PA enhances macrophage inflammatory response to LPS

Many groups have shown that PA alone induces a modest, but highly reproducible increase in the expression and release of inflammatory cytokines in macrophages and monocytes [14,50]. However, it remains unknown if PA can act as a primary inflammatory stimulus to induce a hyper-inflammatory response to a secondary heterologous stimulus in primary cells. Thus, we next wanted to determine if pre-exposure to physiologically relevant concentrations of PA altered the macrophage response to a secondary challenge with LPS. Current literature indicates a wide range of serum PA levels, between 0.3 and 4.1 mM, reflects a high-fat diet in humans [51-55]. We aimed to use a physiologically relevant concentration of PA reflecting a human host for our in vitro studies, thus we treated primary bone marrow-derived macrophages (BMDMs) with and without 1 mM of PA containing 2% bovine serum albumin (BSA) for 12 hr, removed the media, subsequently treated with LPS (10 ng/mL) for an additional 24 hr, and measured expression and release of TNF, IL-6, and IL-1β. Importantly, the BSA dissolved in the media used for PA treatment solutions was endotoxin- and FA-free to ensure aberrant

TLR signaling would not occur via BSA contamination, and fresh PA was conjugated to BSA-containing media immediately prior to use. We found that BMDMs pre-treated with PA (1 mM) for 12 hr expressed significantly higher levels of *Tnf* and *Il6* in response to secondary treatment with LPS, compared to naïve BMDMs (Figure 3E and F). *Il1b* expression was significantly lower in cells pre-treated with PA (Figure 3G); however, secretion of TNF, IL-6, and IL-1β was enhanced in BMDMs pre-treated with PA (1 mM) for 12 hr and challenged with LPS (Figure 3H–J). We found a similar enhanced *Il6* and *Tnf* expression in response to LPS in BMDMs treated with PA (1 mM) for twice the length of exposure (24 hr), and *Il-1b* expression was decreased (Figure 3—figure supplement 2A-C).

Furthermore, we pre-treated BMDMs with a concentration of PA that reflects the lower range of physiologically relevant serum levels and found 0.5 mM of PA induced significantly higher expression of *Tnf, Il6,* and *Il1b* after 12-hr challenge with LPS; however, only *Tnf* and *Il6* were significantly enhanced after 24-hr LPS treatment, compared to naive BMDMs treated with LPS (Figure 3—figure supplement 2D-I).

Importantly, PA treatment can induce apoptosis and pyroptosis in various cell types; however, we found only an average of 3.4 and 4.4% of cell death after a 12-hr or 24-hr incubation, respectively, with PA (1 mM) and subsequent 24 hr of LPS treatment or control media (Figure 3—figure supplement 3A, B) [56-59]. These data demonstrate PA pre-treatment of macrophages induces a hyper-inflammatory response to LPS

independent of cell death, suggesting PA is sensitizing macrophages to secondary inflammatory challenge.

Thus, we conclude that both 12- and 24-hr pre-treatments with 0.5 mM or 1 mM of PA conjugated to 2% BSA are sufficient to induce reprogramming of macrophages and alter the response to stimulation with a heterologous ligand. Additionally, these data demonstrate that even serum concentrations of PA that are at the lower end of the spectrum for humans consuming a high-fat diet pose a risk for inflammatory dysfunction.

Diverting ceramide synthesis inhibits the PA-dependent hyper-inflammatory response to LPS in macrophages

PA treatment of various cell types diverts cellular metabolism toward the synthesis of the toxic metabolic byproducts: diacylglycerols and ceramide [60]. PA-induced ceramide synthesis has specifically been demonstrated to enhance inflammation [20,21,33,61]. Considering this, we wanted to determine the role of enhanced macrophage ceramide production in driving PA-induced hyper-inflammatory response to LPS. Thus, we treated BMDMs simultaneously with PA (0.5 mM) and a ceramide synthase inhibitor Fumonisin B1 (FB1; 10 μM), for 12 hr, removed the media, subsequently treated with LPS (10 ng/mL) for an additional 24 hr, and measured release of TNF, IL-6, and IL-1β. We found that BMDMs pre-treated simultaneously with PA and FB1 for 12 hr expressed significantly lower levels of TNF, IL-6, and IL-1β secretion in response to LPS, compared to BMDMs pre-treated with only PA (Figure 3K–M). We conclude that

ceramide synthesis induced by PA is required for the macrophage hyper-inflammatory response to secondary challenge with LPS.

PA is sufficient to increase endotoxemia severity and systemic hyper-inflammation

Considering the drastic effect of PA on macrophage response to secondary challenge with LPS, we next wanted to understand if exposure to PA alone is sufficient to induce a hyper-inflammatory response during endotoxemia in vivo. We answered this question using age-matched (6–8 weeks) female BALB/c mice fed SC for 2 weeks, by mimicking systemic PA levels found in serum of humans on high-fat diet via a single i.p. injection of ethyl palmitate (750 mM), and then after 12 hr, challenging with LPS i.p. [62]. Similar to previous publications, we find that a 750-mM i.p. injection of ethyl palmitate enhances free PA levels in the serum to 173–425 μM compared to Veh-treated mice with 110–250 μM (Figure 4—figure supplement 1A). Important to note, free PA is only transiently enhanced by systemic application and is quickly (<1 hr) taken up by peripheral tissues; thus, our detected free serum levels are most likely an underestimation of transient systemic PA [63-65].

Interestingly, after LPS challenge, PA-treated mice experienced increased disease severity as indicated by their significant decline in temperature compared to Veh mice (Figure 4A). Similar to WD- and KD-fed mice, PA-treated mice also exhibited enhanced mortality, compared to Veh mice (Figure 4B). Importantly, mice injected with PA for shorter time periods (0, 3, and 6 hr) and then challenged with LPS did not exhibit increased disease severity or poor survival outcome (Figure 4—figure supplement 1B,

C), concluding that a 12-hr pre-treatment with PA is required for an increase in disease severity.

Next, we measured systemic inflammatory status during disease and found similar to KD-fed mice, the 12-hr PA-pre-treated mice showed significantly enhanced expression of *Tnf* (5 hr and 10 hr) and *Il6* (5 hr) post-LPS challenge, compared to Veh control (Figure 4C and D). Expression of *Il-1b* trended upward but was not significantly upregulated in 12-hr PA-pre-treated mice, compared to Veh-treated mice (Figure 4E). Importantly, as a control we looked at LPS-induced hypoglycemia in PA-treated mice, and 12-hr pre-treatment with PA did not alter LPS-induced hypoglycemia (Figure 4—figure supplement 1D), indicating that diet-dependent hypoglycemic shock was not a driver of endotoxemia severity in PA-treated mice. Thus, exposure to PA to mimic systemic levels found in humans eating high-fat diets is sufficient to drive enhanced inflammation and disease severity in mice stimulated with endotoxin, and this effect is dependent on length of PA exposure.

PA induces long-lived hyper-responsiveness to LPS and enhanced clearance of fungal infection

Our data show that pre-treatment with systemic PA alone enhances endotoxemia severity in vivo and inflammatory responses of macrophages to a secondary and heterologous stimulus in vitro. This form of regulation resembles trained immunity; however, it remains unclear if PA is inducing trained immunity in vivo. We first evaluated the basal level expression of *Tnf, Il6,* and *Il1b* in mice treated with 750 mM of PA or Veh i.p. for

12 hr, before stimulation with LPS. Interestingly, we did not see significant differences in *Tnf, Il6,* or *Il1b* expression at 12 hr of exposure with PA (Figure 4F), which suggests that circulating immune cells of these mice is not in a primed state at these time points prior to LPS injection. These data suggest PA induces trained immunity, and not priming; however, the time point of initial inflammation induced by PA remains unknown.

As mentioned previously, canonical inducers of trained immunity (e.g. BCG or β-glucan) induce long-lived enhanced innate immune responses to secondary inflammatory stimuli [23,1]. Thus, we hypothesized that exposure to a PA bolus would enhance disease severity and mortality in mice, and that this phenotype would persist even after mice were rested from PA injections for 1 week. We injected age matched (6–8 weeks), female BALB/c mice fed SC with a vehicle solution (Veh→SC) or PA (750 mM; PA→SC) i.p. once a day for 9 days and then rested the mice for 1 week. When challenged with systemic LPS, PA→SC showed an increase in disease severity and mortality compared to Veh→SC mice (Figure 4G and H), indicating that PA alone can induce long-lived trained immunity that increases susceptibility to inflammatory disease. Importantly, the difference between Veh→SC and PA→SC survival was not significant (Figure 4H), suggesting PA is not the sole driver of the enhanced mortality we see in KD.

Lastly, the most commonly studied models for inducing trained immunity are immunization with BCG or stimulation with β-glucan, and they have been shown to protect mice from systemic *Candida albicans* infection via lymphocyte-independent immunological reprogramming that leads to decreased kidney fungal burden [2].

Therefore, we next tested if PA treatment induces lymphocyte-independent clearance of *C. albicans* infection. For these experiments, *Rag1* knockout (*Rag1*$^{-/-}$) mice were treated with a vehicle or PA solution for 12 hr and subsequently infected intravenously (i.v.) with 2×10^6 *C. albicans*. In accordance with canonical trained immunity models, mice treated with PA for 12 hr showed a significant decrease in kidney fungal burden compared to Veh mice, 24 hr post-infection (Figure 4I). These are the first data to suggest PA enhances innate immune clearance of *C. albicans* in vivo.

OA reverses enhanced disease severity in WD- and KD-fed mice

We have reported here that diversion of ceramide synthesis reverses the PA-dependent hyper-inflammatory response to LPS in macrophages in vitro (Figure 3K–M). Interestingly, OA (C18:1) is a mono-unsaturated fatty acid naturally found in animal fats and vegetable oils, and in the presence of PA, diverts lipid metabolism away from ceramide production [60,66]. Considering OA and PA are the most prevalent fatty acids found in the human diet and in human serum, we wanted to test if OA diversion of ceramide synthesis could reverse the PA-dependent hyper-inflammatory response to LPS in macrophages [60]. Thus, we treated BMDMs with OA (0.2 mM), PA (0.5 mM), or OA and PA together for 12 hr and then with LPS. We found that macrophages simultaneously pre-treated with PA and OA produced significantly lower levels of TNF, IL-6, and IL-1β following subsequent LPS exposure, compared to BMDMs pre-treated with only PA prior to LPS stimulation (Figure 5A–C). These data reveal OA-dependent depletion of intracellular ceramides neutralizes the PA-dependent hyper-inflammatory response to LPS in macrophages.

Considering this, we next wanted to know if i.p. injections of OA in KD-fed mice would mitigate enriched dietary SFA-associated disease severity and mortality. Thus, we fed age-matched (6–8 weeks) female BALB/c mice SC or KD for 2 weeks and injected them i.p. with 300 mM OA or Veh once per day for the final 3 days of feeding. We then injected LPS i.p. and measured hypothermia and survival. Veh-injected KD-fed mice showed significant and prolonged hypothermia starting at 8 hr p.i. compared to SC-fed mice (Figure 5D). In accordance with these findings, KD-fed mice displayed significantly enhanced mortality by 24 hr p.i. compared to 100% survival of SC-fed mice (Figure 5E). Strikingly, for KD-fed mice injected with 300 mM OA prior to LPS treatment, there was minimal temperature loss comparable to SC-fed mice, and 100% survival (Figure 5D and E). Together, these data show systemic OA can abrogate KD-dependent hypothermia and survival defect in response to LPS in mice-fed diets enriched solely in SFA and highlight the fascinating plasticity of dietary fatty acid reprogramming of innate immune cell populations and disease dynamics.

Discussion

In this study, we showed that mice fed diets enriched in SFA exhibit hyper-inflammation during endotoxemia and poorer outcomes, compared with mice fed a standard low-SFA diet, independent of the diet-associated microbiome, ketosis, and the impact of each diet on LPS-induced hypoglycemia (Figure 1; Figure 1—figure supplement 1). Strikingly, we found that before LPS treatment, healthy mice fed a diet solely enriched in SFAs (KD) displayed significant expansion of HSCs, including MPPs, and harbored BMDMs, BM monocytes, and splenocytes that were not inherently more inflamed, but when challenged

with LPS exhibited increased production of inflammatory cytokines (Figure 2; Figure 2—figure supplement 1, Figure 2—figure supplement 2). Since we did not confirm the hyper-inflammatory phenotype in BMDMs, BMMs, and splenocytes with WD, but only from KD-fed mice, and the KD is only enriched in SFAs and contains no sucrose, allowing us to ask questions specifically about SFAs, we chose to focus on the KD for the remainder of the study [1,2].

Considering the immunogenic properties of some dietary SFAs enriched in the KD, and that excess dietary SFAs are found circulating throughout the blood and peripheral tissues, we used lipidomics to identify dietary SFAs that may be directly reprogramming innate immune cells to respond more intensely to secondary inflammatory stimuli. Our study identified enriched PA (C16:0; PA) and PA-associated fatty acids in the blood of KD-fed mice (Figure 3; Figure 3—figure supplement 1). And, when we treated macrophages with physiologically relevant concentrations of PA, we found that PA alone induces a hyper-inflammatory response to secondary challenge with LPS (Figure 3; Figure 3—figure supplement 2). This enhanced production of inflammatory cytokines in response to secondary heterologous stimuli has been shown in previous models of innate immune memory, specifically trained immunity [4,19,67]. Furthermore, our data suggests PA induces trained immunity by showing that circulating inflammatory levels in PA-injected mice were not upregulated or in a primed state prior to LPS stimulation in vivo (Figure 4F), and PA-associated enhanced endotoxemia severity and mortality are still shown in mice rested for 7 days post-PA exposure (Figure 4G–H). Importantly, we have not fully defined the initial inflammatory response to PA in our model, thus our data only

suggests trained immunity is induced by PA exposure. However, we do find that PA exacerbates the acute phase of endotoxin challenge and correlates with increased mortality but also enhances resistance to infection independent of mature lymphocytes (Figure 4). Together, our data concludes PA exposure can lead to hallmark phenotypes associated with canonical trained immunity models in vitro and in vivo.

Interestingly, the in vivo blood expression of cytokines for KD-fed mice following endotoxin challenge is mild in comparison to the cytokine secretion we show for BM monocytes, splenocytes, and BMDMs isolated from KD-fed mice treated with LPS ex vivo (Figure 1; Figure 2; Supplementary file 2). The media used for culturing and treating BM monocytes and splenocytes ex vivo with LPS contained a high-glucose concentration (4.5 g/L; 25 mM). However, high-glucose media does not alter TNF, IL-6, or IL-1β secretion, or mitochondrial metabolic activity, in WT BMDMs treated with LPS following 7 days of differentiation in high-glucose media [68]. Additionally, in these studies, metabolic adaptation likely takes place within 48 hr for BMDMs cultured in high-glucose media; thus, we suggest it is unlikely that high glucose contributed to the significant augmentation of LPS-induced TNF and IL-6 secretion for BMDMs from KD-fed mice compared to controls, following 7 days of differentiation in high-glucose media prior to LPS challenge [68]. However, further studies on the metabolic flexibility of the SC- and KD-BMDMs will be required to answer this question directly.

Additionally, we have previously shown that WD-induced weight gain does not correlate with enhanced endotoxemia severity and mortality in conventional mice [18]. This is

important to address because of the “obesity paradox” that describes the diversity in sepsis severity and mortality exhibited within the obese patient population, with some studies showing that obesity may even be protective in certain disease contexts [69]. Humans on an animal-based KD that contains 76% fat with 30% SFA content, and 10% carbohydrates, experience ketosis within 1–2 weeks characterized by a three- to fourfold elevation in blood BHB levels and exhibit greater energy expenditure and weight loss compared to humans on a low-fat, plant-based diet that contains 10% fat and 75% carbohydrates [70]. Likewise, KD-fed mice do not gain weight but show enhanced energy expenditure after 5 weeks of diet administration, and a trend toward weight loss during 9 weeks of diet exposure, compared to mice fed an SC diet [71]. Thus, neither weight gain nor the obesity paradox is the confounding feature for the data we present here showing that both KD and dietary PA mediate innate immune memory in vivo during endotoxemia.

Furthermore, the metabolism of dietary SFAs is a key element of immune system function, and metabolic intermediates enhanced by SFAs and PA alone, such as ceramide, serve as signaling lipids in diseases of inflammation [72]. Mechanistically, we show that inhibiting ceramide synthesis or diverting metabolism away from ceramide synthesis using OA protects macrophages from PA-induced trained immunity, suggesting that dietary intervention may help regulate inflammatory dysregulation during disease (Figure 5). And, to complement our in vitro mechanistic findings we show that three single i.p. injections of OA prior to endotoxin stimulation protects KD-fed mice from enhanced disease severity and mortality (Figure 5).

Our findings align with the growing body of evidence indicating that trained immunity is a double-edged sword, where the phenomenon can be beneficial for resistance to infection but detrimental in the context of diseases exacerbated by systemic inflammation [73]. Specifically, we show that PA-induced memory is beneficial in that it promotes clearance of *C. albicans* infection in the kidneys of *Rag1*$^{-/-}$ mice (Figure 4I). In stark contrast, PA-induced memory is detrimental in the context of endotoxemia, a disease driven by organ damage due to acute hyper-inflammation (Figure 4G and H) [74-78]. Furthermore, it is known that trained immunity is a key feature of BCG vaccination, which has been shown to enhance resistance to infections, and is a possible mechanism that drives increased resistance to severe COVID-19 in the BCG-vaccinated population [7,79]. Thus, future research in understanding the plasticity of the SFA- and PA-regulated immune memory responses, enhanced pathogen clearance, and the mechanisms that drive this phenomenon, will be of interest to the larger medical community.

Mechanistically, it is appreciated that PA is not acting as a ligand for the pattern recognition receptor TLR4; however, the presence of TLR4 (independent of TLR4 signaling capability) is required for PA-dependent inflammation [14]. Our data and others contribute to the growing evidence that PA is inducing cell intrinsic stress through alterations in metabolism. The crosstalk between glycolytic and oxidative metabolism, and epigenetics, is crucial for trained immunity in human monocytes, and metabolic intermediates of the TCA cycle directly modify histone methylation patterns associated with proinflammatory cytokines upregulated in trained immunity [4,80,81]. While ceramides are known to modify histone acetylation and DNA methylation patterns, the interplay

between ceramide metabolism and epigenetics within innate immune cells has not been explored [82]. Though we have shown that PA-dependent ceramide production leads to innate immune memory, the impact of these alterations on the epigenome remains unknown. Therefore, the influence of ceramide metabolism on epigenetics will be important to consider in future trained immunity studies where PA serves as the primary stimulus.

Interestingly, we find here that immunoparalysis, which is associated with a prolonged septic response and is enhanced in patients with poorer outcomes, is greater in mice-fed diets enriched in SFAs (Figure 1) [37,38]. However, we found that this SFA-dependent enhanced immunoparalysis is abrogated in GF mice, suggesting, for the first time, that the microbial species within the SFA-fed mice may be regulating the late immunoparalytic phase of endotoxin shock. Considering the clinical correlation of immunoparalysis and increased sepsis mortality, it will be imperative to explore the identity of the SFA-dependent microbiome and the host/microbe mechanisms that drive sepsis-associated immunoparalysis.

Importantly, previous seminal studies concluded that mice treated with antibodies to the TNF receptor and challenged with systemic LPS increased survival from 0% to nearly 100%, suggesting that acute inflammation driven by TNF is responsible for endotoxemia-related mortality [74,75]. Furthermore, it has been shown that TNF is required for acute renal failure, lung injury, and liver damage during LPS challenge [76-78]. These data show that acute inflammation, specifically the bioactivity of TNF, drives endotoxemia

mortality and organ damage in conventional mice. It has also been shown that acute inflammation, specifically TNF production, is a driver of endotoxemia in GF mice [83]. Thus, although our conventional mice show increased immunoparalysis, we suggest that early acute systemic inflammation is the driver of disease severity and mortality in both our conventional and GF endotoxemia mouse models; however, the data we present here is not sufficient to make this conclusion.

