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DOI:
10.1016/j.biocel.2021.105972

Document Version
Peer reviewed version

Citation for published version (Harvard):
Kucuk, S, Niven, J, Caamano, J, Jones, S, Camacho-muñoz, D, Nicolaou, A & Mauro, C 2021, 'Unwrapping the mechanisms of ceramide and fatty acid-initiated signals leading to immune-inflammatory responses in obesity', *The International Journal of Biochemistry & Cell Biology*, vol. 135, 105972.
https://doi.org/10.1016/j.biocel.2021.105972

Title: Unwrapping the mechanisms of ceramide and fatty acid-initiated signals leading to immune-inflammatory responses in obesity

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Abstract
Obesity is considered a global epidemic developed in part as a consequence of the overconsumption of a high fat diet. One of the main negative outcomes of obesity is the development of low-grade chronic systemic inflammation, induced by dysregulated immune responses, which can lead to multiple obesity-related diseases. Ceramides are a group of bioactive lipids known to be elevated in obesity and obesity-associated conditions, including cardiovascular disease and type II diabetes. Ceramides may be key players in promoting an obesity–induced inflammatory environment due to their ability to activate key pathways such as TLR4 and Nlrp3, while studies have shown that inhibition of ceramide synthesis gives rise to an anti-inflammatory environment. N-3 polyunsaturated fatty acids (n-3 PUFA) have been of interest due to their anti-inflammatory actions and shown to have beneficial effects in obesity-related diseases. This review will highlight the impact of ceramides in promoting an obesity-induced inflammatory microenvironment and discuss how n-3 PUFA could potentially counteract these responses and have a regulatory effect promoting immune homeostasis.

Keywords:
Ceramides, obesity, inflammation, fatty acids, N-3 polyunsaturated fatty acids

Abbreviations:
Acid sphingomyelinase (ASM), cardiovascular diseases (CVD), dihydroceramide desaturase (DES), dihydroceramide synthase (CerS), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), fatty acid (FA), high fat diet (HFD), obesity-induced inflammatory microenvironment (OIIM), serine palmitoyltransferase (SPT), sphingomyelinases (SMase), Type II diabetes (T2D), western diet (WD), World Health organisation (WHO), ceramide synthase enzyme (CerS)

Introduction

Obesity is one of the major worldwide health concerns of the 21st century. In 2016, 650 million adults were classified as obese, 1.9 billion were classified as overweight and the current rising trend estimates that 1 billion people would be obese by 2025 (WHO, 2016). Obesity-induced inflammatory microenvironment (OIIM) and dysregulated immune responses have been shown to induce, escalate or be associated with most obesity-linked diseases including cardiovascular disease (CVD), type II diabetes (T2D), cancer, osteoarthritis, depression and autoimmune diseases (Hruby et al., 2016). Obesity can also impact upon many other conditions such as the recent severe acute respiratory disease SARS-CoV-2. Meta-analysis of 400,000 SARS-CoV-2 patients has shown that obesity increases infection risk by 46%, hospitalisation risk by 113% and death rate by 48%, while there are concerns that vaccines might be less effective in obese individuals (Popkin et al., 2020). It is therefore crucial to identify the main players driving OIIM and the molecular mechanisms underlying disease initiation. The critical link between obesity and diet is well recognised (Bortolin et al., 2018; Almeida-Suhett et al., 2019). Excessive lipid accumulation because of the overconsumption of high fat (HFD) or western (WD) diets, leads to the expansion of white adipose tissue. Adipose tissue contains many immune cells, including macrophages and T cells. Under lean conditions, adipose tissue maintains an anti-inflammatory environment, with M2 like macrophage populations and regulatory T cells. However, in obese conditions the adipose tissue environment changes, leading to the development of low-grade chronic systemic inflammation, due to shifts in the presence immune cell populations (Donohoe et al., 2016).

Certain bioactive lipids have been shown to be elevated in obesity and modulate multiple critical immune cell processes, affecting obesity-induced inflammation (Chaurasia et al., 2016; Hamada et al., 2014 and Chaurasia et al., 2019). It is crucial to identify the main bioactive lipid species and explore their involvement into the molecular mechanisms leading to OIIM. Sphingolipids, in particular ceramides, have attracted interest due to their immunomodulatory effects. Here, we summarise the immunomodulatory effects of ceramides in the establishment of OIIM and discuss the therapeutic potential of using n-3 polyunsaturated fatty acids (n-3 PUFA) to promote an anti-inflammatory immune response.

The establishment of obesity-induced inflammatory microenvironment

The World Health Organisation (WHO) has defined obesity as abnormal or excessive fat accumulation that presents a risk to health. Diets rich in high fat, sugar, salt and low fibre content such as HFD and WD have been shown to induce obesity (Almeida-Suhett et al., 2019). Overconsumption of these diets with disrupted balance in energy homeostasis leads to the accumulation of excessive lipids in adipose and non-adipose tissue of the body (Donohoe et al., 2016).