In conclusion, this unappreciated role of dietary SFAs, specifically PA, may provide insight into the long-lasting immune reprogramming associated with a high-SFA fed population, and lends insight into the complexity of nutritional immunoregulation. Considering the results in this study, we suggest the potential for SFAs such as PA to directly impact innate immune metabolism and epigenetics associated with inflammatory pathways. Thus, our findings are paramount not only for potential dietary interventions, but also treatment of inflammatory diseases exacerbated by metabolic dysfunction in humans.

Materials and Methods

Cell lines and reagents

RAW 264.7 macrophages (from ATCC), CASP-1KO BMDMs, BMDMs, and BMMs were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) containing L-glutamine, sodium pyruvate, and high glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS; GE Healthcare, SH3039603). BMDMs were also supplemented with 10% macrophage colony-stimulating factor (M-CSF; M-CSF-conditioned media

was collected from National Institutes of Health (NIH) 3T3 cells expressing M-CSF, generously provided by Denise Monack at Stanford University).

Generation of BMDMs, BMMs, and splenocytes

BMDMs and BMMs were harvested from the femurs and tibias of age-matched (6–8 weeks) CO_2-euthanized female BALB/c mice or male and female C57BL/6 J mice. BMDM media was supplemented with 10% M-CSF for differentiation, cells were seeded at 5×10^6 in petri dishes and cultured for 6 days, collected with cold PBS, and frozen in 90% FBS and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen for later use. BMMs were isolated from BMDM fraction using EasySep Mouse Monocyte Isolation Kit (STEMCELL). Spleens were harvested from age-matched (6–8 weeks) CO_2-euthanized female BALB/c mice, tissue was disrupted using the end of a syringe plunger on a 70-µm cell strainer and rinsed with FACS buffer (PBS + 2 mM EDTA). Cells were subjected to red blood cell lysis with RBC lysing buffer (Sigma) followed by neutralization in FACS buffer.

Treatments

After thawing and culturing for 5 days, BMDMs were pelleted and resuspended in DMEM containing 5% FBS, 2% endotoxin- and fatty acid-free BSA (Proliant Biologicals) and 10% M-CSF. Cells were seeded at 2.5×10^5 cells/well in 24-well tissue culture plates, treated with EtOH (1.69%, or 0.83%) 10 ng/mL LPS (Ultrapure LPS, *Escherichia coli* 0111:B4, Invivogen), 500 µM or 1 mM PA (Sigma-Aldrich, PHR1120), 10 uM FB1 (Sigma-Aldrich, F1147), or 200 µM OA (Sigma-Aldrich, O7501) and

incubated at 37°C and 5% CO_2 for 12 or 24 hr. Next, cells were treated with an additional 10 ng/mL LPS and incubated an additional 12 or 24 hr. RAW 264.7 macrophages were thawed and cultured for 3–5 days, pelleted and resuspended in DMEM containing 5% FBS and 2% endotoxin- and fatty acid-free BSA, and treated identical to BMDM treatments. BMMs were seeded immediately after harvesting at 4×10^5 cells/well in 96-well V-bottom plates in DMEM containing 10% FBS and treated with LPS for 2 or 24 hr. Splenocytes were seeded immediately after harvesting at 1×10^5 cells/well in 96-well V-bottom plates in RPMI media with L-glutamine (Cytiva) containing 10% FBS and treated with LPS for 2 or 24 hr. BMDMs for ex vivo treatments were isolated as described above, plated at 2.5×10^5 cells/well in 24-well plates, and stimulated with 10 ng/mL LPS after 12 hr of adherence. For all treatments, supernatant was removed for ELISA analysis, and cells were lysed with TRIzol (ThermoFisher), flash-frozen in liquid nitrogen, and stored at –80°C until qRT-PCR analysis. For all plates, all treatments were performed in triplicate.

Flow cytometry

Modified panel using combined methods from Kaufmann et al., Nowlan et al., and Vasquez et al. Red blood cells were lysed in BM cells using RBC lysis buffer (Biolegend). BM cells (3×10^6 cells) were stained with viability stain Live/Dead Fixable Aqua (ThermoFisher) at the concentration of 1:200 for 30 min at 4°C. Next, cells were washed with FACS buffer (PBS supplemented with 0.5% BSA; Proliant Biologicals, fatty acid free), and incubated with anti-CD16/32 (clone 93, BioLegend) at a concentration of 1:100 in FACS buffer for 10 min at 4°C. The following antibodies were

then used for staining HSCs, and MPPs: anti-Ter-110, anti-CD11b (clone M1/70), anti-CD5 (clone 53–7.3), anti-CD4 (clone RM4-5), anti-CD8a (clone 53–6.7), anti-CD45R (clone RA3-6B2), and anti-Ly6G/C (clone RB6-8C5), all biotin-conjugated (all BD Bioscience), were added at a concentration of 1:100 for 30 min at 4°C and washed with FACS buffer. Streptavidin-APC-Cy7 (eBioscience), anti-CD150-eFluor450 (clone Q38-480, eBioscience), anti-CD48-PerCPeFluor710 (BD Bioscience), anti-Flt3-PE (clone A2F10.1, BD Bioscience), anti-CD34-PEDazzle 594 (clone HM34, BioLegend), anti-CD27-PE-Cy7 (eBioscience), and anti-CD201-APC (eBioscience) were added all at a concentration of 1:100 for 20 min at 4°C. All cells were then washed with FACS buffer before and after incubation in 1% paraformaldehyde for 30 min at 4°C. Cells were acquired on BD flow cytometer (FACSymphony A1 Cell Analyzer) with FACSDiva Software. Analyses were performed using FlowJo software v.10.1. The DownSample version 3.3.1 plugin was used to standardize events for each sample after populations were gated.

Lactate dehydrogenase assays

BMDMs were cultured as stated above with culture media, PA, or ethanol in 96-well tissue-culture plates at a concentration of 5×10^4 cells/well and incubated for 12 hr. Cells were treated with PBS or 10 ng/mL LPS in a phenol-red-free Optimem media (ThermoFisher) and incubated an additional 12 or 24 hr. Supernatants were collected at the specified time points with LDH release quantified with a CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega). Cytotoxicity was measured per well as a

percentage of max LDH release, with background media-only LDH release subtracted. For all plates, all treatments were performed in triplicate.

Measurement of cell viability

Cell viability was determined by 0.4% Trypan Blue dye exclusion test executed by a TC20 Automated Cell Counter (Bio-Rad).

Blood RNA extraction and real-time qPCR

Mice were treated with PBS or LPS, and at specified time points 10–20 μL of blood was collected from the tail vein, transferred into 50 μL of RNALater (ThermoFisher Scientific), and frozen at –80°C. RNA extractions were performed using RNeasy Mini Kit (Qiagen), and cDNA was synthesized from RNA samples using SuperScript III First-Strand synthesis system (Invitrogen). Gene-specific primers were used to amplify transcripts using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). A complete list of all primers used, including the names and sequences, is supplied as Table 2.

Enzyme-linked immunosorbent assay

TNF, IL-6, and IL-1β concentrations in mice serum were measured and analyzed using TNF, IL-6, and IL-1β Mouse ELISA kits (ThermoFisher Scientific), according to the manufacturer's instructions. Absorbances were measured at a wavelength of 450 nm using a microplate reader (BioTek Synergy HTX). Values below the limit of detection (LOD) of the ELISA were imputed with LOD divided by 2 (LOD/2) values.

LPS-induced endotoxemia model

Age-matched (6–8 weeks) female BALB/c mice were anesthetized with isoflurane and injected subcutaneously with ID transponders (Bio Medic Data Systems). 2 weeks post diet change, and 1 week post ID transponder injection, mice were stimulated with a single injection of 6–10 mg/kg LPS reconstituted in endotoxin-free LAL reagent water (Invivogen) and diluted in PBS for a total volume of 200 μL. Control mice received corresponding volumes of PBS. Progression of disease was monitored every 2 hr after LPS injection for clinical signs of endotoxin shock based on weight, coat and eyes appearance, level of consciousness, and locomotor activity. Age-matched (20–21 weeks) female C57BL/6 mice were treated as described above, except for their LPS dose (4.5 mg/kg). Temperature was recorded using a DAS-8007 thermo-transponder wand (Bio Medic Data Systems). For PA injections, a solution of 750 mM ethyl palmitate (Millipore Sigma), 1.6% lecithin (Sigma-Aldrich), and 3.3% glycerol was made in endotoxin-free LAL reagent water (Lonza). The lecithin-glycerol-water solution was used as a vehicle, and mice were injected with 200 μL of the vehicle as a control or ethyl palmitate solution to increase serum PA levels. For OA injections, a solution of 300 mM OA (Sigma-Aldrich) was made using the same solution and vehicle described above. Mice were injected i.p. with 200 μL of the vehicle as a control, or OA solution, between 7 and 9 pm for 3 days prior to LPS exposure.

Mouse diets, glucose, and ketones

Six-week-old female mice were fed soft, irradiated chow (PicoLab Mouse Diet 20, product 5058) and allowed to acclimate to research facility undisturbed for 1 week. Chow

was replaced by WD (Envigo, TD.88137), KD (Envigo, TD.180423), or SC (Envigo, TD.08485), and mice were fed ad libitum for 2 weeks before induction of endotoxemia. For KD, food was changed daily. For WD, food was changed every 72 hr. Ketones and blood glucose were measured weekly and immediately prior to LPS injections with blood collected from the tail vein using Blood Ketone and Glucose Testing Meter (Keto-Mojo), or with urine collected on ketone indicator strips (One Earth Health, Ketone Test Strips).

Statistics analysis

Mann Whitney, Mantel-Cox, and student's t-tests were carried out with GraphPad Prism 9.0 software.

Ethical approval of animal studies

All animal studies were performed in accordance with NIH guidelines, the Animal Welfare Act, and US federal law. All animal experiments were approved by the Oregon Health and Sciences University (OHSU) Department of Comparative Medicine or Oregon State University (OSU) Animal Program Office and were overseen by the Institutional Care and Use Committee (IACUC) under Protocol IDs #IP00002661 and IP00001903 at OHSU and #5091 at OSU. Conventional animals were housed in a centralized research animal facility certified by OHSU. Conventional 6–10-week-aged female BALB/c mice (Jackson Laboratory 000651) were used for the endotoxemia model, and isolation of BMDMs, BMMs, and splenocytes. GF male and female C57BL/6 mice (Oregon State University; bred in house) between 14- and 23-week-old were used for the GF endotoxemia model. BALB/c *Rag1*$^{-/-}$ mice between 8 and 24 weeks were

infected i.v. with 2×10^6 CFUs of *C. albicans* SC5314 (ATCC #MYA-2876), and kidney fungal burden was assessed 24 hr post-infection. Kidneys were harvested 24 hr post-infection, and homogenized organs were plated in serial dilutions on Yeast Peptone Dextrose plates to assess fungal burden.

Lipidomics PCA analysis

Mice on specialized diets were sacrificed at the indicated time points after PBS or LPS treatment with 300–600 μL of blood collected via cardiac puncture into heparinized tubes. Blood samples were centrifuged for 15 min at 2500 rpm at 4°C, and serum was transferred to a new tube before storage at –80°C. Serum samples were analyzed via LC-MS/MS. Lipidomic data sets were scaled using the *scale* function, and PCAs were performed using the *prcomp* function from the stats package in R Version 3.6.2. Visualization of PCAs and biplots was performed with the *fviz_pca_ind* and *fviz_pca_biplot* functions from the factoextra package and with the *ggplot2* package [84,85]. For each diet group, 95% confidence ellipses were plotted around the group mean using the *coord.ellipse* function from the FactoMineR package [86]. Heatmaps were created using the *pheatmap* package [87].

Table 1. Diet compositions (values represent percentage of total kcal).

Diet	Envigo Cat. No.	Fat %	Carb %	Protein %	kcal/g
Standard Chow	TD.08485	13 (3% PA)	67.9	19.1	3.6
Western Diet	TD.88137	42 (12% PA)	42.7	15.2	4.5
Ketogenic Diet	TD.180423	90.5 (23% PA)	0.4	9.1	6.8
PicoLab Mouse Diet 20 (product 5058)	N/A	21.6	55.2	23.2	4.6

Table 2. List of primers used in this study.

Primer	Sequence F (5′-3′)	Sequence R (5′-3′)
TNF	GATCGGTCCCCAAAGGGATG	TGGTTTGTGAGTGTGAGGGTC
IL-1β	AGCTTCCTTGTGCAAGTGTCT	GACAGCCCAGGTCAAAGGTT
IL-6	TCC AGT TGC CTT CTT GGG AC	AGT CTC CTC TCC GGA CTT GT
IL-10	GTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
β-actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTCC

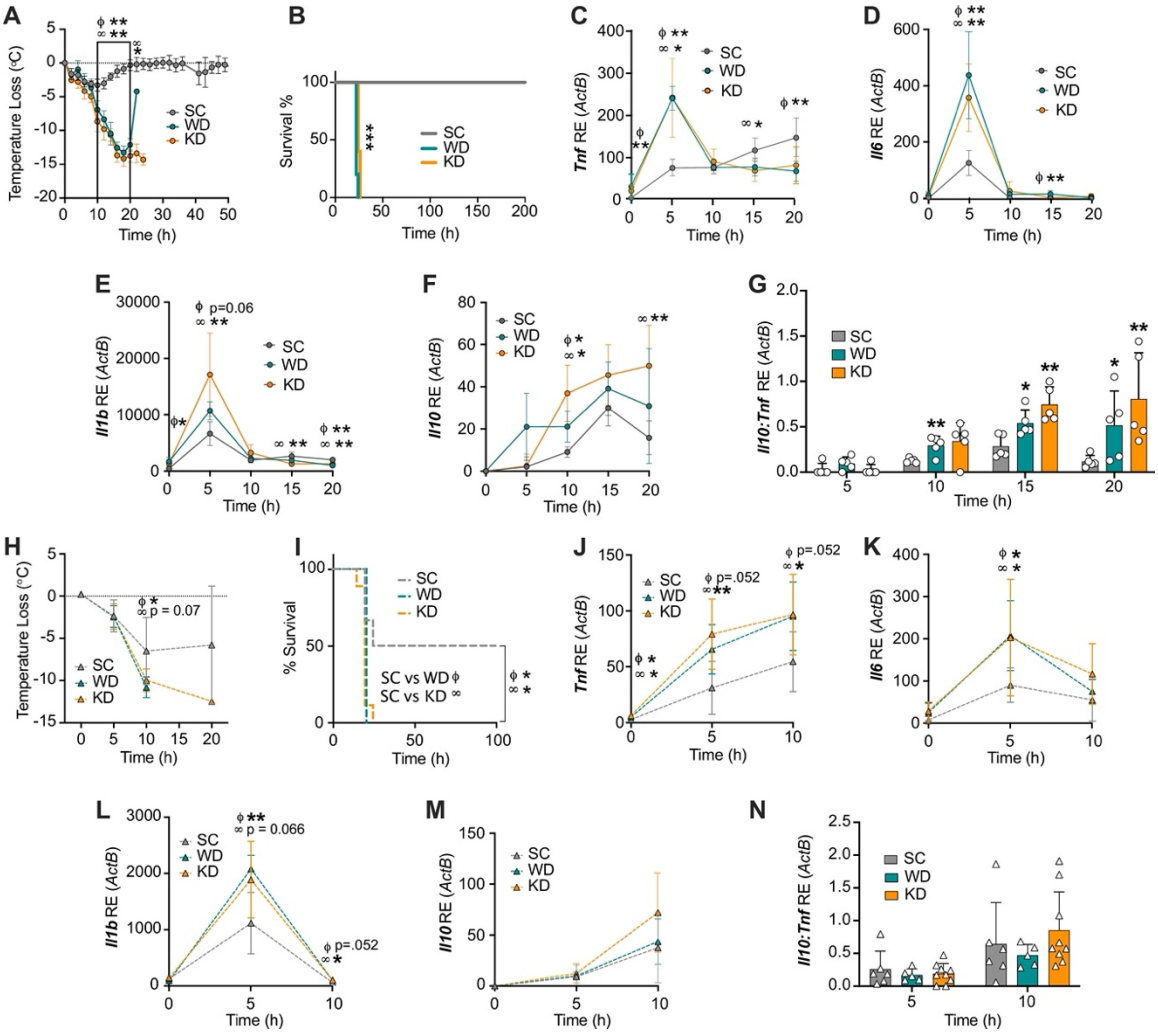

Figure 1. Diets enriched in saturated fatty acids lead to enhanced endotoxemia severity and altered systemic inflammatory profiles, independent of diet-associated microbiome. (**A–G**) Age-matched (6–8 weeks) female BALB/c mice were fed standard chow (SC), Western diet (WD), or ketogenic diet (KD) for 2 weeks and injected intraperitoneal (i.p.) with 6 mg/kg of lipopolysaccharide (LPS). (**A**) Temperature loss and (**B**) survival were monitored every 2 hr. At indicated times, 10–20 µL of blood was drawn via the tail vein, RNA was collected, and samples were assessed for expression of (**C**) *Tnf,* (**D**) *Il6,* (**E**) *Il1b,* and (**F**) *Il10* via qRT-PCR. (**G**) *Il10:Tnf* ratio was calculated for 5, 10, 15, and 20 hr post-injection (p.i.) with LPS. (**H–N**) Next, 19–23-week-old female and 14–23-week-old male and female germ-free C57BL/6 mice were fed SC, WD, or KD for 2 weeks and injected i.p. with 50 mg/kg of LPS. (**H**) Temperature loss and (**I**) survival were monitored every 5 hr p.i. (**J–N**) At indicated times, 10–20 µL of blood was drawn via the tail vein, RNA was collected, and samples were assessed for expression of (**J**) *Tnf,* (**K**) *Il6,* (**L**) *Il1b,* and (**M**) *Il10* via qRT-PCR. (**N**) *Il10:Tnf* ratio was calculated for 5 and 10 hr p.i. with LPS. For (**A–G**), all experiments were run three times, and data are representative of one experiment, n=5 per diet group. For (**H–N**) SC, n=6; WD, n=5; and KD, n=9; and data are representative of one experiment. For (**A, C–G, H**, and **J–N**) a Mann Whitney test was used for pairwise comparisons. For (**B**) and (**I**) a log-rank Mantel-Cox test was used for survival curve comparison. For all panels, *p<0.05; **p<0.01; ***p<0.001. For (**C–E**), Φ symbols indicate WD significance, and ∞ symbols indicate KD significance. Error bars shown mean ± SD.

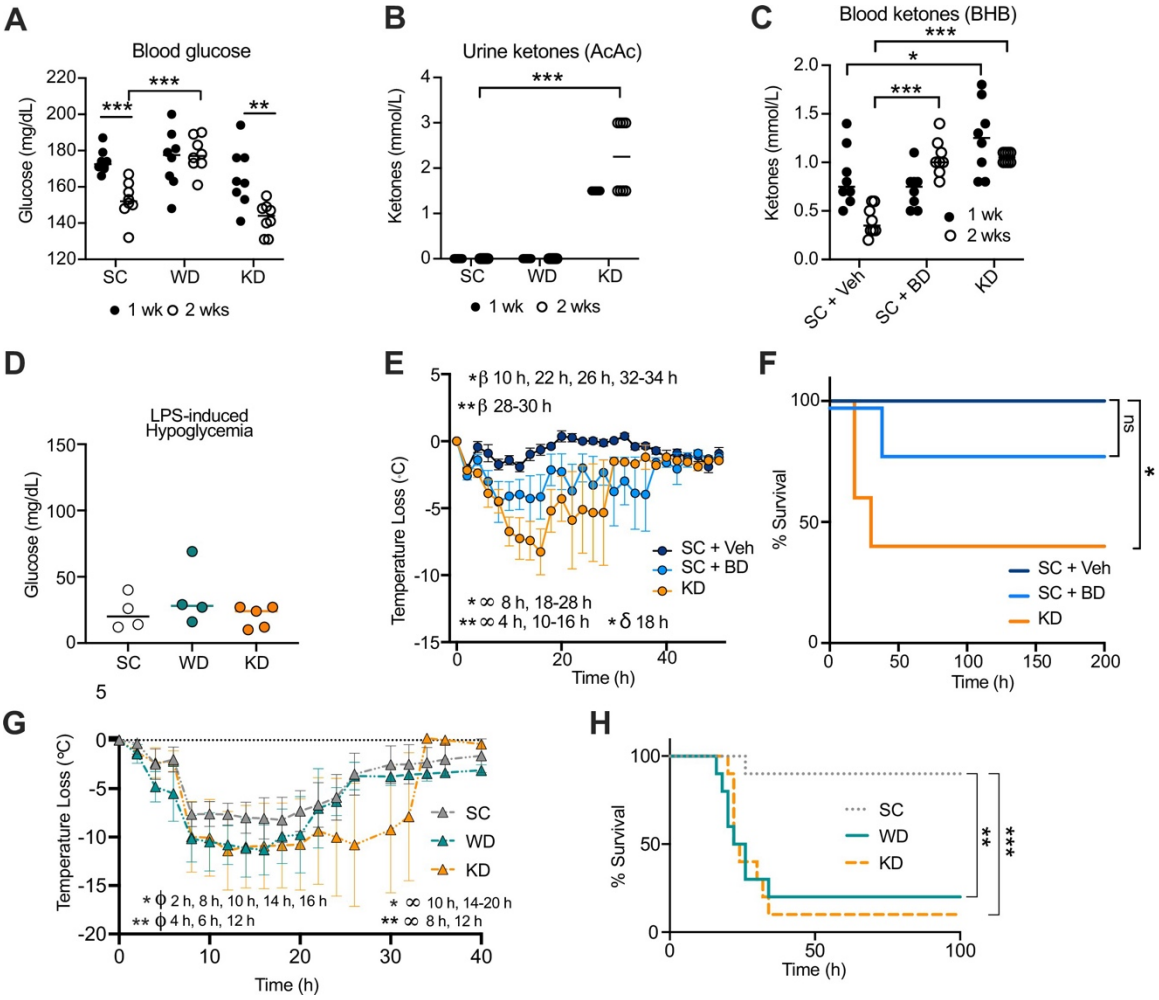

Figure 1—figure supplement 1. Increase in disease severity in ketogenic diet (KD) mice is independent of ketosis. Age-matched (6–8 weeks) female BALB/c mice were fed standard chow (SC), Western diet (WD), or KD for 2 weeks. At 1 week and 2 weeks, (**A**) blood was collected via the tail vein to measure blood glucose levels using a glucose testing meter (Keto-Mojo), and (**B**) urine was collected on ketone indicator strips to measure levels of systemic acetoacetate (AcAc). Age-matched (6–8 weeks) female BALB/c mice were fed SC supplemented with 1,3-butanediol (SC + BD) or with a saccharine vehicle solution as a control (SC + Veh), or KD for 2 weeks. At 1 week and 2 weeks, (**C**) blood was collected via the tail vein to measure levels of systemic β-hydroxybutyrate (BHB) using a ketone testing meter (Keto-Mojo). At 2 weeks, SC-, WD-, and KD-fed mice were injected intraperitoneal (i.p.) with lipopolysaccharide (LPS; 6 mg/kg) and (**D**) 25 hr post-injection (p.i.), blood glucose levels were measured as stated in (A). (**E**) Temperature loss and (**F**) survival were monitored every 2 hr for mice treated as in (**C**) followed by i.p. injection with LPS (10 mg/kg). Age-matched (20–21 weeks) female C57BL/6 mice were fed SC, WD, or KD for 2 weeks followed by i.p. injection with LPS (4.5 mg/kg). (**G**) Temperature loss and (**H**) survival were monitored every 2 hr. For (**A, B, D**), all experiments were run three times, and data are representative of one experiment, n=5–8 mice/group. For (**C, E, F**), all experiments were run three times, and data are representative of one experiment, n=5–8 mice/group. For (**G, H**), data are representative of one experiment, n=10 mice/group. For (**A–C, E, G**), a Mann-Whitney U test was used for pairwise comparisons. For (**F, H**), a log-rank Mantel-Cox test was used for survival curve comparison. For (**E**), β

symbols indicate SC +Veh vs. SC + BD significance, ∞ symbols indicate SC + Veh vs. KD significance, and δ symbols indicate SC + BD vs. KD significance. For (**G**), ϕ symbols indicate SC vs. WD significance, and ∞ symbols indicate SC vs. KD significance. For all panels, * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. Error bars show mean ± SD.

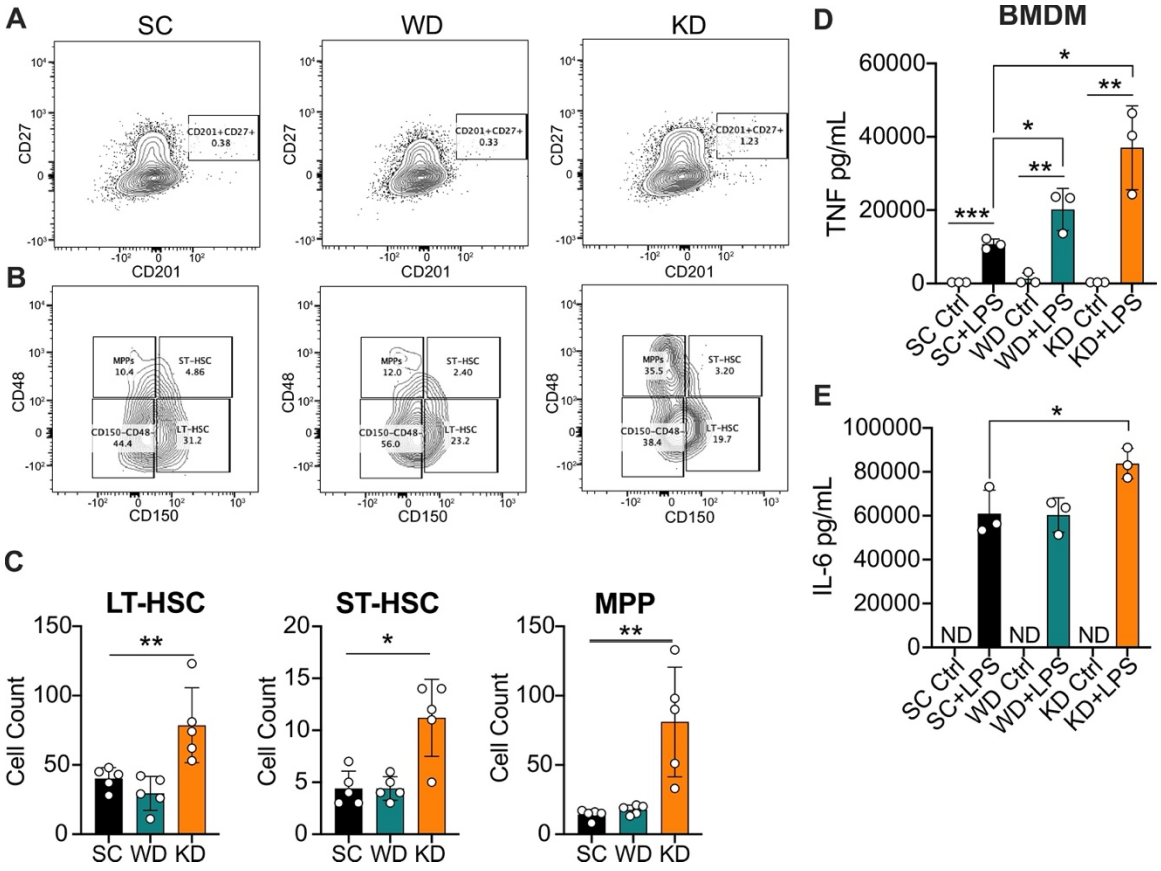

Figure 2. Ketogenic diet (KD) feeding alters HSC populations and bone marrow-derived macrophages (BMDMs) from KD-fed mice show a hyper-inflammatory response to lipopolysaccharide (LPS) *ex vivo*. Bone marrow was extracted from the femurs and tibias of age-matched (6–8 weeks) female BALB/c mice fed standard chow (SC), Western diet (WD), or KD for 2 weeks. (**A**) Fluorescence-Activated Cell Sorting (FACS) plots of total HSCs ($CD201^{+}CD27^{+}$) and (**B**) LT-HSCs, ST-HSCs, and multipotent progenitors (MPPs) from mice fed SC, WD, or KD for 2 weeks. Quantification of (**C**) the total numbers of LT- and ST-HSCs, and MPPs in bone marrow from mice fed SC, WD, or KD for 2 weeks. Next, BMDMs were plated at 5×10^6 cells/mL and differentiated for 7 days in media supplemented with macrophage colony-stimulating factor. Cells were split and plated in 24-well plates to adhere for 12 hr and treated with media (Ctrl) or LPS (24 hr; 10 ng/mL). Supernatants were assessed via ELISA for (**D**) TNF and (**E**) IL-6 secretion at 24 hr post-LPS treatment. IL-6 Ctrl supernatants were below the limit of detection; ND = no data. For (**A-E**), all experiments were run three times, and data are representative of one experiment, n=5 per diet group. (**C**) A Mann Whitney test was used for pairwise comparisons. (**D, E**) For all plates, all treatments were performed in triplicate, and a student's t-test was used for statistical significance. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. Error bars show the mean ± SD.

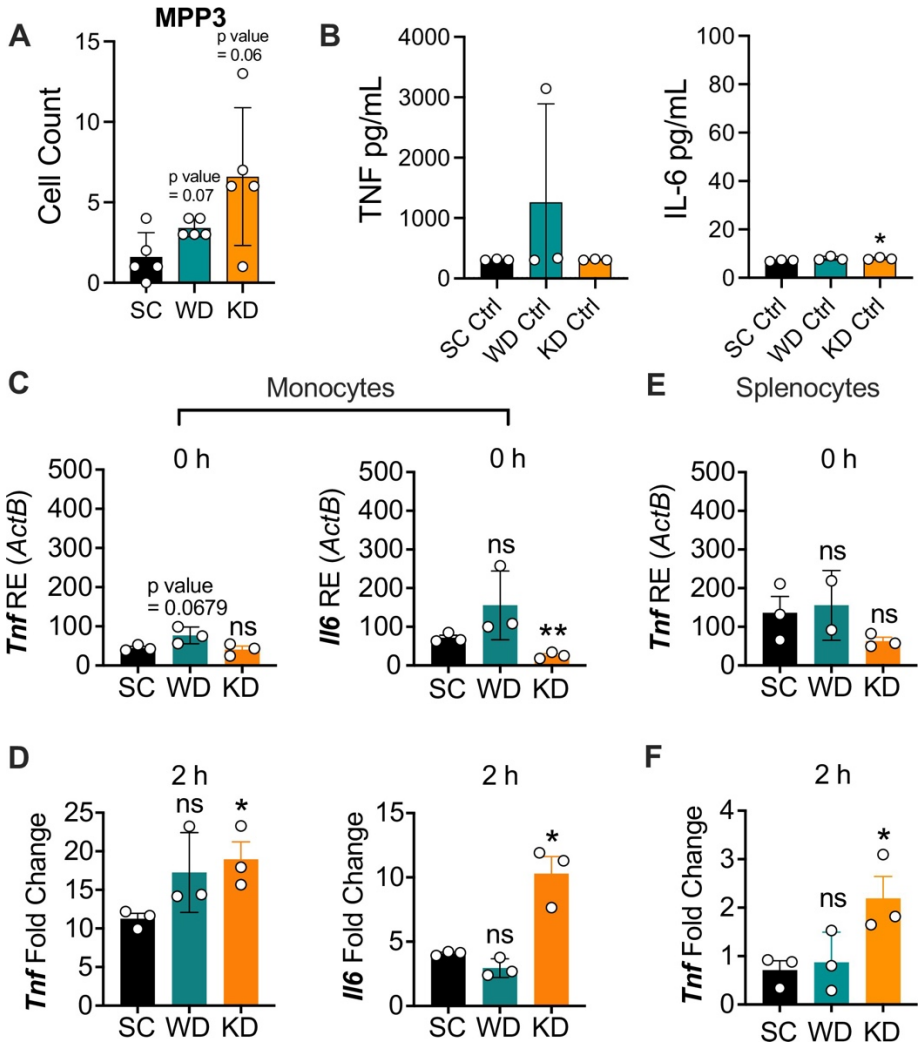

Figure 2—figure supplement 1. Ketogenic diet (KD) does not alter multipotent progenitor (MPP) differentiation or basal inflammation in bone marrow-derived macrophages (BMDMs), and monocytes and splenocytes show a hyper-inflammatory response to lipopolysaccharide (LPS) *ex vivo*. Age-matched (6–8 weeks) conventional, wild-type, female BALB/c mice were fed standard chow (SC), Western diet (WD), or KD for 2 weeks. Bone marrow was extracted from the femurs and tibias of mice, HSCs were isolated via FACS, and (**A**) MPPs were quantified. BMDMs were plated at 5×10^6 cells/mL and differentiated for 7 days in media supplemented with macrophage colony-stimulating factor. Cells were split and plated in 24-well plates to adhere for 12 hr and treated with media (Ctrl) for 24 hr. Supernatants were assessed via ELISA for (**B**) TNF and IL-6 secretion. Monocytes were isolated from the femurs and tibias of mice and plated at 2×10^6 cells/mL. RNA was extracted from (**C**) untreated monocytes (0 hr) or (**D**) monocytes with LPS (10 ng/mL) for 2 hr. Expression of *Tnf* and *Il6* was analyzed via qRT-PCR. Splenocytes were isolated and plated at 1×10^6 cells/mL. RNA was isolated from (**E**) untreated splenocytes (0 hr) or (**F**) splenocytes treated with LPS (10 ng/mL) for 2 hr. Expression of *Tnf* and *Il6* was analyzed via qRT-PCR. For (**A, B**), all experiments were run three times, and data are representative of one experiment, n=5 per diet group. For (**C-F**), all experiments were run twice, and data are representative of one experiment, n=3 per diet group. (**A**) A Mann Whitney test was used for pairwise comparisons. (**B-F**) For all

plates, all treatments were performed in triplicate, and a student's t-test was used for statistical significance. For all panels, * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. Error bars show mean ± SD.

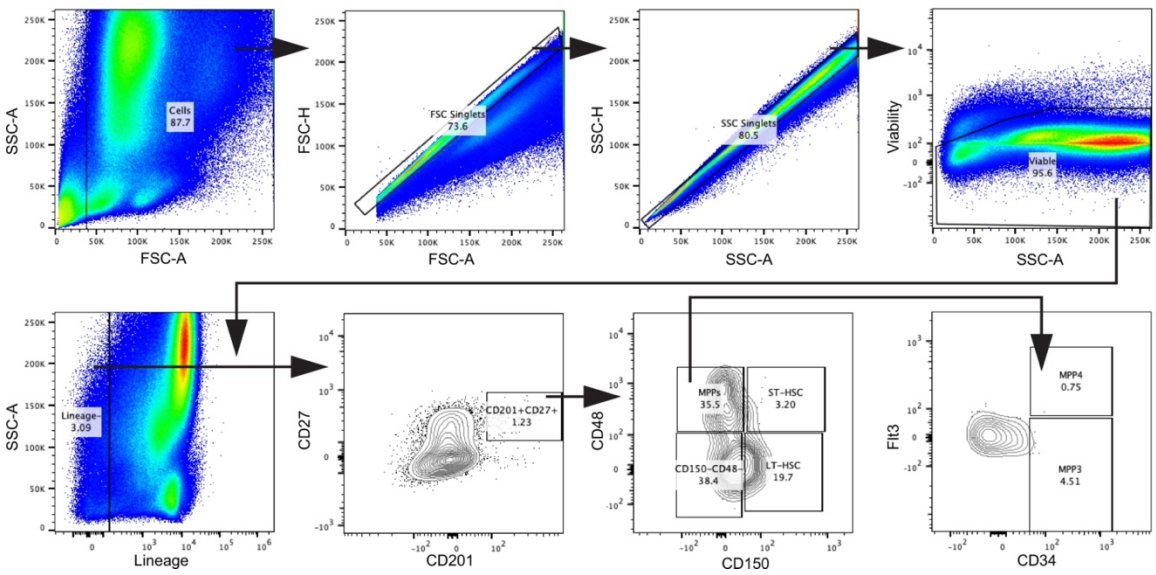

Figure 2—Figure supplement 2. Gating strategy for HSCs, related to Figure 2. Cells were gated in FSC-A against SSC-A. Doublets were excluded using FSC-A against FSC-H and subsequently SSC-A against SSC-H. Viable cells were gated, and lineage-committed cells were excluded. Within the lineage-negative cells, the $CD201^{+}CD27^{+}$ population was gated. In a CD150 against CD48 plot, the $CD201^{+}CD27^{+}$ cells were divided into LT-HSC, ST-HSC, multipotent progenitor (MPP), and the remaining $CD150^{-}CD48^{-}$ population. MPPs were characterized as MPP3 and MPP4 by their surface expression of CD34 and Flt3.