The infiltration of macrophages and lymphocytes into adipose tissues leads to an immune tipping point, with the production of inflammatory cytokines and lipid mediators giving rise to a chronic low-grade inflammatory environment (Yang et al., 2010 & Christ et al., 2018). Murine models of diet-induced obesity have shown naïve adipose-resident T cells decrease and give rise to effector-memory populations, in which the TCR-Vβ repertoire were also impacted (Yang et al., 2010). Importantly, these mice had elevated levels of IFN-γ^{+}, granzyme and other pro-inflammatory mediators which account for the obesity-induced chronic inflammation (Yang et al., 2010). WD was shown to be misinterpreted as a threat to the host, in which innate immune system cells became hyperactive through inflammasome activation, resulting in the production of pro-inflammatory cytokine response via IL-1b forming a hyperactive inflammatory environment (Christ et al., 2018). These studies illustrated an extensive pro-inflammatory effect linked to the diet which 'reprogram' or 'prime' both innate and adaptive immune cells towards a low-grade chronic inflammatory microenvironment observed in obesity. Recent developments in lipidomic techniques have allowed lipid profiling of tissues from overweight and obese individuals, identifying ceramides which could potentially act on immune cell processes and promote obesity-induced inflammation.

Ceramide Biosynthesis

Ceramides are sphingolipids, synthesised in the endoplasmic reticulum (ER), that have been considered as key players in promoting the OIIM. Ceramides are derivatives of long-chain bases (e.g. sphingosine) and FAs of varying lengths (Hannun & Obeid, 2017). Not only are they a universal component of the cellular membrane,

they also play crucial roles in cell proliferation, apoptosis, migration, senescence, autophagy and inflammation (Hannun & Obeid, 2017, Chaurasia & Summers, 2020).

Dysregulation in sphingolipid metabolism has been associated with various inflammatory diseases including cancer, obesity and autoimmune conditions (Chaurasia & Summers, 2020). Elevated ceramide levels within specific tissues have been associated with different pathological conditions (Table 1). Lipidomic analysis has shown ceramides to accumulate in multiple tissues including liver, skeletal muscle, heart and adipose tissue in obesity (Kien et al., 2013, Chaurasia & Summers, 2020).

Ceramide biosynthesis is regulated by three pathways; *de novo* synthesis, sphingomyelin hydrolysis and the salvage pathway. The *de novo* pathway is the main biosynthetic route, starting with the condensation of palmitate and serine, although other saturated fatty acids and amino acids can be involved (Hannun & Obeid, 2017). The main enzymes of the *de novo* pathway are serine palmitoyltransferase (SPT), that is also the rate limiting step, 3- ketodihydrosphingosine reductase, dihydroceramide synthase (CerS) and dihydroceramide desaturase (DES) (Figure 1). The other two pathways involved in ceramide biosynthesis mainly function as recycling pathways which break down surplus complex sphingolipids via catabolic enzymes including various sphingomyelinases (SMase). Acid sphingomyelinase (ASM) is one member of the hydrolase enzymes that catalyses the breakdown of sphingomyelin to create ceramide (Xiong et al., 2016). Targeting the ceramide biosynthetic pathways can be used to assess the impact of these bioactive lipids on specific immune cell populations (Table 2).

Overconsumption of obesity promoting diets containing the key substrates, palmitoyl-CoA and serine, for ceramide synthesis is likely to promote this elevation. In addition, inflammatory modulators, which are elevated in the OIIM, can regulate cellular ceramide metabolism elevating cellular ceramide levels. Inflammatory cytokines IL-1β and TNFα have been shown to promote the accumulation of ceramides (Gill & Sattar, 2009, Haus et al., 2009, Holland et al., 2011). TNFα has been shown to specifically induce genes involved in the ceramide synthesis pathway, such as serine palmitoyltransferase (SPT) in murine fibroblast (L929) (Meyer& de Groot, 2003). Sphingomyelinase (SMase), a key enzyme in the sphingomyelin hydrolysis pathway for ceramide production has been shown to not only promote ceramide accumulation in bovine cerebral endothelial cells (Xu et al., 1998) but recently, inhibition of neural sphingomyelinase-2 resulted in down regulation of the TNFα-mediated expression of CD11c and secretion of inflammatory mediators IL1β and MCP-1 in monocytes/macrophages via the phosphorylation of JNK, p38 and NF-κB/ NF-kB/AP-1 pathways (Al-Rashed et al., 2020). These *in vitro* observations are also supported by *in vivo* research where intratracheal administration of TNFα boosted ceramide levels via inducing neutral sphingomyelinase in murine models (Mallampalli et al., 1999). Additionally, it is known that TNFα can inhibit insulin signalling via stimulation of p55 TNF receptor and sphingomyelinase activity, which plays a critical role in obesity-induced insulin resistance (Peraldi et al., 1996). In addition, it is well documented that the accumulation of adipose tissue during obesity leads to decrease in adiponectin, an important adipokine and their receptors (Nigro et al., 2014). Adiponectin has been described to regulate ceramide metabolism via the adiponectin receptor, expressed endogenously by various cells including T cells (Holland et al., 2011). Previous reports have shown that activation of the adiponectin receptor leads to ceramidase activity and consequently to a decrease of ceramide levels, acting as a negative regulator in cardiomyocyte and pancreatic β cell. As adiponectin levels are reduced in obesity, this may lead to increased ceramide accumulation (Holland et al., 2017).

TLR-4-stimulated macrophages activate the transcription factor NF-κB via IκB kinase (IKKβ). A knockout of IKKβ which inhibited the pro-inflammatory signalling mediated via TLR-4, led to a significant reduction in ceramide synthesis (Holland et al., 2011). To further confirm the role of TLR-4-mediated ceramide synthesis, macrophages were supplemented with palmitate but even the palmitate supplementation was not able to restore ceramide levels. Signalling pathways modulated by ceramides to induce a pro-inflammatory response can also enhance ceramide biosynthesis creating an inflammatory feed forward loop, which could be one of the critical mechanisms maintaining a low-grade chronic inflammation.

Immunomodulatory mechanisms of ceramide action

Ceramides have been shown to modulate the function of immune system cells. Initially, studies have focused on cells of the innate immune system while recent studies have suggested a link between ceramides and the adaptive immune cell response (Chaurasia et al., 2016, Hamada et al., 2014, Turpin et al., 2014). Although the precise mechanisms are still not fully understood, below we will discuss the immunomodulatory roles ceramides play in principal immune cells and how their modulation impacts their function and fate contributing to OIIM (Figure 2).

Ceramide modulation on macrophages

In obesity, macrophages are pivotal players in maintaining the tissue homeostatic microenvironment, but can also promote the OIIM via polarising to a pro-inflammatory fate, producing inflammatory cytokines such as IL-1β and TNFα (Chaurasia et al., 2016 & Haus et al., 2009). A clear association between these pro-inflammatory cytokines and increased ceramides levels in obesity, particularly in adipose tissue and circulating serum has been described (Haus et al., 2009). In peripheral adipose tissue, both genetic and pharmacological inhibition (via myriocin) of serine palmitoyltransferase (Sptlc) enzyme and ceramide synthesis pathway altered macrophage populations, with a reduction of pro-inflammatory cytokines, including IL-6 and TNFα and an increase in the M2 anti-inflammatory macrophage markers, such as IL-10 (Chaurasia et al., 2016).

Inhibition of ceramide biosynthesis may elicit, including improved glucose tolerance, increased insulin sensitivity, amelioration of hepatic steatosis and reduction in adipocyte size (Chaurasia et al., 2019). Alterations of the M1/M2 macrophage ratio with associated altered gene and cytokine expression, can push OIIM towards an anti-inflammatory homeostatic environment (Chaurasia et al., 2016). Injection of nanoliposome-loaded C6-ceremide to mice models was able to enhance the M1 cytokine production (including IL12, IFN-γ and TNF-α) and inhibit M2 cytokine production in bone marrow-derived macrophages. This observation is important to conceive the flexibility of the immune response in relation to ceramides, which can be easily driven to pro/anti-inflammatory states (Li et al., 2018). Although ceramide synthase enzyme (CerS)-6 deficient mice present reduced macrophage infiltration within gonadal white adipose tissue and improved insulin homeostasis, macrophage-specific CerS6 deletion did not specifically reduce the body weight/adiposity nor improve insulin sensitivity (Turpin et al., 2014). These observations highlight the crucial pathophysiological role of ceramides, demonstrate immunomodulatory effects on OIIM and illustrate the complexity underlying these processes that involve multiple tissues and immune cell types. In addition, reduced macrophage infiltration into white adipose tissue of CerS5 knockout mice fed HFD was observed, as well as a reduction in pro-inflammatory gene expression such as Caspase1, TNFα and IL-1β (Gosejacob et al., 2016).

The Nod Like Receptor 3 (Nlrp3) has been shown to be a critical link between ceramide accumulation and macrophage activation leading to OIIM. Nlrp3 can sense bioactive lipids including ceramides, which trigger the macrophages activation and infiltration to the peripheral OIIMs and ablation of Nlrp3 prevented the obesity–induced inflammasome activation in fat depots and liver together with enhanced insulin–signalling. Additionally, when Nlrp3 ceramide sensing is interrupted, the levels of pro-inflammatory cytokines (IL-18, IFNγ) are reduced alongside with the number of effector adipose tissue T cells. Importantly, the number of naïve T lymphocytes ($CD4^+CD62L^+CD44^-$, $CD8^+CD62L^+CD44^-$) are increased suggesting an ameliorative anti-inflammatory effect on OIIM and obesity-associated pathologies, also supporting the role of ceramides in both innate and adaptive immune systems (Vandanmagsar et al. 2011).