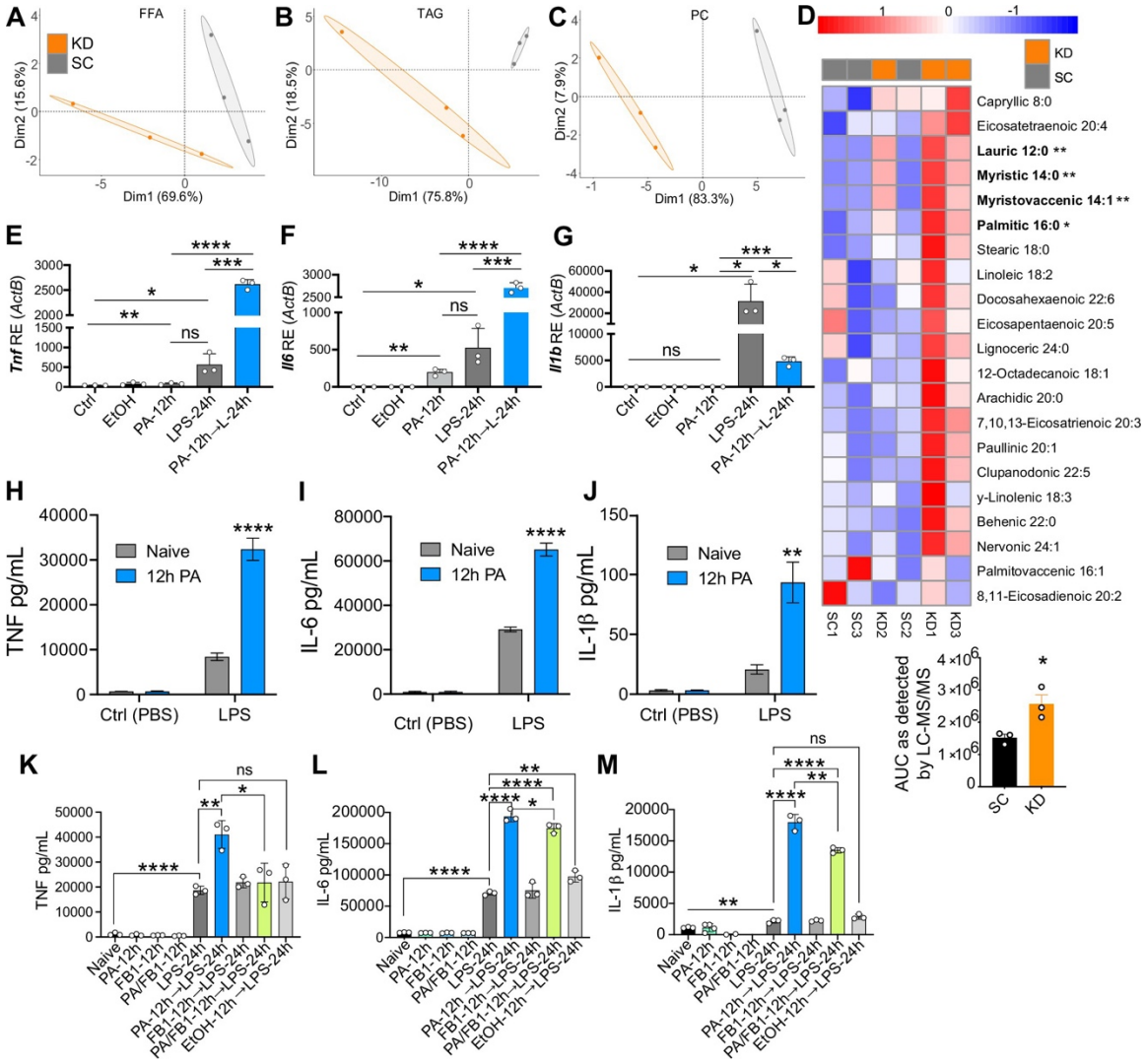

Figure 3. Ketogenic diet (KD) alters lipid profiles, and palmitic acid (PA) is mediating a hyper-inflammatory response to secondary challenge with lipopolysaccharide (LPS). Data points represent single animal samples, and colors represent groups fed standard chow (SC; gray) or KD (orange) diets for 2 weeks. A 95% confidence ellipse was constructed around the mean point of each group for (**A**) free fatty acids (FFA), (**B**) triglycerides (TAG), and (**C**) phosphatidylcholines (PC). (**D**) Heatmap analysis of FFA in SC and KD mice. Components that are significantly different between the two groups are in bold. Below the heatmap is a comparison of PA 16:0 peak area detected by Liquid Chromatography Quadruple Time of Flight Mass Spectrometry (LC-QToF MS/MS) between SC and KD groups; AUC = area under the curve. Statistical significance is determined by unpaired two-tailed t-test between SC and KD groups with n=3 per group. Primary bone marrow-derived macrophages (BMDMs) were isolated from age-matched (6–8 weeks) C57BL/6 female and male mice. BMDMs were plated at 1×10^6 cells/mL and treated with either ethanol (ethanol (EtOH); media with 0.83% ethanol), media (Ctrl for LPS), or LPS (10 ng/mL) for 12 hr, or PA (PA stock diluted in 0.83% EtOH; 1 mM PA conjugated to 2% bovine serum albumin [BSA]) for 12 hr, with and without a secondary challenge with LPS. After indicated time points, RNA was isolated, and expression of (**E**) *Tnf*, (**F**) *Il6*, and (**G**) *Il1b* was measured via qRT-PCR. BMDMs were plated at 1×10^6 cells/mL and treated with either ethanol (EtOH; media with 0.83% ethanol), media (Naïve), or 1 mM PA

for 12 hr followed by PBS (control) or LPS (10 ng/mL). Supernatants were assessed via ELISA for (**H**) TNF, (**I**) IL-6, and (**J**) IL-1β secretion. Next, BMDMs were plated at 1×10^6 cells/mL and treated with either media (Ctrl), LPS (10 ng/mL) for 24 hr, PA (PA stock diluted in 0.83% EtOH; 0.5 mM PA conjugated to 2% BSA) for 12 hr, Fumonisin B1 (FB1; 10 μM; diluted in 0.14% EtOH) or EtOH (0.97% to mimic simultaneous PA/FB1 treatment). Controls for all treatments are shown next to experimental groups treated additionally with LPS (10 ng/mL) for 24 hr. Supernatants were assessed via ELISA for (**K**) TNF, (**L**) IL-6, and (**M**) IL-1β secretion. For all plates, all treatments were performed in triplicate. For all panels, a student's t-test was used for statistical significance. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Error bars show the mean ± SD

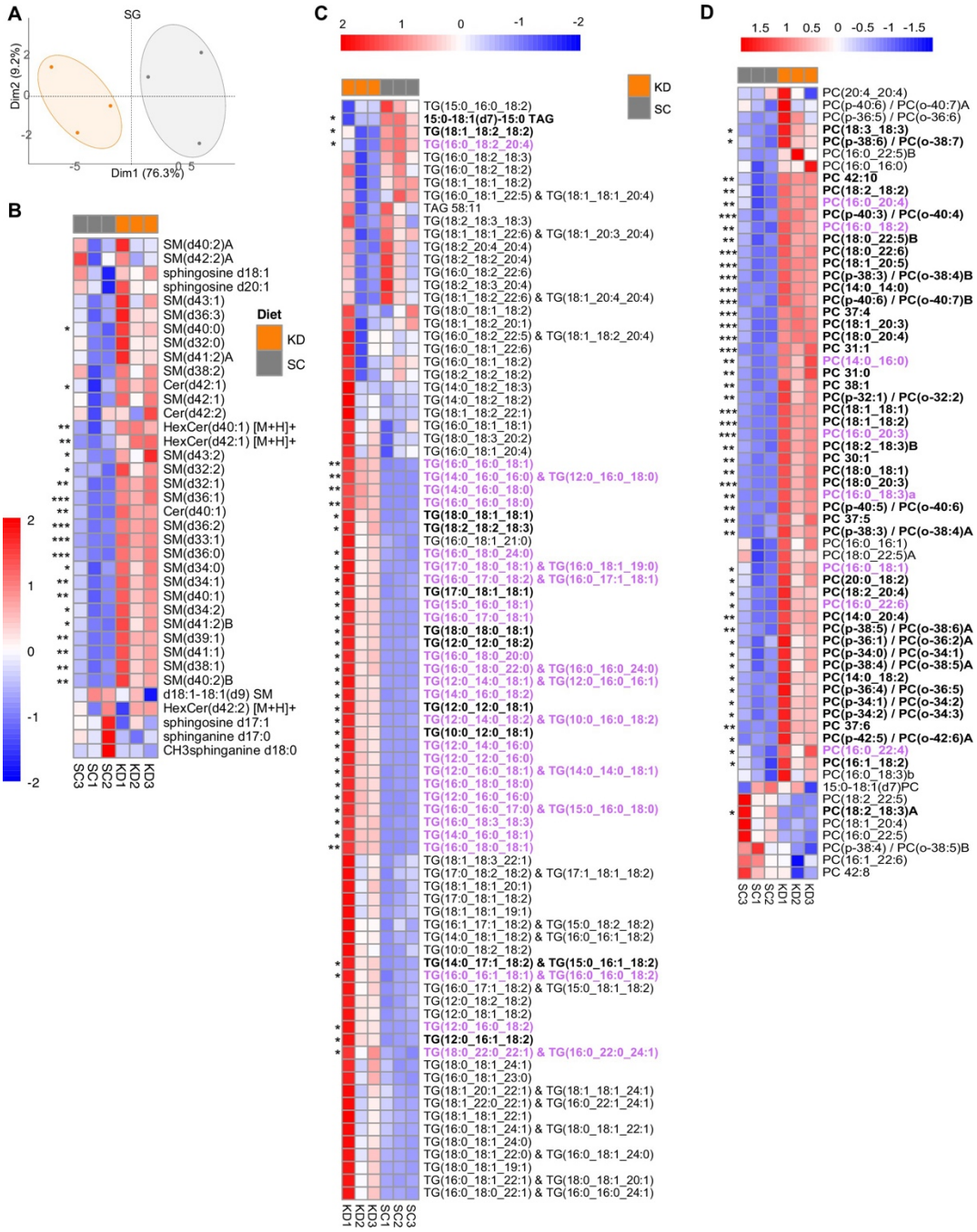

Figure 3—figure supplement 1. Principal component analysis and heatmap analysis of sphingolipid lipidomic data in mouse serum samples. (**A**) Data points represent single animal samples, and colors represent groups fed standard chow (SC; gray) or Ketonic diet (KD; orange) diets for 2 weeks, and a 95% confidence ellipse was constructed around the mean point of each group. Heatmap analysis of (**B**) sphingolipids (SM), (**C**) triglycerides (TG), and (**D**) phosphatidylcholines (PC) in SC and KD groups. Lipid components containing 16:0 palmitic chains are highlighted in purple, and components that are significantly different between the two groups are in bold. Statistical significance determined by unpaired

two-tailed t-test between SC and KD groups. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. n=3 per group.

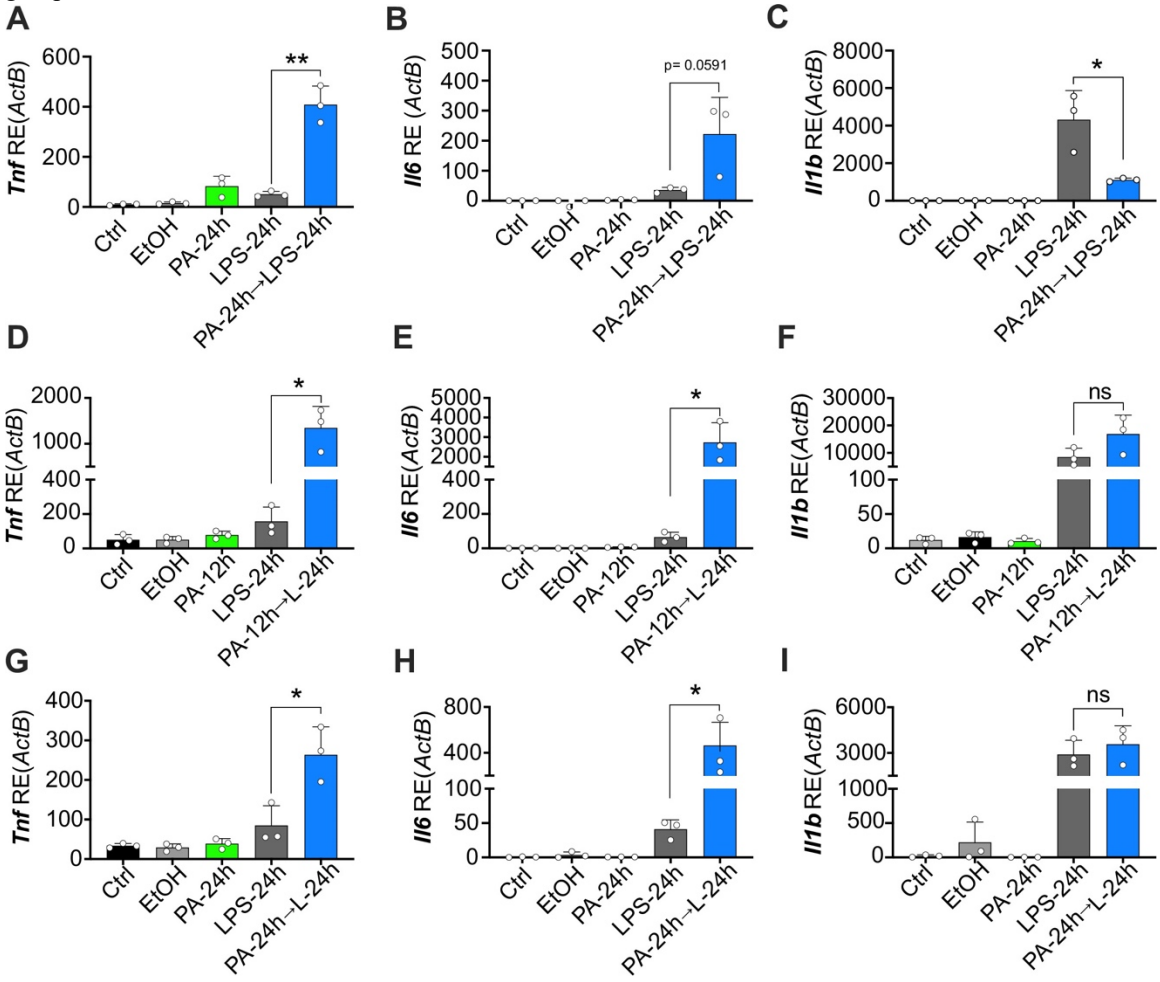

Figure 3—figure supplement 2. Physiological levels of palmitic acid (PA) induce a hyper-inflammatory response to secondary challenge with lipopolysaccharide (LPS) in macrophages. Primary bone marrow-derived macrophages (BMDMs) were isolated from age-matched (6–8 weeks) female and male mice. (**A–C**) BMDMs were plated at 1×10^6 cells/mL and treated with ethanol (EtOH; media with 1.69% ethanol), media (Ctrl for LPS), or PA (1 mM; diluted in 1.69% EtOH) for 12 hr. Next, PA-treated cells were treated with LPS (10 ng/mL) for 24 hr, and all other wells were given fresh media. (**D–I**) BMDMs were plated at 1×10^6 cells/mL and treated with PA (0.5 mM; diluted in 1.69% EtOH) for 12 or 24 hr. Next, PA-treated cells were treated with LPS (10 ng/mL) for 24 hr, and all other wells were given fresh media. After indicated time points, RNA was isolated and expression of (**A, D, G**) *Tnf,* (**B, E, H**) *Il6*, and (**C, F, I**) *Il1b* was measured via qRT-PCR. For all plates, treatments were performed in triplicate. For all panels, a student's t-test was used for statistical significance. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. Error bars show mean ± SD.

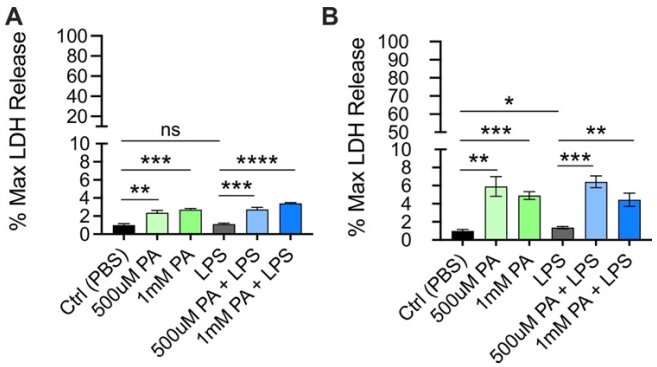

Figure 3—figure supplement 3. Cytotoxicity as determined by lactate dehydrogenase (LDH) release from bone marrow-derived macrophages (BMDMs) pre-treated with palmitic acid (PA) followed by lipopolysaccharide (LPS) stimulation. BMDMs from age-matched (6–8 weeks) male and female C57BL/6 mice were plated in 96-well plates at 5×10^4 cells/well and incubated for 12 hr with PA (0.5 mM or 1 mM). Next, media was removed, and cells were treated with PBS for 10 ng/mL LPS in phenol-red-free Opti-MEM media and incubated for an additional 24 hr. Supernatants were collected, and LDH release was quantified using CytoTox96 non-radioactive cytotoxicity assay. (**A, B**) Cytotoxicity is shown as percentage of max LDH release. For all plates, all treatments were performed in triplicate, and a student's t-test was used for statistical significance. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Error bars show mean ± SD.

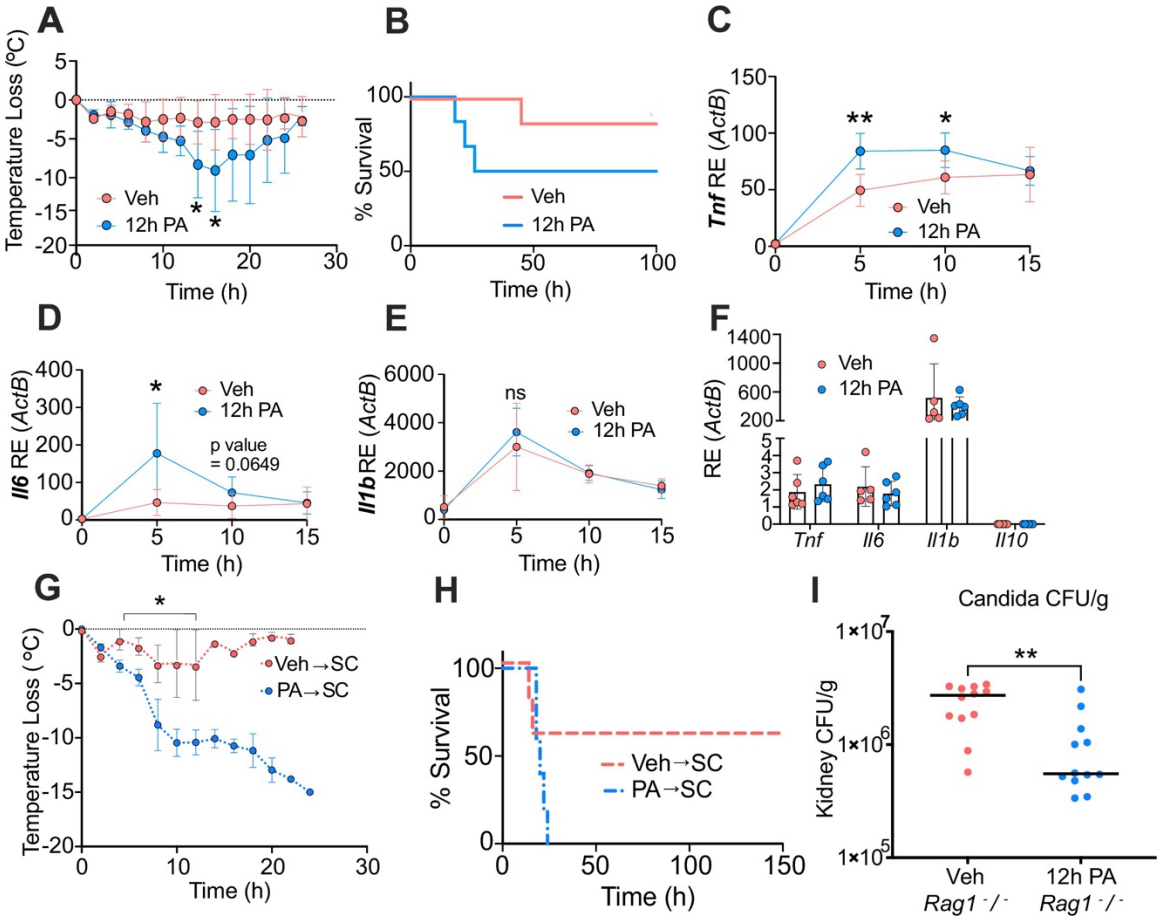

Figure 4. Palmitic acid (PA) acts as a novel mediator of trained immunity by inducing a hyper-inflammatory response lipopolysaccharide (LPS)-induced endotoxemia and enhancing clearance of *Candida albicans* infection. Age-matched (6–8 weeks) female BALB/c mice were fed standard chow (SC) for 2 weeks and injected intraperitoneal (i.p.) with ethyl palmitate (PA, 750 mM) or vehicle (Veh) solutions 12 hr before i.p. LPS injections (10 mg/kg). (**A**) Temperature loss was monitored every 2 hr as a measure of disease severity or (**B**) survival. At indicated times blood was collected via the tail vein, RNA was extracted, and samples were assessed for expression of (**C**) *Tnf,* (**D**) *Il6,* and (**E**) *Il1b* via qRT-PCR. (**F**) Blood was collected via the tail vein from Veh and PA pre-treated (12-hr PA) mice immediately prior to LPS injection, and samples were assessed for expression of *Tnf, Il6, Il1b*, and *Il10* via qRT-PCR. Additionally, age-matched (6–8 weeks) female BALB/c mice fed SC, injected i.p. with ethyl palmitate (PA, 750 mM) or Veh solutions every day for 9 days, and then rested for 7 days before i.p. LPS injections (10 mg/kg) (**G**) Temperature loss and (**H**) survival were monitored during endotoxemia. (**I**) Age-matched (8–9 weeks) female *Rag1*$^{-/-}$ mice were injected i.p. with ethyl palmitate (PA, 750 mM) or Veh solutions 12 hr before intravenous *C. albicans* infection. Fungal burden of kidneys from Veh and PA pre-treated (12-hr PA) mice 24 hr after *C. albicans* infection. For (**A–F**), experiments were run three times, and data are representative of one experiment, n=3 mice/group. For (**G, H**), experiments were run twice, and data are representative of one experiment, n=5 mice/group. For (**I**), experiments were run three times, and data are representative of one experiment, n=6 mice/group. For (**A**), (**C–E**), (**G**), and (**I**), a Mann Whitney test was used for pairwise comparisons. For (**B**) and (**H**), a log-rank Mantel-Cox test was used for survival curve comparison. For all panels, *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. Error bars shown mean ± SD.