Ceramide modulation on Neutrophils

In addition to macrophage infiltration into adipose tissue, other cells of the innate immune system have been shown to infiltrate, promoting the OIIM. Neutrophils have been shown to be elevated in obese individuals (Xu et al., 2015) and infiltrate into adipose tissue at the early stages of HFD consumption (Elgazar-Carmon et al., 2008 & Hadad et al., 2013). Levels of basal superoxide production and also formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated superoxides were found to be elevated in neutrophils isolated from obese indivuals (Brotfain et al., 2015). Both in stimulated and unstimulated conditions, neutrophils from obese

microenvironment were primed to increase superoxide production and chemotactic activity that can drive OIIM (Brotfain et al., 2015). Early studies have shown in vitro priming of neutrophils with C2 ceramide lead to enhanced superoxide levels followed by fMLP treatment (Richard et al., 1996). However, this response may be concentration dependent, as in an additional study, in vitro activated neutrophils showed C2 ceramide to increase (< 1 microM) and inhibit (> 1 microM) superoxide generation (Wong K., Li X. B., & Hunchuk N., 1995). Neutrophil extracellular traps (NETs), DNAs components that are known to activate immune responses, have been shown at elevated levels in obese individuals (D'Abbondanza et al., 2019). One study has shown how the enhancement of NET production can occur via a ceramide/PKCζ-mediated pathway, and treatment with synthetic ceramide is sufficient to promote NET formation (Corriden et al., 2015). Furthermore, intracellular C16 and C24 levels shown to contribute to spontaneous neutrophil apoptosis via caspase activation, *in vitro*. Pharmacological inhibition of de novo pathway, reducing ceramide accumulation, created an anti-apoptotic effect on neutrophils (Seumois et al., 2007).

Ceramide modulation on T cells

T cells have also shown to play a role in obesity and promotion of the OIIM. In HFD murine models, elevated $CD8^+$ effector T cells were found to infiltrate obese epididymal adipose tissue even before macrophage accumulation, with reduced numbers of $CD4^+$ helper and regulatory T cells (Nishimura et al., 2009). Genetic depletion of $CD8^+$ T cells inhibits OIIM cascade with reduced macrophage infiltration and improved systemic insulin resistance. It is now clear that $CD8^+$ T cells play crucial roles in macrophage differentiation, activation and infiltration which contribute to the initiation and maintenance of OIIM (Nishimura et al., 2009). Ceramide biosynthesis has been shown to impact effective TCR signalling, activation and effector responses. In graft versus host murine models, acid sphingomyelinase (ASMase) deletion resulted in reduced $CD8^+$ activation and effector responses (Rotolo et al., 2009). Sofi et al. showed that CerS6 was as required for optimal T cell response, with reduced allogeneic responses observed due to impaired C16 ceramide production (Sofi et al., 2017). The ceramide biosynthesis pathways have also been shown to impact CD8 granule-mediated cytotoxicity, in which sphingomyelinase (ASMase)-deficient mice are defective in exocytosis of cytolytic effector molecules (Herz et al., 2009).

Other studies have suggested that ceramides can promote T cell differentiation towards a specific pro- or anti-inflammatory fate, in a concentration and tissue-specific manner (Martín-Leal et al., 2020). Palmitate, a 16-carbon saturated FA, is a main product of de novo fatty acid biosynthesis and common dietary component of HFD. In both humans and rodents (including obese and insulin resistance murine models) elevated systemic FA availability via acute infusion, can increase plasma ceramide concentrations (Watt et al., 2012 and Tran et al., 2016). Palmitate has been shown to promote obesity related low-grade chronic inflammation and can directly prime $CD4^+$ T cell differentiation into a $CD44^{hi}$-$CCR7^{lo}$-$CD62L^{lo}$-$CXCR3^+$-$LFA1^+$ effector memory-like phenotype (Mauro et al., 2017). This bias in $CD4^+$ T cell differentiation leads to a preferential trafficking to non-lymphoid periphery sites with an increased effector Th1/Th17 pro-inflammatory function, contributing to the creation of OIIM. Palmitate was shown to modulate $CD4^+$ T cells via enhanced activation of PI3K p110d-Akt-dependent pathway (Mauro et al., 2017). Furthermore, ceramides can push homeostasis towards a pro-inflammatory environment via reducing the $Foxp3^+$ Treg population (Zhou et al., 2016 and Hollmann et al., 2016). As mentioned, ASM is a major enzyme in the synthesis of ceramides from sphingomyelin. In ASM specific knock out mice, *in vitro* and *in vivo* assays illustrated that ASM ablation leads to a higher number of Tregs (Zhou et al., 2016). In addition to elevated Treg frequency, the suppressive activity of Tregs was also enhanced both in the ASM knock out mice and in the presence of amitriptyline, a ASM pharmacological inhibitor (Hollmann et al., 2016).

Ceramide modulation on B cells

Although changes in macrophages and T cells have been the main focus on the development on the OIIM, B cells also contribute to the inflammatory environment. In diet-induced obese mice, Winer *et al* showed how B cells accumulate in visceral adipose tissue. Depletion of B cells resulted in the suppression of M1 macrophage activation, polarisation and $CD8^+$ T cell activation with significant reduction in TNF-α and IFN-γ pro-inflammatory cytokine levels in adipose tissue stromal vascular cells (Winer et al., 2011). Importantly, B cells