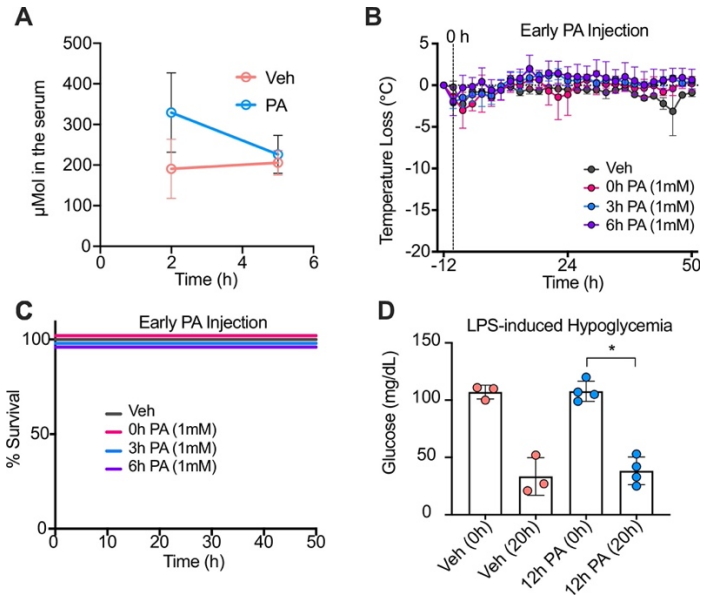

Figure 4—figure supplement 1. Palmitic acid (PA) intraperitoneal (i.p.) injections enhance serum PA concentrations, and PA-induced trained immunity is time-dependent. Conventional wild-type, age-matched (6–8 weeks), female BALB/c mice were fed standard chow (SC) for 2 weeks and injected i.p. with ethyl palmitate (PA 750 mM in 1.6% lecithin and 3.3% glycerol in endotoxin-free limulus amebocyte lysate [LAL] reagent water) or a vehicle solution (Veh, 1.6% lecithin and 3.3% glycerol in endotoxin-free LAL reagent water). (**A**) Serum was collected via cardiac punctures from mice 2 hr and 5 hr post-injection (p.i.). Serum samples were analyzed for absolute PA concentrations using qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry. At 0, 3, and 6 hr after PA injection, endotoxemia was induced via a single i.p. injection of lipopolysaccharide (LPS; 10 mg/kg). (**B**) Temperature loss and (**C**) survival were monitored every 2 hr. (**D**) Blood was collected via the tail vein to measure blood glucose levels at 0 and 20 hr p.i. with LPS using a glucose testing meter (Keto-Mojo). For (**D**), a Mann Whitney test was used for pairwise comparisons. Data is representative of one experiment, n=3–4 mice/group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Error bars show mean ± SD.

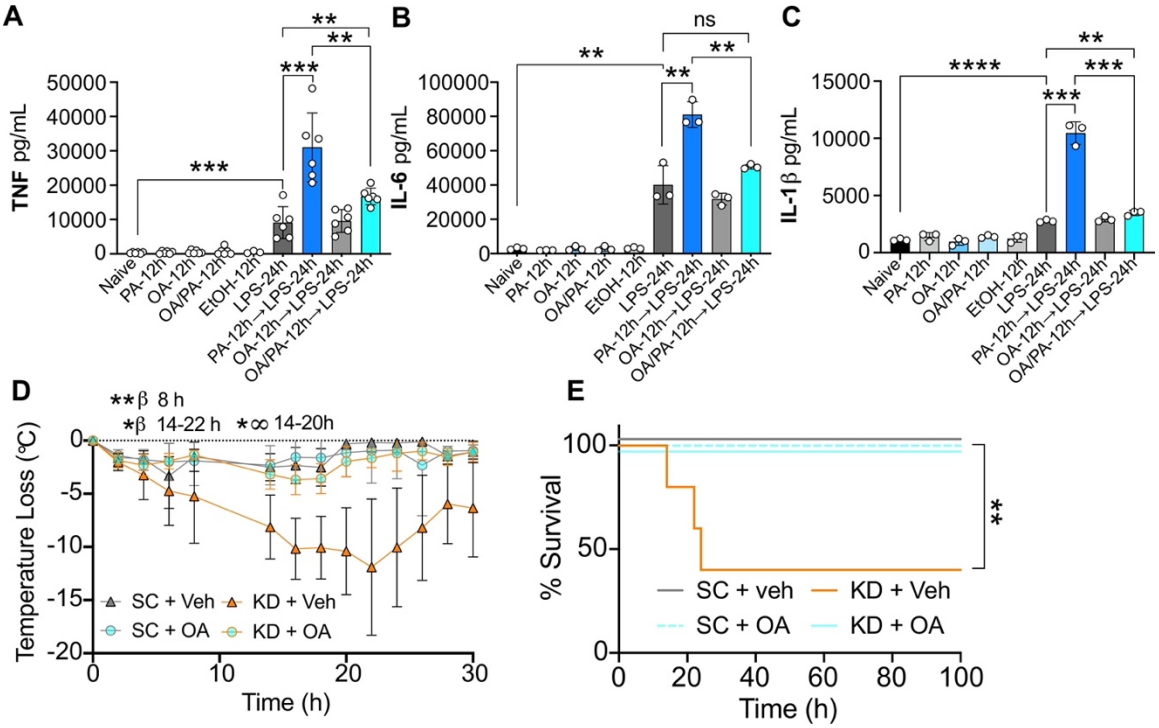

Figure 5. Oleic acid (OA) reverses palmitic acid (PA)-dependent hyper-inflammation in response to lipopolysaccharide (LPS) in vitro, and PA-dependent enhanced endotoxemia disease severity *in vivo*. Primary bone marrow-derived macrophages (BMDMs) were isolated from age-matched (6–8 weeks) C57BL/6 female and male mice. BMDMs were plated at 1×10^6 cells/mL and treated with either media (Ctrl), LPS (10 ng/mL) for 24 hr, PA (PA stock diluted in 0.83% EtOH; 0.5 mM PA conjugated to 2% bovine serum albumin) for 12 hr, or OA (200 μM; diluted in endotoxin-free water). Controls for all treatments are shown next to experimental groups treated additionally with LPS (10 ng/mL) for 24 hr. Supernatants were assessed via ELISA for (**A**) TNF, (**B**) IL-6, and (**C**) IL-1β secretion. Age-matched (6–8 weeks) female BALB/c mice were fed standard chow (SC) or ketonic diet (KD) for 2 weeks and injected intraperitoneal with 7 mg/kg LPS. (**D**) Temperature loss and (**E**) survival were monitored every 2 hr. For (**A–C**), experiments were run three times and data are representative of (**A**) two experiments and (**B, C**) one experiment. For all plates, all treatments were performed in triplicate, and a student's t-test was used for statistical significance. For (**D**), a Mann Whitney test was used for pairwise comparisons. For (**E**), a log-rank Mantel-Cox test was used for survival curve comparison. For (**D, E**), experiments were run three times, and data are representative of one experiment, n=5 mice/group. β symbols indicate KD + Veh vs KD + OA significance, and ∞ symbols indicate KD + Veh vs. SC + Veh. For all panels, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Error bars shown mean ± SD.

REFERENCES:

[1] Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, Joosten LAB, van der Meer JWM, Mhlanga MM, Mulder WJM, Riksen NP, Schlitzer A, Schultze JL, Stabell Benn C, Sun JC, Xavier RJ, Latz E (2020) Defining trained immunity and its role in health and disease Nature Reviews. Immunology 20:375–388.

[2] Kleinnijenhuis J, Quintin J, Preijers F, Joosten LAB, Ifrim DC, Saeed S, Jacobs C, van Loenhout J, de Jong D, Stunnenberg HG, Xavier RJ, van der Meer JWM, van Crevel R, Netea MG (2012) Bacille calmette-guerin induces nod2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes PNAS 109:17537–17542.

[3] Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, Jacobs L, Jansen T, Kullberg B-J, Wijmenga C, Joosten LAB, Xavier RJ, van der Meer JWM, Stunnenberg HG, Netea MG (2012) Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes Cell Host & Microbe 12:223–232.

[4] Saeed S, Quintin J, Kerstens HHD, Rao NA, Aghajanirefah A, Matarese F, Cheng S-C, Ratter J, Berentsen K, van der Ent MA, Sharifi N, Janssen-Megens EM, Ter Huurne M, Mandoli A, van Schaik T, Ng A, Burden F, Downes K, Frontini M, Kumar V, Giamarellos-Bourboulis EJ, Ouwehand WH, van der Meer JWM, Joosten LAB, Wijmenga C, Martens JHA, Xavier RJ, Logie C, Netea MG, Stunnenberg HG (2014) Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity Science 345:1251086.

[5] Lanz-Mendoza H, Contreras-Garduño J (2022) Innate immune memory in invertebrates: concept and potential mechanisms Developmental and Comparative Immunology 127:104285.

[6] Katzmarski N, Domínguez-Andrés J, Cirovic B, Renieris G, Ciarlo E, Le Roy D, Lepikhov K, Kattler K, Gasparoni G, Händler K, Theis H, Beyer M, van der Meer JWM, Joosten LAB, Walter J, Schultze JL, Roger T, Giamarellos-Bourboulis EJ, Schlitzer A, Netea MG (2021) Transmission of trained immunity and heterologous resistance to infections across generations Nature Immunology 22:1382–1390.

[7] Netea MG, Joosten LAB, Latz E, Mills KHG, Natoli G, Stunnenberg HG, O'Neill LAJ, Xavier RJ (2016) Trained immunity: a program of innate immune memory in health and disease Science 352:aaf1098.

[8] Covián C, Retamal-Díaz A, Bueno SM, Kalergis AM (2020) Could BCG vaccination induce protective trained immunity for SARS-cov-2? Frontiers in Immunology 11:970.

[9] Meena DK, Das P, Kumar S, Mandal SC, Prusty AK, Singh SK, Akhtar MS, Behera BK, Kumar K, Pal AK, Mukherjee SC (2013) Beta-Glucan: an ideal immunostimulant in aquaculture (a review) Fish Physiology and Biochemistry 39:431–457.

[10] van Splunter M, van Osch TLJ, Brugman S, Savelkoul HFJ, Joosten LAB, Netea MG, van Neerven RJJ (2018) Induction of trained innate immunity in human monocytes by bovine milk and milk-derived immunoglobulin G Nutrients 10:E1378.

[11] Swinburn BA, Sacks G, Hall KD, McPherson K, Finegood DT, Moodie ML, Gortmaker SL (2011) The global obesity pandemic: shaped by global drivers and local environments Lancet 378:804–814.

[12] Popkin BM (2006) Global nutrition dynamics: the world is shifting rapidly toward a diet linked with noncommunicable diseases The American Journal of Clinical Nutrition 84:289–298.

[13] Christ A, Latz E (2019) The Western lifestyle has lasting effects on metaflammation Nature Reviews. Immunology 19:267–268.

[14] Lancaster GI, Langley KG, Berglund NA, Kammoun HL, Reibe S, Estevez E, Weir J, Mellett NA, Pernes G, Conway JRW, Lee MKS, Timpson P, Murphy AJ, Masters SL, Gerondakis S, Bartonicek N, Kaczorowski DC, Dinger ME, Meikle PJ, Bond PJ, Febbraio MA (2018) Evidence that TLR4 is not a receptor for saturated fatty acids but mediates lipidinduced inflammation by reprogramming macrophage metabolism Cell Metabolism 27:1096–1110.

[15] Lumeng CN, Bodzin JL, Saltiel AR (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization The Journal of Clinical Investigation 117:175–184.

[16] Meikle PJ, Summers SA (2017) Sphingolipids and phospholipids in insulin resistance and related metabolic disorders Nature Reviews. Endocrinology 13:79–91.

[17] Reyes AWB, Huy TXN, Vu SH, Kim HJ, Lee JJ, Choi JS, Lee JH, Kim S (2021) Protection of palmitic acid treatment in RAW264.7 cells and BALB/c mice during Brucella abortus 544 infection Journal of Veterinary Science 22:e18.

[18] Napier BA, Andres-Terre M, Massis LM, Hryckowian AJ, Higginbottom SK, Cumnock K, Casey KM, Haileselassie B, Lugo KA, Schneider DS, Sonnenburg JL, Monack DM (2019) Western diet regulates immune status and the response to LPS-driven sepsis independent of diet-associated microbiome PNAS 116:3688–3694.

[19] Christ A, Günther P, Lauterbach MAR, Duewell P, Biswas D, Pelka K, Scholz CJ, Oosting M, Haendler K, Baßler K, Klee K, Schulte-Schrepping J, Ulas T, Moorlag SJC, Kumar V, Park MH, Joosten LAB, Groh LA, Riksen NP, Espevik T, Schlitzer A, Li Y, Fitzgerald ML, Netea MG, Schultze JL, Latz E (2018) Western diet triggers NLRP3-dependent innate immune reprogramming Cell 172:162–175.

[20] Schilling JD, Machkovech HM, He L, Sidhu R, Fujiwara H, Weber K, Ory DS, Schaffer JE (2013) Palmitate and lipopolysaccharide trigger synergistic ceramide production in primary macrophages The Journal of Biological Chemistry 288:2923–2932.

[21] Zhang Y, Rao E, Zeng J, Hao J, Sun Y, Liu S, Sauter ER, Bernlohr DA, Cleary MP, Suttles J, Li B (2017) Adipose fatty acid binding protein promotes saturated fatty acid-induced macrophage cell death through enhancing ceramide production Journal of Immunology 198:798–807.

[22] Divangahi M, Aaby P, Khader SA, Barreiro LB, Bekkering S, Chavakis T, van Crevel R, Curtis N, DiNardo AR, Dominguez-Andres J, Duivenvoorden R, Fanucchi S, Fayad Z, Fuchs E, Hamon M, Jeffrey KL, Khan N, Joosten LAB, Kaufmann E, Latz E, Matarese G, van der Meer JWM, Mhlanga M, Moorlag SJC, Mulder WJM, Naik S, Novakovic B, O'Neill L, Ochando J, Ozato K, Riksen NP, Sauerwein R, Sherwood ER, Schlitzer A, Schultze JL, Sieweke MH, Benn CS, Stunnenberg H, Sun J, van de Veerdonk FL, Weis S, Williams DL, Xavier R, Netea MG (2021) Trained immunity, tolerance, priming and differentiation: distinct immunological processes Nature Immunology 22:2–6.

[23] Kaufmann E, Sanz J, Dunn JL, Khan N, Mendonça LE, Pacis A, Tzelepis F, Pernet E, Dumaine A, Grenier J-C, Mailhot-Léonard F, Ahmed E, Belle J, Besla R, Mazer B, King IL, Nijnik A, Robbins CS, Barreiro LB, Divangahi M (2018) Bcg educates

hematopoietic stem cells to generate protective innate immunity against tuberculosis Cell 172:176–190.

[24] Napier BA, Brubaker SW, Sweeney TE, Monette P, Rothmeier GH, Gertsvolf NA, Puschnik A, Carette JE, Khatri P, Monack DM (2016) Complement pathway amplifies caspase-11-dependent cell death and endotoxin-induced sepsis severity The Journal of Experimental Medicine 213:2365–2382.

[25] Saito H, Sherwood ER, Varma TK, Evers BM (2003) Effects of aging on mortality, hypothermia, and cytokine induction in mice with endotoxemia or sepsis Mechanisms of Ageing and Development 124:1047–1058.

[26] Raetzsch CF, Brooks NL, Alderman JM, Moore KS, Hosick PA, Klebanov S, Akira S, Bear JE, Baldwin AS, Mackman N, Combs TP (2009) Lipopolysaccharide inhibition of glucose production through the toll-like receptor-4, myeloid differentiation factor 88, and nuclear factor kappa B pathway Hepatology 50:592–600.

[27] Filkins JP, Cornell RP (1974) Depression of hepatic gluconeogenesis and the hypoglycemia of endotoxin shock The American Journal of Physiology 227:778–781.

[28] Goldberg EL, Molony RD, Kudo E, Sidorov S, Kong Y, Dixit VD, Iwasaki A (2019) Ketogenic diet activates protective γδ T cell responses against influenza virus infection Science Immunology 4:eaav2026.

[29] Lewis AJ, Seymour CW, Rosengart MR (2016) Current murine models of sepsis Surgical Infections 17:385–393.

[30] Wang A, Huen SC, Luan HH, Yu S, Zhang C, Gallezot J-D, Booth CJ, Medzhitov R (2016) Opposing effects of fasting metabolism on tissue tolerance in bacterial and viral inflammation Cell 166:1512–1525.

[31] Radzyukevich YV, Kosyakova NI, Prokhorenko IR (2021) Participation of monocyte subpopulations in progression of experimental endotoxemia (ee) and systemic inflammation Journal of Immunology Research 2021:1762584.

[32] Zhang L, Cardinal JS, Pan P, Rosborough BR, Chang Y, Yan W, Huang H, Billiar TR, Rosengart MR, Tsung A (2012) Splenocyte apoptosis and autophagy is mediated by interferon regulatory factor 1 during murine endotoxemia Shock 37:511–517.

[33] Schwartz EA, Zhang W-Y, Karnik SK, Borwege S, Anand VR, Laine PS, Su Y, Reaven PD (2010) Nutrient modification of the innate immune response: a novel mechanism by which saturated fatty acids greatly amplify monocyte inflammation Arteriosclerosis, Thrombosis, and Vascular Biology 30:802–808.

[34] Fang C, Wang L, Qiao J, Chang L, He Q, Zhang X, Liu M (2022) Differential regulation of lipopolysaccharide-induced IL-1β and TNF-α production in macrophages by palmitate via modulating TLR4 downstream signaling International Immunopharmacology 103:108456.

[35] Cheng S-C, Scicluna BP, Arts RJW, Gresnigt MS, Lachmandas E, Giamarellos-Bourboulis EJ, Kox M, Manjeri GR, Wagenaars JAL, Cremer OL, Leentjens J, van der Meer AJ, van de Veerdonk FL, Bonten MJ, Schultz MJ, Willems PHGM, Pickkers P, Joosten LAB, van der Poll T, Netea MG (2016) Broad defects in the energy metabolism of leukocytes underlie immunoparalysis in sepsis Nature Immunology 17:406–413.

[36] Nedeva C, Menassa J, Puthalakath H (2019) Sepsis: inflammation is a necessary evil Frontiers in Cell and Developmental Biology 7:108.

[37] Gogos CA, Drosou E, Bassaris HP, Skoutelis A (2000) Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options The Journal of Infectious Diseases 181:176–180.