were shown to promote systemic as well as local (visceral adipose tissue) inflammation in obesity-associated diseases such as insulin resistance and glucose intolerance. B^{null} mice showed improved glucose/insulin sensitivity, adipose tissue inflammation, reduced adipose tissue hypertrophy and no impact on circulating adiponectin levels (DeFuria et al., 2013). Consistent with the reduction observed in inflammatory B- and T-cell cytokine levels, reduced inflammation in obese/insulin resistant B cell-null mice associated with an increased percentage of anti-inflammatory Tregs (DeFuria et al., 2013). Additionally, ceramides were linked to B cell-associated apoptotic program known as activation induced cell death triggered by B cell receptor. Specifically, long chain ceramides C16, C18 and C24 synthesised via de novo pathway were shown to cross-link with B cell receptor. This cross-linking triggers a specific sequence of biochemical evets leading to caspase-dependent apoptosis which potentially involves a mitochondrial damage and loss of function (Kroesen et al., 2001 & Kroesen et al., 2003). Furthermore, ceramides (C2) are known to inactivate the anti-apoptotic protein B cell lymphoma 2 (Bcl2) via dephosphorylating and reducing the mRNA levels of Bcl2 (Ruvolo et al., 1999 & Chen et al., 1995).

Therapeutic potential of n-3 PUFA on immune function and OIIM

N-3 PUFA could be used to counteract the inflammatory response resulting from the over consumption of a HFD. Although the extent of their effects is still debated, n-3 PUFA have been shown to have overall anti-inflammatory effects, for example, n-3 PUFA supplementation has been shown to lower blood pressure and reduce the levels of triglycerides, leading to a reduced risk of CVD (Hu et al., 2019). In inflammatory diseases, such as type II diabetes, n-3 PUFA improved insulin sensitivity reduced the body fat ratio (Martins et al., 2018 and Gutiérrez et al., 2019).

The molecular mechanism of the anti-inflammatory action of n-3 PUFA remains of interest and is subject of on-going investigations. Increasingly, evidence suggests that n-3 PUFA act in three major ways to help the restoration of homeostasis in OIIM. Firstly, n-3 PUFA are recognised by distinct receptors such as TLR4, Nlrp3 and G protein-coupled receptors, mainly GRP120 expressed by immune cells (such as macrophages) leading to reduced inflammatory cytokine and chemokine responses (Oh et al., 2010 and Mildenberger et al., 2017). Secondly, n-3 PUFA can modulate the differentiation and motility of T lymphocytes (Cucchi et al., 2019). Thirdly, n-3PUFA dilute the availability of pro-inflammatory n-6PUFA substrates (Nicolaou et al., 2014 and Kendall et al., 2019)

As discussed, the saturated FA palmitate and ceramides can directly recognise and activate macrophages via TLR4 receptor (Liu et al., 2013 and Eraky et al., 2018). This is a crucial point in which n-3 PUFA could act to rebalance the immune response and promote an anti-inflammatory environment and potentially ameliorate obesity-associated diseases such as T2D (Eraky et al., 2018). In a human clinical trial, overweight/obese pregnant women were daily supplemented with n-3 PUFA which led to down-regulation of TLR4 expression and reduction of IL-6, IL-8, and TNFα production in both adipose and placental tissues (Haghiac et al., 2015). Further, *in vitro* assays on cells isolated from maternal subcutaneous adipose tissue and placenta illustrated that palmitate was responsible for a 10-30-fold increase in the expression of TLR4, IL-6 and IL-8. Whereas n-3 PUFA supplementation in an ex-vivo cell culture, significantly reduced the transcription of TLR4, IL-6, IL-8 induced by palmitate, reducing its inflammatory effect by 70% (Haghiac et al., 2015). N-3 PUFA recognition by GPR120 significantly reduced the production of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α which is mediated by TLR4 (Haghiac et al., 2015 and Liu et al., 2013). Additionally, activation of GPR120 by n3- PUFA reduced the levels of pro-inflammatory cytokines IL-1b, TNFα, MCP-1 and also lead to IL-10-mediated promotion of M2 anti-inflammatory genes in adipose tissue (Yan et al., 2013 and Lee et al., 2019). Consistently, GPR120 knockout in both murine model and *in vitro* monocytes and macrophages abrogated the anti-inflammatory effects of n3- PUFA. After activation by n3-PUFA, the GPR120 coupled to β-arrestin2 leading to the inhibition of TAB1-mediated activation of TAK1, which may account for the inhibition of both the TLR and TNFα pro-inflammatory signalling pathways (Oh et al., 2010).
Nlrp3 is another important innate immune cell receptor which can drive the anti-inflammatory effect of n-3 PUFA on OIIM, via inhibition of inflammasome activation (Kumar et al., 2016) and subsequent expression of pro-inflammatory cytokines such as IL-1b, TNFα, MCP-1 (Yan et al. 2013). Importantly, n-3 PUFA-dependent

inhibition of Nlrp3 activation has been shown to exert beneficial effects on HFD-induced T2D murine models via reconstitution of a homeostatic cellular microenvironment (Kumar et al., 2016 & Yan et al., 2013) and in clinical trials via downregulation of the expression of various pro-inflammatory cytokines such as IL-18 and IL-1β in adipocyte and macrophages (Lee et al., 2019).

Furthermore, n-3 PUFA limit ceramide synthesis via diverting their major substrate palmitate (Tachtsis et al., 2020) but also via directly inhibiting the ceramide biosynthetic enzyme genes serine palmitoyl transferase long chain base (Sptlc) and degenerative spermatocyte homolog 2 (Des2), as shown in the liver of a murine model of hepatic steatosis (Dong et al., 2017).

Final remarks

A growing body of evidence implicates various immune cells in sensing exogenous and endogenous metabolic signals raised by metabolic dysregulation and obesogenic diets. Ceramides, potent signalling lipids involved in energy homeostasis, are found to mediate a number of the cellular and pathophysiological process linked with OIIM and other obesity-associated diseases. Increasing evidence suggests that n-3 PUFA can partly create an anti-inflammatory environment acting via inflammatory receptors, such as TLR4 and Nlrp3 expressed on macrophages. As these pathways are activated by ceramides, n-3PUFA could be a tool to counteract their pro-inflammatory activities (Figure 3). The extensive modulatory effects ceramides have on various cells and tissues, suggests the presence of many more mechanistic interactions still to be unrecovered.

Acknowledgements

This work is supported by funds of the Versus Arthritis- and Medical Research-funded Centre for Musculoskeletal Ageing Research at the University of Birmingham to SK, funds of the British Heart Foundation Accelerator Award at the University of Birmingham to JN and a University of Birmingham Professorial Research Fellowship to CM.

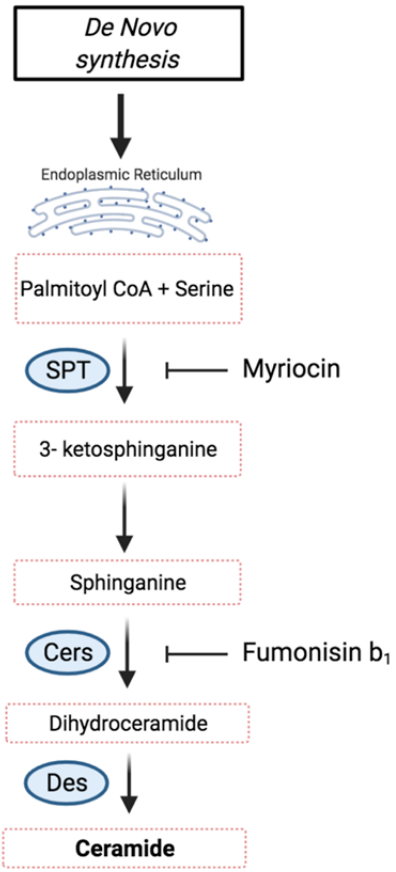

Figure 1. Ceramide *de novo* biosynthesis pathway. Biosynthesis of ceramides begins in the endoplasmic reticulum with condensation of palmitate and serine by serine palmitoyltransferase (SPT), followed by a series of reactions catalysed by 3-dehydrosphinganine reductase, dihydroceramide synthase (CerS) and dihydoriceramide desaturase (Des). Highlighting Myriocin and Fumonisin $b_{1,}$ commonly used inhibitors of SPT and CerS respectively.