[38] van Dissel JT, van Langevelde P, Westendorp RG, Kwappenberg K, Frölich M (1998) Anti-inflammatory cytokine profile and mortality in febrile patients Lancet 351:950–953.

[39] De Zuani M, Frič J (2022) Train the trainer: hematopoietic stem cell control of trained immunity Frontiers in Immunology 13:827250.

[40] Vazquez SE, Inlay MA, Serwold T (2015) CD201 and cd27 identify hematopoietic stem and progenitor cells across multiple murine strains independently of kit and sca-1 Experimental Hematology 43:578–585.

[41] Nowlan B, Williams ED, Doran MR, Levesque JP (2020) Cd27, cd201, FLT3, CD48, and CD150 cell surface staining identifies long-term mouse hematopoietic stem cells in immunodeficient non-obese diabetic severe combined immune deficient-derived strains Haematologica 105:71–82.

[42] Dougherty RM, Galli C, Ferro-Luzzi A, Iacono JM (1987) Lipid and phospholipid fatty acid composition of plasma, red blood cells, and platelets and how they are affected by dietary lipids: a study of normal subjects from Italy, Finland, and the USA The American Journal of Clinical Nutrition 45:443–455.

[43] Skeaff CM, Hodson L, McKenzie JE (2006) Dietary-Induced changes in fatty acid composition of human plasma, platelet, and erythrocyte lipids follow a similar time course The Journal of Nutrition 136:565–569.

[44] Zöllner N, Tatò F (1992) Fatty acid composition of the diet: impact on serum lipids and atherosclerosis The Clinical Investigator 70:968–1009.

[45] Choi J, Yin T, Shinozaki K, Lampe JW, Stevens JF, Becker LB, Kim J (2018) Comprehensive analysis of phospholipids in the brain, heart, kidney, and liver: brain phospholipids are least enriched with polyunsaturated fatty acidsMolecular and Cellular Biochemistry 442:187–201.

[46] I S Sobczak A, A Blindauer C, J Stewart A (2019) Changes in plasma free fatty acids associated with type-2 diabetes Nutrients 11:E2022.

[47] Sokolowska E, Blachnio-Zabielska A (2019) The role of ceramides in insulin resistance Frontiers in Endocrinology 10:577.

[48] Papadimitriou-Olivgeris M, Aretha D, Zotou A, Koutsileou K, Zbouki A, Lefkaditi A, Sklavou C, Marangos M, Fligou F (2016) The role of obesity in sepsis outcome among critically ill patients: a retrospective cohort analysis BioMed Research International 2016:5941279.

[49] Mancini A, Imperlini E, Nigro E, Montagnese C, Daniele A, Orrù S, Buono P (2015) Biological and nutritional properties of palm oil and palmitic acid: effects on health Molecules 20:17339–17361.

[50] Korbecki J, Bajdak-Rusinek K (2019) The effect of palmitic acid on inflammatory response in macrophages: an overview of molecular mechanisms Inflammation Research 68:915–932.

[51] Abdelmagid SA, Clarke SE, Nielsen DE, Badawi A, El-Sohemy A, Mutch DM, Ma DWL (2015) Comprehensive profiling of plasma fatty acid concentrations in young healthy Canadian adults PLOS ONE 10:e0116195.

[52] Liu L, Feng R, Guo F, Li Y, Jiao J, Sun C (2015) Targeted metabolomic analysis reveals the association between the postprandial change in palmitic acid, branched-chain amino acids and insulin resistance in young obese subjects Diabetes Research and Clinical Practice 108:84–93.

[53] Gallego SF, Hermansson M, Liebisch G, Hodson L, Ejsing CS (2018) Total fatty acid analysis of human blood samples in one minute by high-resolution mass spectrometry Biomolecules 9:E7.

[54] Buchanan CDC, Lust CAC, Burns JL, Hillyer LM, Martin SA, Wittert GA, Ma DWL (2021) Analysis of major fatty acids from matched plasma and serum samples reveals highly comparable absolute and relative levels Prostaglandins, Leukotrienes, and Essential Fatty Acids 168:102268.

[55] Perreault M, Zulyniak MA, Badoud F, Stephenson S, Badawi A, Buchholz A, Mutch DM (2014) A distinct fatty acid profile underlies the reduced inflammatory state of metabolically healthy obese individuals PLOS ONE 9:e88539.

[56] Borradaile NM, Han X, Harp JD, Gale SE, Ory DS, Schaffer JE (2006) Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death Journal of Lipid Research 47:2726–2737.

[57] Li H, Xiao Y, Tang L, Zhong F, Huang G, Xu J-M, Xu A-M, Dai R-P, Zhou Z-G (2018) Adipocyte fatty acid-binding protein promotes palmitate-induced mitochondrial dysfunction and apoptosis in macrophages Frontiers in Immunology 9:81.

[58] Ly LD, Xu S, Choi S-K, Ha C-M, Thoudam T, Cha S-K, Wiederkehr A, Wollheim CB, Lee I-K, Park K-S (2017) Oxidative stress and calcium dysregulation by palmitate in type 2 diabetes Experimental & Molecular Medicine 49:e291.

[59] Tao L, Yi Y, Chen Y, Zhang H, Orning P, Lien E, Jie J, Zhang W, Xu Q, Li Y, Ding Z, Wu C, Ding Q, Wang J, Zhang J, Weng D (2021) Rip1 kinase activity promotes steatohepatitis through mediating cell death and inflammation in macrophages Cell Death and Differentiation 28:1418–1433.

[60] Palomer X, Pizarro-Delgado J, Barroso E, Vázquez-Carrera M (2018) Palmitic and oleic acid: the yin and yang of fatty acids in type 2 diabetes mellitus Trends in Endocrinology and Metabolism 29:178–190.

[61] Jin J, Zhang X, Lu Z, Perry DM, Li Y, Russo SB, Cowart LA, Hannun YA, Huang Y (2013) Acid sphingomyelinase plays a key role in palmitic acid-amplified inflammatory signaling triggered by lipopolysaccharide at low concentrations in macrophages American Journal of Physiology. Endocrinology and Metabolism 305:E853–E867.

[62] Eguchi K, Manabe I, Oishi-Tanaka Y, Ohsugi M, Kono N, Ogata F, Yagi N, Ohto U, Kimoto M, Miyake K, Tobe K, Arai H, Kadowaki T, Nagai R (2012) Saturated fatty acid and TLR signaling link β cell dysfunction and islet inflammation Cell Metabolism 15:518–533.

[63] Black DD (2007) Development and physiological regulation of intestinal lipid absorption. I. development of intestinal lipid absorption: cellular events in chylomicron assembly and secretion American Journal of Physiology. Gastrointestinal and Liver Physiology 293:G519–G524.

[64] Mansbach CM, Gorelick F (2007) Development and physiological regulation of intestinal lipid absorption. II. dietary lipid absorption, complex lipid synthesis, and the intracellular packaging and secretion of chylomicrons American Journal of Physiology. Gastrointestinal and Liver Physiology 293:G645–G650.

[65] Karavolos MH, Spencer H, Bulmer DM, Thompson A, Winzer K, Williams P, Hinton JCD, Khan CMA (2008) Adrenaline modulates the global transcriptional profile of Salmonella revealing a role in the antimicrobial peptide and oxidative stress resistance responses BMC Genomics 9:458.

[66] Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Ory DS, Schaffer JE (2003) Triglyceride accumulation protects against fatty acid-induced lipotoxicity PNAS 100:3077–3082.

[67] Bekkering S, Quintin J, Joosten LAB, van der Meer JWM, Netea MG, Riksen NP (2014) Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes Arteriosclerosis, Thrombosis, and Vascular Biology 34:1731–1738.

[68] Ayala TS, Tessaro FHG, Jannuzzi GP, Bella LM, Ferreira KS, Martins JO (2019) High glucose environments interfere with bone marrow-derived macrophage

inflammatory mediator release, the tlr4 pathway and glucose metabolism Scientific Reports 9:11447.

[69] Ng PY, Eikermann M (2017) The obesity conundrum in sepsis BMC Anesthesiology 17:147.

[70] Hall KD, Guo J, Courville AB, Boring J, Brychta R, Chen KY, Darcey V, Forde CG, Gharib AM, Gallagher I, Howard R, Joseph PV, Milley L, Ouwerkerk R, Raisinger K, Rozga I, Schick A, Stagliano M, Torres S, Walter M, Walter P, Yang S, Chung ST (2021) Effect of a plant-based, low-fat diet versus an animal-based, ketogenic diet on AD libitum energy intake Nature Medicine 27:344–353.

[71] Kennedy AR, Pissios P, Otu H, Xue B, Asakura K, Furukawa N, Marino FE, Liu F-F, Kahn BB, Libermann TA, Maratos-Flier E (2007) A high-fat, ketogenic diet induces a unique metabolic state in mice American Journal of Physiology-Endocrinology and Metabolism 292:E1724–E1739.

[72] Galadari S, Rahman A, Pallichankandy S, Galadari A, Thayyullathil F (2013) Role of ceramide in diabetes mellitus: evidence and mechanisms Lipids in Health and Disease 12:98.

[73] DiNardo AR, Netea MG, Musher DM (2021) Postinfectious epigenetic immune modifications-a double-edged sword The New England Journal of Medicine 384:261–270.

[74] Beutler B, Milsark IW, Cerami AC (1985) Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin Science 229:869–871.

[75] Mohler KM, Torrance DS, Smith CA, Goodwin RG, Stremler KE, Fung VP, Madani H, Widmer MB (1993) Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists Journal of Immunology 151:1548–1561.

[76] Cunningham PN, Dyanov HM, Park P, Wang J, Newell KA, Quigg RJ (2002) Acute renal failure in endotoxemia is caused by TNF acting directly on TNF receptor-1 in kidney Journal of Immunology 168:5817–5823.

[77] Chen Y, Guo F, Jachak A, Kim S-P, Datta D, Liu J, Kulaots I, Vaslet C, Jang HD, Huang J, Kane A, Shenoy VB, Hurt RH (2012) Aerosol synthesis of cargo-filled graphene nanosacks Nano Letters 12:1996–2002.

[78] Zhong W, Qian K, Xiong J, Ma K, Wang A, Zou Y (2016) Curcumin alleviates lipopolysaccharide induced sepsis and liver failure by suppression of oxidative stress-related inflammation via PI3K/Akt and NF-κB related signaling Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie 83:302–313.

[79] Escobar LE, Molina-Cruz A, Barillas-Mury C (2020) Bcg vaccine protection from severe coronavirus disease 2019 (COVID-19) PNAS 117:17720–17726.

[80] Arts RJW, Novakovic B, Ter Horst R, Carvalho A, Bekkering S, Lachmandas E, Rodrigues F, Silvestre R, Cheng S-C, Wang S-Y, Habibi E, Gonçalves LG, Mesquita I, Cunha C, van Laarhoven A, van de Veerdonk FL, Williams DL, van der Meer JWM, Logie C, O'Neill LA, Dinarello CA, Riksen NP, van Crevel R, Clish C, Notebaart RA, Joosten LAB, Stunnenberg HG, Xavier RJ, Netea MG (2016) Glutaminolysis and fumarate accumulation integrate immunometabolic and epigenetic programs in trained immunity Cell Metabolism 24:807–819.

[81] Ryan DG, O'Neill LAJ (2020) Krebs cycle reborn in macrophage immunometabolism Annual Review of Immunology 38:289–313.

[82] Silva G da, B Coeli-Lacchini F, Leopoldino AM (2022) How do sphingolipids play a role in epigenetic mechanisms and gene expression? Epigenomics 14:219–222.

[83] Souza DG, Vieira AT, Soares AC, Pinho V, Nicoli JR, Vieira LQ, Teixeira MM (2004) The essential role of the intestinal microbiota in facilitating acute inflammatory responses Journal of Immunology 173:4137–4146.

[84] Kassambara & F. Mundt (2017) Factoextra: extract and visualize the results of multivariate data analyses. R package 268 version 1, 337-354.

[85] Wickham H (2016) Ggplot2: Elegant Graphics for Data Analysis Springer Cham.

[86] Lê W (2008) FactoMineR:an R package for multivariate analysis Journal of Statistical Software 1:25.

[87] Kolde R (2018) Pheatmap: pretty heatmaps, version 1.0.10 R Package.

CHAPTER 4:

Additional data and future directions

Additional data:

A. Identifying the primary inflammatory event induced by palmitic acid.

A reoccurring quest throughout my project was to identify the time point of initial inflammation induced by palmitic acid (PA) *in vitro*. The first consequence of trained immunity is described as a metabolic and epigenetic reprogramming that occurs following a primary inflammatory stimulus, and importantly, this inflammation returns to baseline prior to secondary stimulation [1]. My data, however, suggests that PA does not induce primary inflammatory secretion *in vitro* prior to secondary stimulation with lipopolysaccharide (LPS; refer to Chapter 3, Figure 3E-M). Instead, I show that treating bone marrow-derived macrophages (BMDMs) with PA (0.5 mM) significantly enhances transcription of tumor necrosis factor (*Tnf)* at 2 and 4 hours (h), interleukin-6 (*Il-6)* at 6 and 12 h, and interleukin 1β (*Il-1β*) at 1, 2, 4, and 6 h post-treatment, compared to naïve and ethanol-treated (EtOH; 0.25%) controls (Figure 1A-C). In the same experiments, I measured secretion of TNF, IL-6 and IL-1β and find no significant alterations at these time points (Figure 1D-F). Currently, it is unknown if transcriptional modification alone is sufficient to induce metabolic and epigenetic rewiring and mediate trained immunity in macrophages.

I considered the possibility that PA could be inducing a different form of memory

than canonical trained immunity, with the only difference being the absence of primary inflammatory cytokine secretion [1]. However, I found that others have described trained immunity inducers with varying levels of potency [2]. For example, microbial inducers of trained immunity like β-glucan and BCG are considered to have greater potential for altering inflammatory effector function of human monocytes compared to lipids like oxidized low-density lipoprotein (OxLDL) [2]. Moreover, similar to the data I present here showing the absence of initial inflammatory cytokine secretion by PA in BMDMs, OxLDL has been shown to have no effect on secretion of TNF, IL-6, or IL-1β following 24 h treatment of human monocytes [3]. Still, a 24 h OxLDL treatment is sufficient to induce innate immune memory *in vitro*, and is designated a non-microbial, and therefore a less potent, inducer of trained immunity [4]. Together, these data suggest that non-microbial lipids are capable of inducing trained immunity even when the initial inflammatory stimulus is absent.

PA exposure alone via intraperitoneal (i.p.) injection is shown in this work to exacerbate endotoxemia severity and survival outcome in mice, and significantly enhanced circulating *Tnf* and *Il-6* following LPS-injection (refer to Chapter 3, Figure 4C, D). However, these phenotypes were not as robust compared to those of KD-fed mice during endotoxemia, and PA-injected mice did not show enhanced circulating *Il-1β* as KD-fed mice did following LPS treatment (refer to Chapter 3, Figure 4E). Additionally, I did not find a primary inflammatory spike, though I looked at multiple time points, in the circulations of PA-injected mice prior to endotoxemia, and these mice did not exhibit metaflammation due to PA exposure (refer to Chapter 3, Figure 4F). While these data

support my hypothesis that PA induces trained immunity, and inflammation returned to baseline prior to secondary stimulation, I was unable to determine a time point at which primary stimulation occurs *in vivo*.

Future research regarding the inflammatory impact of PA *in vivo* should ask the following questions: 1) Are there other constituents of the KD, in addition to PA, that drive significant disease severity and survival defects, and enhanced circulating *Il-1β*, during LPS-induced endotoxemia in mice? 2) Do PA-injected mice more closely resemble parenteral (intravenous nutrition) or enteral feeding tubes? Will this be important to consider when administering these types of feeding for patients with sepsis, *i.e.,* is parenteral PA potentially more harmful than enteral PA for systemic inflammation?

B. Untangling the regulation of macrophage metabolism during the palmitic acid-induced trained immune response.

Trained immunity induced by β-glucan is known to exhibit metabolic rewiring of macrophages, specifically accumulation of the TCA cycle metabolites succinate and fumarate that occurs via glutamine anaplerosis (*i.e.,* metabolite replenishment) [5,6]. Accumulation of fumarate leads to its export from the mitochondria, and translocation into the nucleus where it directly inhibits lysine-specific demethylase 5 (KDM5), and this leads to trimethylation of histone 3 lysine 4 (H3K4me3) at the promoters of *Tnf* and *Il-1β*, enhancing transcription of these genes (Figure 5) [7]. Interestingly, fumarate treatment alone induced trained immunity in this model system [7].

If PA is to be established as an inducer of trained innate immune memory in monocytes and macrophages it is important to address the following questions: 1) What are the metabolic and epigenetic mechanisms underlying primary stimulation with PA and is transcriptional regulation of pro-inflammatory cytokines both necessary and sufficient to induce memory? 2) What specific metabolites (*e.g.,* succinate, fumarate, and itaconate) are responsible for epigenetic modifications, and which inflammatory genes are regulated in this way?

In order to probe these questions and determine if our *in vitro* model of PA-induced hyperinflammation in BMDMs alters TCA metabolites important for trained immunity, I measured succinate and fumarate concentrations in the cell supernatants, and show that a 12 h pre-treatment with PA (0.5 mM) induces significantly enhanced succinate and fumarate compared to naïve and EtOH (0.5%) controls (Figure 2A, B). Moreover, cells pre-treated with PA (12 h; 0.5 mM) and subsequent LPS (10 ng/mL; 24 h) show significantly enhanced succinate and fumarate concentrations compared to LPS treatment alone, suggesting that elevation of these metabolites coincides with the hyperinflammation shown in Chapter 3 using this same model (Figure 2A, B; refer to Chapter 3, Figure 3E-J).

β-glucan-induced trained immunity in human monocytes inhibits expression of immune responsive gene 1 (*Irg1*), which encodes the enzyme responsible for itaconate synthesis via the TCA cycle [8]. In this way, β-glucan reverses the mechanism underlying LPS induced tolerance whereby itaconate inhibits succinate dehydrogenase (SDH) and breaks the TCA cycle (Figure 4) [6]. I hypothesized that, similar to β-glucan-induced

trained immunity, PA-induced memory would inhibit *Irg1* expression *in vitro.* Surprisingly, I show that pre-treating BMDMs with PA (0.5 mM; 12 h) significantly enhanced expression of *Irg1* compared to naïve and EtOH (0.5%) control treatments (Figure 2C). Moreover, cells pre-treated with PA (12 h; 0.5 mM) and subsequent LPS (10 ng/mL; 24 h) show significantly enhanced *Irg1* expression compared to LPS treatment alone, suggesting that enhanced itaconate synthesis may coincide with PA-mediated hyperinflammation following subsequent LPS treatment (refer to Chapter 3, Figure 3E-J).

I next probed the impact of PA on *Irg1* expression *in vivo*, and show that circulating expression of *Irg1* is elevated in mice fed KD for 2 weeks compared to SC-fed mice, and that daily oleic acid (OA) treatment via i.p. injection for the final 3 days of feeding reverses this phenotype (Figure 2D). 5 h post LPS injection, *Irg1* expression is significantly enhanced in KD-fed mice compared to SC-fed controls, suggesting that KD-mediated hyperinflammation during endotoxemia (refer to Chapter 3; Figure 1C-E) coincides with enhanced itaconate synthesis *in vivo* (Figure 2E). I find that germ-free (GF) mice fed a KD for 2 weeks (wk) do not show enhanced circulating *Irg1* expression 5 h post-LPS, indicating that itaconate synthesis *in vivo* may be regulated by KD-specific microbiota during endotoxemia (Figure 2F).

I next asked if dietary PA specifically could be regulating the enhanced circulating *Irg1* expression shown in KD-fed mice in Figure 2. Mice were fed a PA-enriched diet (PAe) or a SC control diet for 2 wk and tail vein blood was collected prior to diet changes (0 d) and 14 d following diet change. Although I show that circulating *Irg1* is enhanced in PAe-fed mice 14 d post-diet change compared to SC-fed mice, it is

not significant (Figure 3A). Mice were then injected intraperitoneally (i.p.) with LPS, and tail vein blood was collected at 5 h post-injection. In contrast to KD-fed mice, PAe-fed mice do not exhibit enhanced circulating *Irg1* at 5 h post-injection with LPS compared to SC-fed mice (Figure 3B). PAe-fed mice show significantly enhanced hypothermia after LPS-injection compared to mice fed a SC, however, the hypothermia was not as extreme as PA-injected and KD-fed mice experienced (Figure 3C; refer to Chapter 3, Figures 1A and 4A, G). Moreover, PAe-fed mice did not experience circulating pro-inflammatory cytokine augmentation following LPS, as PA-injected and KD-fed mice did (Figure 3D-F; refer to Chapter 3, Figures 1C-E and 4C, D).

Altogether, these data suggest that some part of the KD-induced microbiota that is perhaps unrelated to the PA content could be regulating *Irg1* expression in the blood following LPS injection. Specifically, *Irg1* expression is upregulated *in vitro* following PA treatment alone and LPS treatment alone, and increasingly more so with PA pre-treatment and subsequent LPS (Figure 2C). Circulating *Irg1* expression *in vivo* is upregulated after 2 wk of KD feeding and augmented following LPS-injection, compared to controls, and this was independent of diet-induced microbiota (Figure 2D-F). While there is a trend toward enhanced circulating *Irg1* expression with 2 wk of PAe feeding, no change in expression occurs following LPS-injection, indicating potential KD-specific microbiota regulation of *Irg1* during endotoxemia (Figure 3A, B). These data mark the beginning stages of our knowledge of PA-mediated metabolic reprogramming in macrophages and mice, and show that the *in vitro* and *in vivo* models of PA- and KD-

mediated trained immunity presented are unique from other types of trained immunity described previously.

Future studies should investigate the cell and molecular signaling pathways, metabolic pathways, and resultant epigenetic alterations in order to interpret the distinct mechanisms underlying PA-induced memory. For example, BCG and β-glucan are known to signal through the macrophage PRRs, NOD2 (nucleotide binding and oligomerization domain 2) and Dectin-1 respectively [9,10]. In contrast, OxLDL enters through the macrophage scavenger receptor, CD36, and is subsequently taken up by the phagolysosome where lysosomal membrane sphingomyelins are hydrolyzed and converted to ceramide [11,12]. Beyond cell entry via CD36 and mitochondrial passage via the carnitine shuttle, there are many aspects of PA-mediated transport and signaling that remain mysterious in macrophages [13,14]. My data suggests PA induces accumulation of TCA cycle metabolites succinate and fumarate, and even the byproduct of TCA cycle metabolism, itaconate (Figure 4). These data are reminiscent of LPS-induced alterations to macrophage metabolism, and considering how different concentrations of LPS can induce training as opposed to tolerance, it will be important to compare these underlying mechanisms with the effects of varying concentrations of PA [15]. Although it was not emphasized, we show that different concentrations of PA impact transcriptional regulation of *Il-1β* in an opposing manner, while IL-1β secretion remains elevated in both contexts (refer to Chapter 3, Figure 3G, J). These confounding dynamics will need to be carefully parsed out if one aims to pursue lipid modulation as a therapeutic strategy for the treatment of disease.

Future directions: bringing my research to healthcare

A. Disease markers

Many human studies have identified associations between insulin resistance and ceramide concentrations in the serum [16-21]. One study of 200 men and women showed a low-fat Nordic Diet reduced plasma levels of ceramide species known to induce to insulin resistance, compared to subjects fed a control diet [22]. A smaller scale study of 28 men and women showed that a low PA/high OA diet resulted in lower serum and muscle ceramide concentrations, suggesting that the ratio of PA:OA in the diet may impact diabetes risk in humans [23]. This yin-yang feature of PA and OA in the serum shows promising therapeutic potential for type 2 diabetes (T2D) patients [24].

Lipidomics may be useful for identifying and categorizing biomarkers of disease. Many of the lipids upregulated in KD-fed mice serum are known to occur at excessive concentrations in human serum of patients with obesity, T2D, cardiovascular disease (CVD), and autoimmunity; and when studied *in vitro*, exhibit lipotoxicity and inflammation in macrophages, TCA cycle dysfunction, and reprogramming of cholesterol metabolism [25-30]. Specifically, a study using a cohort of 2583 patients with coronary artery disease (a subtype of CVD) and 733 healthy controls (70% male patients all with a mean age of 67 years) identified in a plasma lipidomics panel that PA-containing ceramide species showed the strongest positive association with CVD-associated mortality of 484 patients [31]. In a cohort of 30 patients, serum PA levels were

significantly elevated in obese individuals with insulin resistance [32]. Moreover, an Artificial Neural Network (ANN) model identified serum PA, among other saturated fatty acids (SFA), to be a biomarker and potential predictor of autoimmune disease (AD) among 163 healthy controls and 240 patients diagnosed with AD, including rheumatoid arthritis, thyroid disease, multiple sclerosis, vitiligo, psoriasis, and inflammatory bowel disease [33].

Currently, common blood lipid panels used in the clinic lack detail regarding specific lipid species found in the blood, measuring only low-density, very-low-density, and high-density lipoprotein cholesterol and total triglycerides (TAG) in order to identify hyperlipidemia, CVD risk and metabolic syndrome [34-35]. Although plasma cholesterol and total TAG concentrations are useful in the prevention and identification of hyperlipidemia, CVD, and metabolic syndrome, many other circulating lipids exist that are known to correlate with metabolic and inflammatory dysfunction. Within lipid classes, specific lipid species may also be useful as biomarkers for early detection of CVD, COVID-19, sepsis, cancer, neurodegenerative diseases, and chronic obstructive pulmonary disease [36-41].

B. Hospital diagnostics

Currently, the World Health Organization reports that sepsis patients in intensive care units (ICUs) worldwide have a 42% mortality rate [42]. It is known that diets high in saturated fats are associated with obesity, metabolic dysfunction, and systemic inflammation [43]. Patients with these conditions show significantly enhanced

susceptibility to infection, and are predisposed to significantly higher mortality rates in the ICU compared to patients who are metabolically healthy prior to being admitted to the ICU [44]. The nutritional status of a patient upon admittance to the ICU is used to measure the risk of mortality for individuals with enhanced metabolic stress due to disease [45]. Measuring nutritional risk of ICU patients is critical to understand when determining feeding tube formulas for dietary intervention strategies that will sufficiently support the metabolic needs of the patient during treatment. Currently there are two scoring systems used by hospitals to understand nutritional risk of patients: 1) The Nutritional Risk Score (NRS), and 2) the median Nutrition risk in critically ill (NUTRIC) score [45,46]. The NUTRIC score is used for sepsis patients to screen for pre-illness malnutrition and nutritional risk, and it was recently shown to predict risk of death in hospitalized COVID-19 patients [47,48]. The scores inform patient caretakers what type of nutrition therapies to use to support the patients via feeding tubes with adequate macronutrients, including fatty acids or lipids. Currently, immune modulating ingredients of feeding tube formulas used for hospitalized critically ill patients with sepsis include omega-3 fatty acids and various levels of SFAs including PA (*e.g.*, Oxepa made by Abbott Nutrition) [47]. Personalized formulas based on patient nutritional status, however, do not yet exist. My research and the work of others presented here suggest that PA and PA-containing lipids may influence disease outcome. Thus, modulation of PA in feeding tube formulas based on patient-specific blood lipid panels may benefit hospitalized patients suffering from inflammatory dysregulation (*e.g.*, sepsis, CVD, COVID-19).

Conclusion

Research in personalized nutrition is still in its infancy and many unknowns continue to challenge what we understand about the interplay between diet and the human immune system [49,50]. In order to illuminate the deep mysteries that remain between the food we eat, the resulting mechanisms within innate immune cells, and ultimately how this impacts disease outcome, we must "allow for the unexpected discovery," as Tuvok proposed in the Star Trek: Voyager episode titled, One Small Step. The effect of PA on macrophage metabolism and the subsequent response to microbial stimuli may indeed be a memory phenomenon, and the characteristics established for well-known trained immunity inducers are suitable guides for pursuing this hypothesis. Formulating creative questions and recognizing unexpected findings while remaining logical will be the challenge.

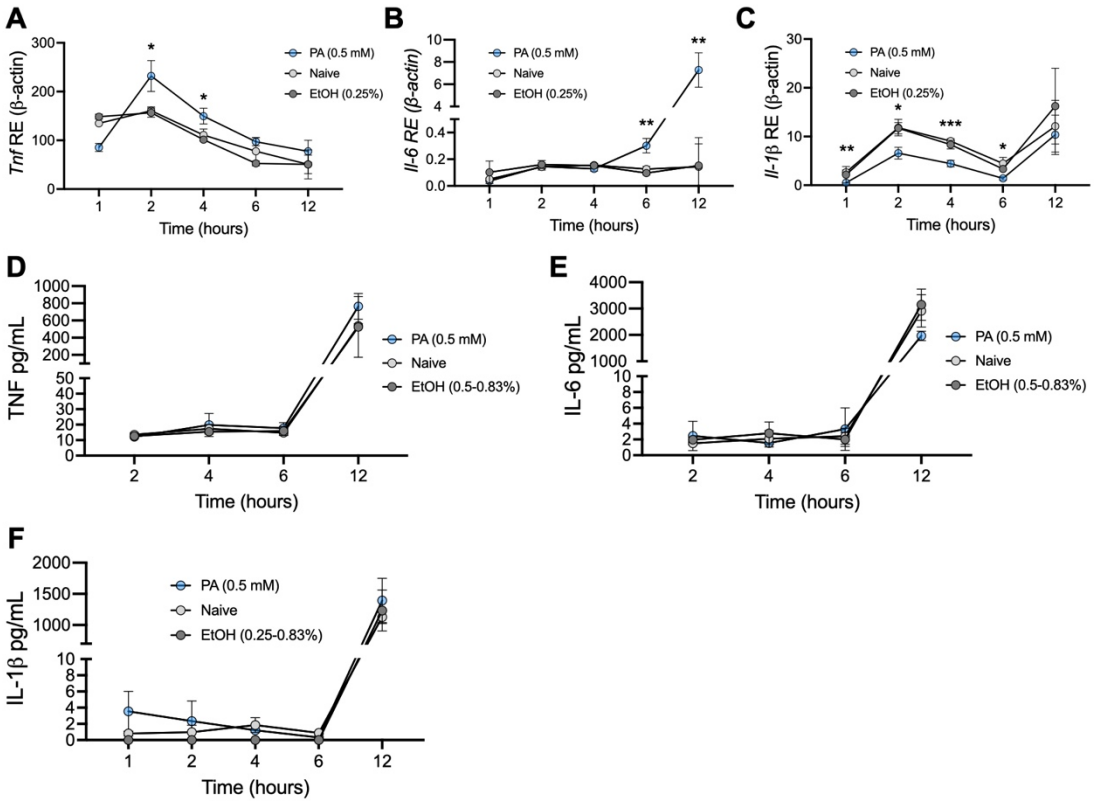

Figure 1. PA upregulates transcription of pro-inflammatory cytokines in BMDMs, but not protein secretion. Primary bone marrow-derived macrophages (BMDMs) were isolated from age-matched (6–8 weeks) female and male mice. (**A–C**) BMDMs were plated at 1×10^6 cells/mL and treated with ethanol (EtOH; media with 0.25% ethanol), media (Naïve ctrl), or PA (0.5 mM; diluted in 0.25% EtOH) for 1, 2, 4, 6, or 12 h. After indicated time points, RNA was isolated and expression of *Tnf, Il6*, and *Il1β* was measured via qRT-PCR. (**D-F**) BMDMs were plated and treated as in A-C. For 12 h treatments, 0.83% EtOH was used. Supernatants were assessed via ELISA for (**D**) TNF, (**E**) IL-6, and (**F**) IL-1β secretion. For all plates, treatments were performed in triplicate. For all panels, a student's t-test was used for statistical significance. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Error bars show mean ± SD.

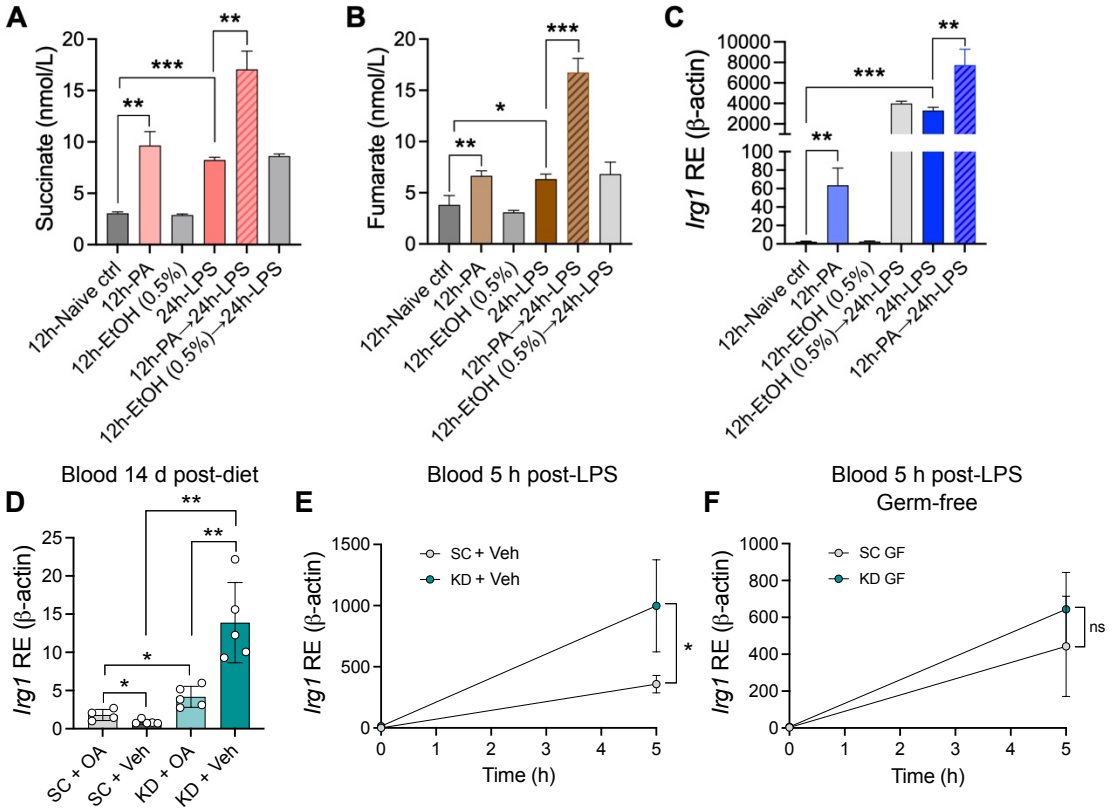

Figure 2. PA regulates TCA cycle metabolites *in vitro* and *in vivo*. Primary bone marrow-derived macrophages (BMDMs) were isolated from age-matched (6–8 weeks) female and male mice. (**A–C**) BMDMs were plated at 1×10^6 cells/mL and treated with ethanol (EtOH; media with 0.5% ethanol), media (Ctrl for LPS), or PA (0.5 mM; diluted in 0.5% EtOH) for 12 h. Next, PA-pre-treated cells were treated with LPS (10 ng/mL) for 24 h, and all other wells were given fresh media. For (**A**) and (**B**), succinate and fumarate were measured in the supernatants using a microplate reader with colorimetric kits (Sigma). For (**C**) RNA was isolated and expression of *Irg1* was measured via qRT-PCR. (**D, E**) Age-matched (6–8 weeks) female BALB/c mice were fed standard chow (SC) or ketogenic diet (KD) for 2 weeks and injected intraperitoneal (i.p.) with oleic acid (OA) daily for 3 days, following by i.p. injection with 7 mg/kg LPS. At 0 h (14 d post-diet; **D**) and 5 h post-LPS (**E**), 10–20 µL of blood was drawn via the tail vein, RNA was collected, and samples were assessed for expression of *Irg1* via qRT-PCR. (**F**) 19–23-week-old female and 14–23-week-old male and female germ-free C57BL/6 mice were fed SC or KD for 2 weeks and injected i.p. with 50 mg/kg of LPS. At 5 h post-LPS, 10–20 µL of blood was drawn via the tail vein, RNA was collected, and samples were assessed for expression of *Irg1* via qRT-PCR. For (**A–C**), experiments were run three times and data are representative of one experiment. For all plates, all treatments were performed in triplicate, and a student's t-test was used for statistical significance. For (**D**) and (**E**), a Mann Whitney test was used for pairwise comparisons. Experiments were run three times, and data are representative of one experiment, n=5 mice/group. For (**F**), a Mann Whitney test was used for pairwise comparisons. Experiment was run once, and data are representative of one experiment, n=6-9 mice/group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Error bars show mean ± SD.

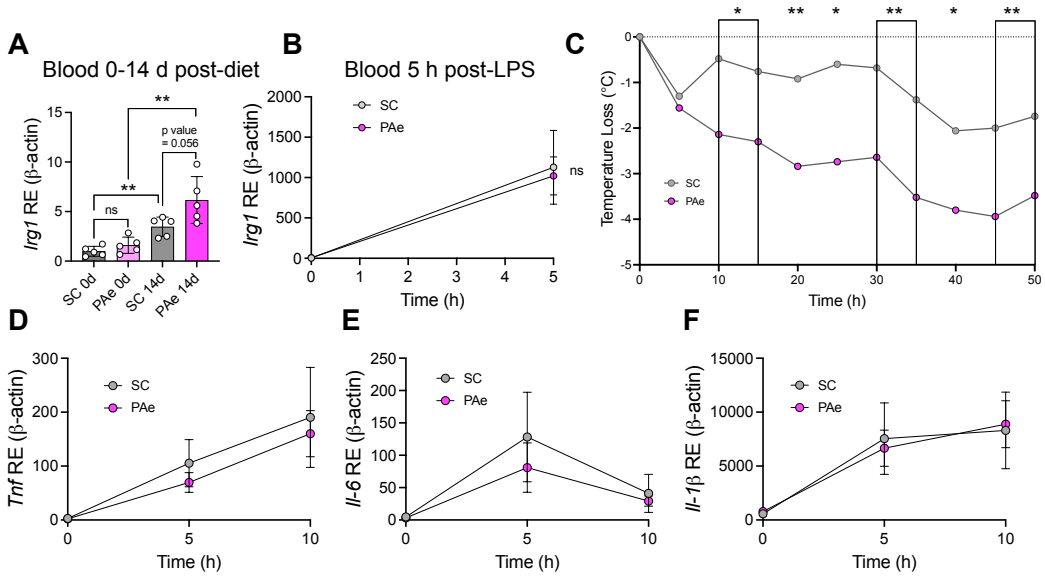

Figure 3. A diet enriched in PA alone (PAe) leads to hypothermia during LPS-induced endotoxemia, but does not enhance expression of *Irg1* or pro-inflammatory cytokines in the blood post-LPS. Age-matched (6–8 weeks) female BALB/c mice were fed standard chow (SC) or PA-enriched diet (PAe) for 2 weeks and injected intraperitoneal (i.p.) with 7 mg/kg LPS. (**A**) At 0 d and 14 d post-diet, and (**B**) 5 h post-LPS, 10–20 µL of blood was drawn via the tail vein, RNA was collected, and samples were assessed for expression of *Irg1* via qRT-PCR. (**C**) Temperature loss was monitored every 5 h. (**D-F**) At 5 and 10 h post-LPS, 10–20 µL of blood was drawn via the tail vein, RNA was collected, and samples were assessed for expression of *Tnf*, *Il-6*, and *Il-1β* via qRT-PCR. For (**A–F**), experiment was run once, n=5 mice/group. A Mann Whitney test was used for pairwise comparisons. For panels (**A**) and (**C**), *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. Error bars shown mean ± SD.