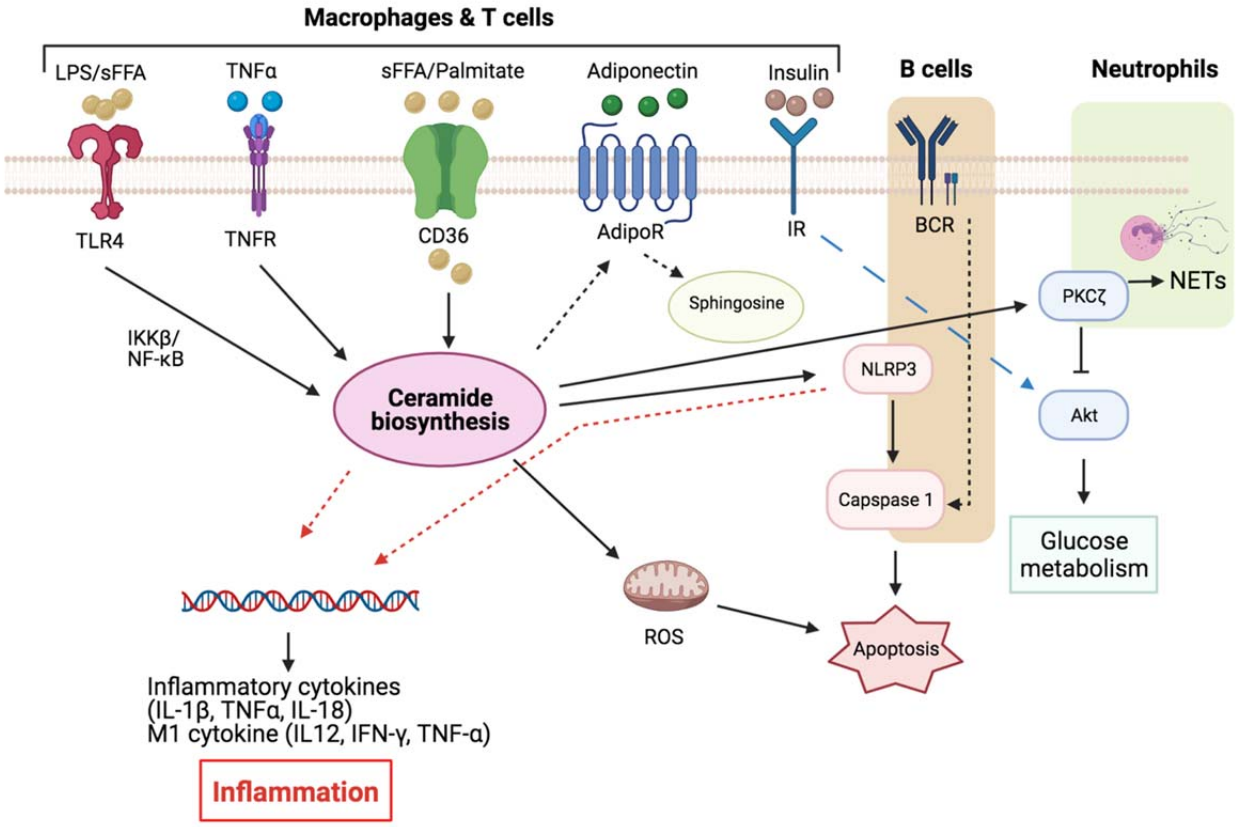

Figure 2. Schematic representation of interactions between ceramide biosynthesis and intracellular signalling in key immune cells. Ceramide overload triggered by key substrates such as sFFA, palmitate and LPS via TLR4, TNF and CD36 receptors leads to excessive ceramide biosynthesis. Ceramide elicits deleterious effects on insulin signalling and glucose metabolism via inhibiting Akt with PKCζ. Ceramide also induces mitochondrial stress via blocking lipid oxidation and creating ROS that induce cellular apoptosis. Activation of Nlrp3 via ceramides also triggers apoptosis via caspase 1 in addition to its role in obesity-induced inflammatory action. (Akt, Protein Kinase B; CD-36, cluster of differentiation 36; AdipoR, Adiponection receptor; IKK, Ikappa kinase; IL, interleukin; IR, Insulin receptor; LPS, lipopolysaccharide, NF-kß, Nuclear factor kappa-light-chain-enhancer of activated B cells; Net, Neutrophil extracellular traps; Nlrp3, NLR family, pyrin domain containing 3; PKC, protein kinase C; ROS, reactive oxygen species; sFFA, Saturated fatty acids; TLR4, Toll like receptor-4; TNF-α, Tumour necrosis factor alpha; Tumour necrosis factor alpha receptor).

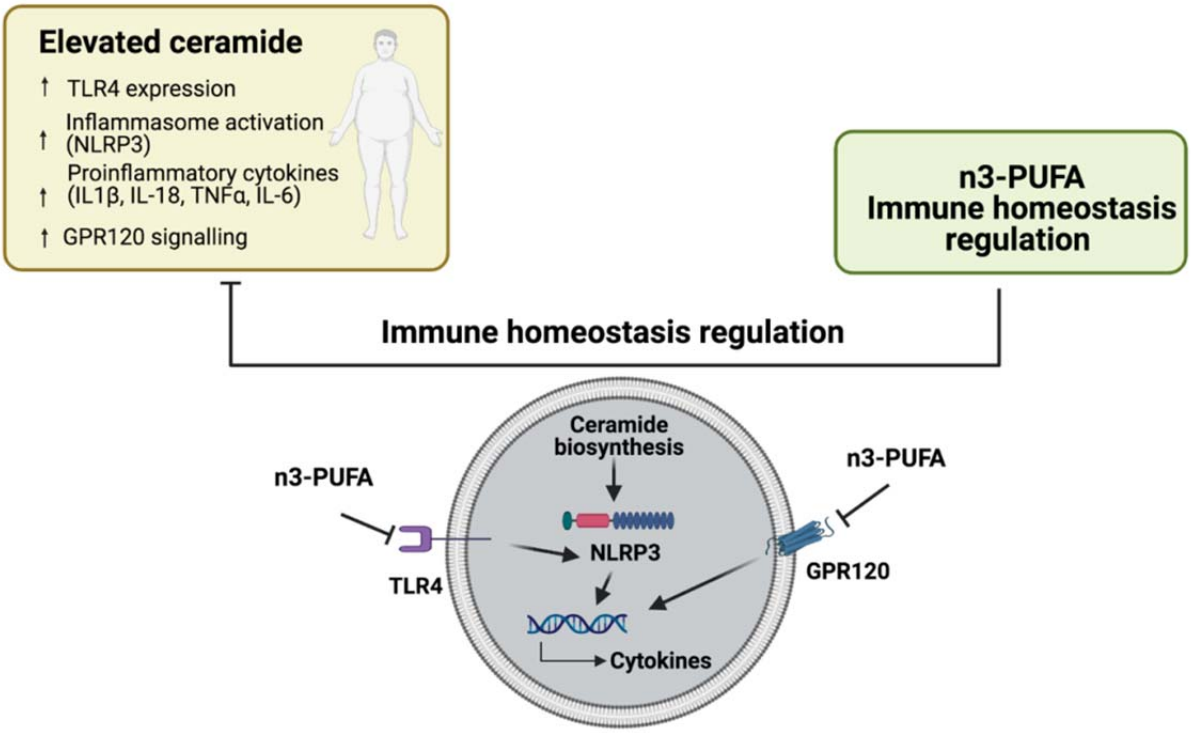

Figure 3. Schematic illustration highlighting the potential converge of ceramides and n-3 PUFA. Ceramides, when elevated within an obese environment, promote inflammation through the activation of TLR4 and GPR120 receptors on immune cells. N-3 PUFA can regulate this environment, inducing and anti-inflammatory response via potentially blocking the corresponding receptors of ceramides and restore immune homeostasis.