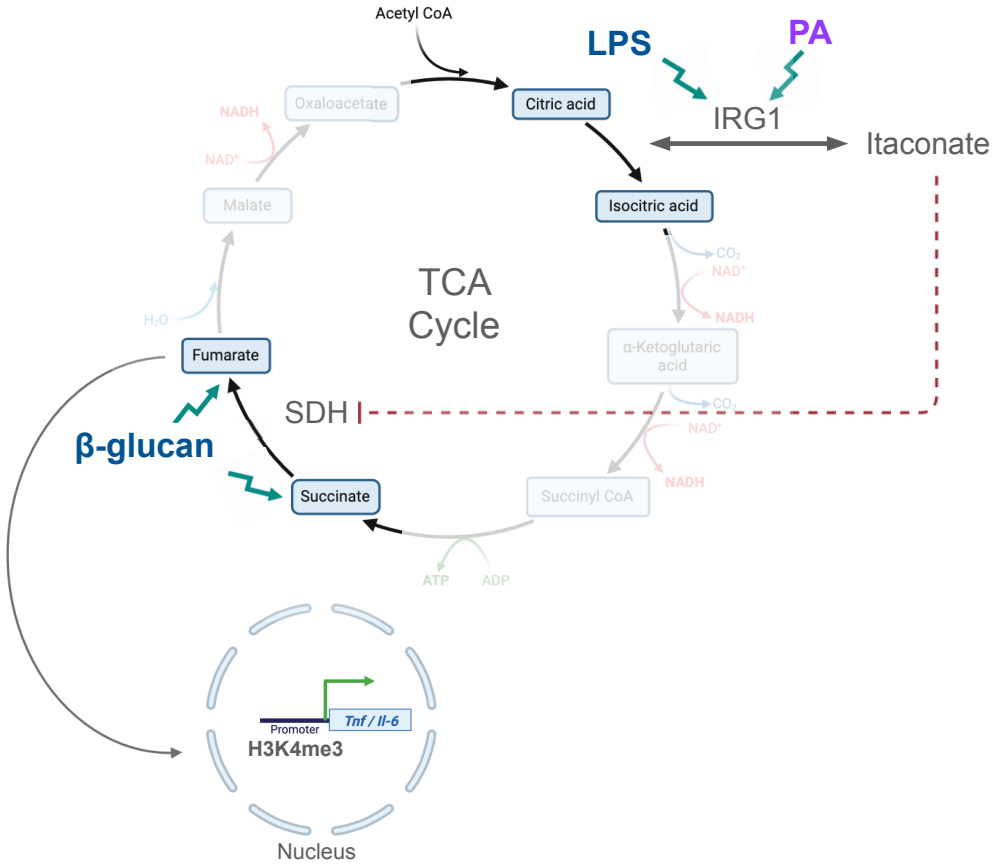

Figure 4. A hypothetical model of palmitic acid-enhanced IRG1 and resultant impacts on the TCA cycle and *Tnf* and *Il-6* transcription. Palmitic acid (PA) enhances expression of immune responsive gene 1 (IRG1), and this catalyzes itaconate synthesis. Itaconate inhibits succinate dehydrogenase (SDH) leading to the accumulation of succinate as it is unable to be converted to fumarate [51]. Fumarate is replenished via glutamine anaplerosis and is translocated from the mitochondrion to the nucleus and inhibits lysine-specific demethylase 5 (KDM5) leading to trimethylation of histone 3 lysine 4 (H3K4me3) at *Tnf* and *Il-6* promoters, inducing transcription of these genes [52]. Lipopolysaccharide (LPS) induces IRG1 expression and similarly upregulates *Tnf* and *Il-6* [51]. Exposure to PA and subsequent LPS leads to hyperinflammatory monocyte and macrophage responses *in vitro* and *in vivo* driven by TNF and IL-6 release.

REFERENCES:

[1] Divangahi M, Aaby P, Khader SA, Barreiro LB, Bekkering S, Chavakis T, van Crevel R, Curtis N, DiNardo AR, Dominguez-Andres J, Duivenvoorden R, Fanucchi S, Fayad Z, Fuchs E, Hamon M, Jeffrey KL, Khan N, Joosten LAB, Kaufmann E, Latz E, Matarese G, van der Meer JWM, Mhlanga M, Moorlag SJCFM, Mulder WJM, Naik S, Novakovic B, O'Neill L, Ochando J, Ozato K, Riksen NP, Sauerwein R, Sherwood ER, Schlitzer A, Schultze JL, Sieweke MH, Benn CS, Stunnenberg H, Sun J, van de Veerdonk FL, Weis S, Williams DL, Xavier R, Netea MG. Trained immunity, tolerance, priming and differentiation: distinct immunological processes. Nat Immunol. 2021 Jan;22(1):2-6.

[2] Bekkering S, Domínguez-Andrés J, Joosten LAB, Riksen NP, Netea MG. Trained Immunity: Reprogramming Innate Immunity in Health and Disease. Annu Rev Immunol. 2021 Apr 26;39:667-693.

[3] Bekkering S, Quintin J, Joosten LA, van der Meer JW, Netea MG, Riksen NP. Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes. Arterioscler Thromb Vasc Biol. 2014 Aug;34(8):1731-8.

[4] Keating ST, Groh L, Thiem K, Bekkering S, Li Y, Matzaraki V, van der Heijden CDCC, van Puffelen JH, Lachmandas E, Jansen T, Oosting M, de Bree LCJ, Koeken VACM, Moorlag SJCFM, Mourits VP, van Diepen J, Strienstra R, Novakovic B, Stunnenberg HG, van Crevel R, Joosten LAB, Netea MG, Riksen NP. Rewiring of glucose metabolism defines trained immunity induced by oxidized low-density lipoprotein. J Mol Med (Berl). 2020 Jun;98(6):819-831.

[5] Riksen NP, Netea MG. Immunometabolic control of trained immunity. Mol Aspects Med. 2021 Feb;77:100897.

[6] Ryan DG, O'Neill LAJ. Krebs Cycle Reborn in Macrophage Immunometabolism. Annu Rev Immunol. 2020 Apr 26;38:289-313.

[7] Arts RJW, Novakovic B, Ter Horst R, et al. Glutaminolysis and fumarate accumulation integrate immunometabolic and epigenetic programs in trained immunity. Cell Metab. 2016;24:807–19.

[8] Domínguez-Andrés J, Novakovic B, Li Y, Scicluna BP, Gresnigt MS, Arts RJW, Oosting M, Moorlag SJCFM, Groh LA, Zwaag J, Koch RM, Ter Horst R, Joosten LAB, Wijmenga C, Michelucci A, van der Poll T, Kox M, Pickkers P, Kumar V, Stunnenberg H, Netea MG. The Itaconate Pathway Is a Central Regulatory Node Linking Innate Immune Tolerance and Trained Immunity. Cell Metab. 2019 Jan 8;29(1):211-220.e5.

[9] Kleinnijenhuis J, Quintin J, Preijers F, Joosten LAB, Ifrim DC, Saeed S, Jacobs C, van Loenhout J, de Jong D, Stunnenberg HG, Xavier RJ, van der Meer JWM, van Crevel R, Netea MG (2012) Bacille calmette-guerin induces nod2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes PNAS 109:17537–17542.

[10] Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, Jacobs L, Jansen T, Kullberg B-J, Wijmenga C, Joosten LAB, Xavier RJ, van der Meer JWM, Stunnenberg HG, Netea MG (2012) Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes Cell Host & Microbe 12:223–232.

[11] Lorey MB, Öörni K, Kovanen PT. Modified Lipoproteins Induce Arterial Wall Inflammation During Atherogenesis. Front Cardiovasc Med. 2022 Mar 3;9:841545.

[12] Kinscherf R, Claus R, Deigner HP, Nauen O, Gehrke C, Hermetter A, Russwurm S, Daniel V, Hack V, Metz J. Modified low density lipoprotein delivers substrate for ceramide formation and stimulates the sphingomyelin-ceramide pathway in human macrophages. FEBS Lett. 1997 Mar 17;405(1):55-9.

[13] Korbecki J, Bajdak-Rusinek K. The effect of palmitic acid on inflammatory response in macrophages: an overview of molecular mechanisms. Inflamm Res. 2019 Nov;68(11):915-932.

[14] Carta G, Murru E, Banni S, et al. Palmitic acid: physiological role, metabolism and nutritional implications. Front Physiol. 2017;8:902.

[15] Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, Frezza C, Bernard NJ, Kelly B, Foley NH, Zheng L, Gardet A, Tong Z, Jany SS, Corr SC, Haneklaus M, Caffrey BE, Pierce K, Walmsley S, Beasley FC, Cummins E, Nizet V, Whyte M, Taylor CT, Lin H, Masters SL, Gottlieb E, Kelly VP, Clish C, Auron PE, Xavier RJ, O'Neill LA. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. Nature. 2013 Apr 11;496(7444):238-42.

[16] Summers SA, Chaurasia B, Holland WL. Metabolic Messengers: ceramides. Nat Metab. 2019 Nov;1(11):1051-1058.

[17] Bergman BC, Brozinick JT, Strauss A, Bacon S, Kerege A, Bui HH, Sanders P, Siddall P, Kuo MS, Perreault L. Serum sphingolipids: relationships to insulin sensitivity and changes with exercise in humans. Am J Physiol Endocrinol Metab. 2015 Aug 15;309(4):E398-408.

[18] Boon J, Hoy AJ, Stark R, Brown RD, Meex RC, Henstridge DC, Schenk S, Meikle PJ, Horowitz JF, Kingwell BA, Bruce CR, Watt MJ. Ceramides contained in LDL are elevated in type 2 diabetes and promote inflammation and skeletal muscle insulin resistance. Diabetes. 2013 Feb;62(2):401-10.

[19] de Mello VD, Lankinen M, Schwab U, Kolehmainen M, Lehto S, Seppänen-Laakso T, Oresic M, Pulkkinen L, Uusitupa M, Erkkilä AT. Link between plasma ceramides, inflammation and insulin resistance: association with serum IL-6 concentration in patients with coronary heart disease. Diabetologia. 2009 Dec;52(12):2612-5.

[20] Haus JM, Kashyap SR, Kasumov T, Zhang R, Kelly KR, Defronzo RA, Kirwan JP. Plasma ceramides are elevated in obese subjects with type 2 diabetes and correlate with the severity of insulin resistance. Diabetes. 2009 Feb;58(2):337-43.

[21] Lopez X, Goldfine AB, Holland WL, Gordillo R, Scherer PE. Plasma ceramides are elevated in female children and adolescents with type 2 diabetes. J Pediatr Endocrinol Metab. 2013;26(9-10):995-8.

[22] Lankinen M, Schwab U, Kolehmainen M, Paananen J, Nygren H, Seppänen-Laakso T, Poutanen K, Hyötyläinen T, Risérus U, Savolainen MJ, Hukkanen J, Brader L, Marklund M, Rosqvist F, Hermansen K, Cloetens L, Önning G, Thorsdottir I, Gunnarsdottir I, Åkesson B, Dragsted LO, Uusitupa M, Orešič M. A Healthy Nordic Diet Alters the Plasma Lipidomic Profile in Adults with Features of Metabolic Syndrome in a Multicenter Randomized Dietary Intervention. J Nutr. 2015 Apr 1;146(4):662-672.

[23] Kien CL, Bunn JY, Poynter ME, Stevens R, Bain J, Ikayeva O, Fukagawa NK, Champagne CM, Crain KI, Koves TR, Muoio DM. A lipidomics analysis of the relationship between dietary fatty acid composition and insulin sensitivity in young adults. Diabetes. 2013 Apr;62(4):1054-63.

[24] Palomer X, Pizarro-Delgado J, Barroso E, Vázquez-Carrera M. Palmitic and Oleic Acid: The Yin and Yang of Fatty Acids in Type 2 Diabetes Mellitus. Trends Endocrinol Metab. 2018 Mar;29(3):178-190.

[25] Guerra IMS, Ferreira HB, Melo T, Rocha H, Moreira S, Diogo L, Domingues MR, Moreira ASP. Mitochondrial Fatty Acid β-Oxidation Disorders: From Disease to Lipidomic Studies-A Critical Review. Int J Mol Sci. 2022 Nov 11;23(22):13933.

[26] Cardoso D, Perucha E. Cholesterol metabolism: a new molecular switch to control inflammation. Clin Sci (Lond). 2021 Jun 11;135(11):1389-1408.

[27] Jennings W, Epand RM. CDP-diacylglycerol, a critical intermediate in lipid metabolism. Chem Phys Lipids. 2020 Aug;230:104914.

[28] Snider SA, Margison KD, Ghorbani P, LeBlond ND, O'Dwyer C, Nunes JRC, Nguyen T, Xu H, Bennett SAL, Fullerton MD. Choline transport links macrophage phospholipid metabolism and inflammation. J Biol Chem. 2018 Jul 20;293(29):11600-11611.

[29] Qin X, Qiu C, Zhao L. Lysophosphatidylcholine perpetuates macrophage polarization toward classically activated phenotype in inflammation. Cell Immunol. 2014 May-Jun;289(1-2):185-90.

[30] Geckin B, Konstantin Föhse F, Domínguez-Andrés J, Netea MG. Trained immunity: implications for vaccination. Curr Opin Immunol. 2022 Aug;77:102190.

[31] Sigruener A, Kleber ME, Heimerl S, Liebisch G, Schmitz G, Maerz W. Glycerophospholipid and sphingolipid species and mortality: the Ludwigshafen Risk and Cardiovascular Health (LURIC) study. PLoS One. 2014 Jan 17;9(1):e85724.

[32] Perreault M, Zulyniak MA, Badoud F, Stephenson S, Badawi A, Buchholz A, Mutch DM. A distinct fatty acid profile underlies the reduced inflammatory state of metabolically healthy obese individuals. PLoS One. 2014 Feb 10;9(2):e88539.

[33] Tsoukalas D, Fragoulakis V, Sarandi E, Docea AO, Papakonstaninou E, Tsilimidos G, Anamaterou C, Fragkiadaki P, Aschner M, Tsatsakis A, Drakoulis N, Calina D. Targeted Metabolomic Analysis of Serum Fatty Acids for the Prediction of Autoimmune Diseases. Front Mol Biosci. 2019 Nov 1;6:120.

[34] Lee Y, Siddiqui WJ. Cholesterol Levels. 2023 Jul 24. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024

[35] Sarwar N, Danesh J, Eiriksdottir G, Sigurdsson G, Wareham N, Bingham S, Boekholdt SM, Khaw KT, Gudnason V. Triglycerides and the risk of coronary heart disease: 10,158 incident cases among 262,525 participants in 29 Western prospective studies. Circulation. 2007 Jan 30;115(4):450-8.

[36] Ference BA, Ginsberg HN, Graham I, Ray KK, Packard CJ, Bruckert E, Hegele RA, Krauss RM, Raal FJ, Schunkert H, Watts GF, Borén J, Fazio S, Horton JD, Masana L, Nicholls SJ, Nordestgaard BG, van de Sluis B, Taskinen MR, Tokgözoglu L, Landmesser U, Laufs U, Wiklund O, Stock JK, Chapman MJ, Catapano AL. Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. Eur Heart J. 2017 Aug 21;38(32):2459-2472.

[37] Kubicek-Sutherland JZ, Vu DM, Mendez HM, Jakhar S, Mukundan H. Detection of Lipid and Amphiphilic Biomarkers for Disease Diagnostics. Biosensors (Basel). 2017 Jul 4;7(3):25.

[38] Kohno S, Keenan AL, Ntambi JM, Miyazaki M. Lipidomic insight into cardiovascular diseases. Biochem Biophys Res Commun. 2018 Oct 7;504(3):590-595.

[39] Liu D, Meister M, Zhang S, Vong CI, Wang S, Fang R, Li L, Wang PG, Massion P, Ji X. Identification of lipid biomarker from serum in patients with chronic obstructive pulmonary disease. Respir Res. 2020 Sep 21;21(1):242.

[40] Casas-Fernández E, Peña-Bautista C, Baquero M, Cháfer-Pericás C. Lipids as Early and Minimally Invasive Biomarkers for Alzheimer's Disease. Curr Neuropharmacol. 2022;20(8):1613-1631.

[41] Ciccarelli M, Merciai F, Carrizzo A, Sommella E, Di Pietro P, Caponigro V, Salviati E, Musella S, Sarno VD, Rusciano M, Toni AL, Iesu P, Izzo C, Schettino G, Conti V, Venturini E, Vitale C, Scarpati G, Bonadies D, Rispoli A, Polverino B, Poto S, Pagliano P, Piazza O, Licastro D, Vecchione C, Campiglia P. Untargeted lipidomics reveals specific lipid profiles in COVID-19 patients with different severity from Campania region (Italy). J Pharm Biomed Anal. 2022 Aug 5;217:114827.

[42] Global report on the epidemiology and burden of sepsis: current evidence, identifying gaps and future directions. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO.

[43] Christ A, Latz E. The Western lifestyle has lasting effects on metaflammation. Nat Rev Immunol. 2019 May;19(5):267-268.

[44] Vincent JL, Marshall JC, Namendys-Silva SA, François B, Martin-Loeches I, Lipman J, Reinhart K, Antonelli M, Pickkers P, Njimi H, Jimenez E, Sakr Y; ICON investigators. Assessment of the worldwide burden of critical illness: the intensive care over nations (ICON) audit. Lancet Respir Med. 2014 May;2(5):380-6.

[45] Kondrup J, Rasmussen HH, Hamberg O, Stanga Z; Ad Hoc ESPEN Working Group. Nutritional risk screening (NRS 2002): a new method based on an analysis of controlled clinical trials. Clin Nutr. 2003 Jun;22(3):321-36.

[46] Heyland DK, Dhaliwal R, Jiang X, Day AG. Identifying critically ill patients who benefit the most from nutrition therapy: the development and initial validation of a novel risk assessment tool. Crit Care. 2011;15(6):R268.

[47] Wischmeyer PE. Nutrition Therapy in Sepsis. Crit Care Clin. 2018 Jan;34(1):107-125.

[48] Li G, Zhou CL, Ba YM, Wang YM, Song B, Cheng XB, Dong QF, Wang LL, You SS. Nutritional risk and therapy for severe and critical COVID-19 patients: A multicenter retrospective observational study. Clin Nutr. 2021 Apr;40(4):2154-2161.

[49] Barabási, AL, Menichetti G, Loscalzo, J. The unmapped chemical complexity of our diet. Nature Food. 2019; 33-37.

[50] Bland JS. The Dark Matter of Nutrition: *Dietary Signals Beyond Traditional Nutrients*. Integr Med (Encinitas). 2019 Apr;18(2):12-15.

[51] Domínguez-Andrés J, Novakovic B, Li Y, Scicluna BP, Gresnigt MS, Arts RJW, Oosting M, Moorlag SJCFM, Groh LA, Zwaag J, Koch RM, Ter Horst R, Joosten LAB, Wijmenga C, Michelucci A, van der Poll T, Kox M, Pickkers P, Kumar V, Stunnenberg H, Netea MG. The Itaconate Pathway Is a Central Regulatory Node Linking Innate Immune Tolerance and Trained Immunity. Cell Metab. 2019 Jan 8;29(1):211-220.e5.

[52] Arts RJW, Novakovic B, Ter Horst R, et al. Glutaminolysis and fumarate accumulation integrate immunometabolic and epigenetic programs in trained immunity. Cell Metab. 2016;24:807–19.