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TABLE 1: Recent publications highlighting the association between obesity- induced ceramide accumulation and various pathological conditions.

Tissue	Experimental model (in vivo/ in vitro)	Ceramide species	Ceramide concentration	Change in OIIM	Change in pathology	Ref.
Liver	*In vitro*-Human HepG2 liver cells, 0.5 mmol/l palmitate for 4-8 hrs.	Total intracellular and exracellular ceramide	Increased	NA	Obesity and diet-induced diabetes	Watt et al. 2012
	C57BL/6J mice on 60% HFD for 18 weeks	C16:0	Increased	NA	Glucose intolerance and obesity induced type II diabetes	Raichur et al. 2019
	C57BL/6J mice on 15% ethanol diet for 4 weeks and human VL17A cells	C14, C20, C20:1, C22, C24, and C26:1	Increased	NA	Promotes alcoholic steatosis	Williams et al. 2018
	Clinical trial- 980 free living human on mediterranian diet followed for 7.4 years	C16:0, C22:0, C24:0 and C24:1 in Plasma	Increased	NA	Higher risk for CVD	Wang et al. 2017
Cardiovascular	Clinical trial- 462 individuals with familial coronary artery disease	Total serum ceramides (30 out of 32 tested), C16:0, C18:1 and C24:1 most strongly associated	Increased	NA	Coronary artery disease risk	Poss et al. 2020
	In vitro Human AC16 cardiomyocytes, 0.5 mM palmitate and In vivo C57BL/6 mice on 60% for 8 weeks	C16:0, C24:0, C24:1	Increased	NA	Heart failure- Mitochondrial dysfunction, oxidative stress and cell death in cardiomyocytes.	Law et al. 2018
	Clinical Trial- Obese individuals (n=439)- white adipose tissue	C16:0, C18:0	Increased	NA	Obesity, insulin resistance, diet-induced diabetes	Turpin et al. 2014
Adipose tissue	In Vitro- Human 3T3-L1 adipocytes and RAW264.7 macrophages	Total ceramide and C2:0	Increased	Upregulation of IL6, IFNγ, TNFα and MCP1	Creation of OIIM	Hamada et al. 2014
	Wistar rats on 60% HFD for 8 weeks	C18:0, C20:0, and C24:0	Increased	Upregulation of TNFα	Insulin resistance and obesity-induced type II diabetes	Blachnio-Zabielska et al. 2018
	Clinical trial- Obese women (n=28) visceral adipose tissue	Total ceramide	Increased	Upregulation of TNFα	Metabolic syndrome	Choromańska et al. 2019
Plasma	Rhesus monkeys on high-fat and high-fructose (HFFD) 'western' diet for 8 months to 5 years	C14:0, C16:0, C22:0, C24:0	Increased	NA	Associated with obesity-induced type II diabetes	Brozinick et al. 2013
	C57BL/6J mice on 60% HFD for 18 weeks	C16:0	Increased	NA	Glucose intolerance and obesity induced type II diabetes	Raichur et al. 2019
	Obese children (n=80, aged 7–17 years)	Total ceramide including C14:0, C16:0, C16:1, C18:0, C18:1, C22:0, C24:0	Increased	NA	Nonalcoholic fatty liver disease	Wasilewska et al. 2018
	C57BL/6 mice on 55.2% HFD for 17 weeks	C14:0 and C18:0	Increased	NA	Contributes to obesity- associated insulin resistance	Turpin et al. 2019
	C57BL/6 mice on 60% HFD for 12 weeks	Total ceramide	Increased	NA	Associated with obesity-induced type II diabetes	Bandet et a. 2018
	Clinical trial-106 individuals with obesity and T2D	C18:1, C:20, C:22, C:24 and C:24:1	Increased (by two-fold)	NA	Associated with obesity-induced type II diabetes	Broskey et al. 2018
Skeletal muscle	In Vitro- Human C2C12 myotubes palmitate treatment (0.75 mmol/L) for 16 h	Total ceramide	Increased (60%)	NA	Associated with obesity-induced type II diabetes	Bandet et a. 2018
	In Vitro- Rat L6 myotubes and mouse C2C12 muscle cells, 0.5 mM palmitate	Total ceramide	Increased	Increased the expression of pro-inflammatory, Il6 and Ccl2	Associated with obesity-induced type II diabetes	Pillon et al. 2018
	Wistar rats on 60% HFD for 8 weeks	Total ceramide including C14:0, C18:0, C18:1, C24:1 and C24:0	Increased	NA	Associated with skeletal muscle insulin sensitivity	Blachnio-Zabielska et al. 2016

TABLE 2: Intervention methods to target the *de novo* ceramide biosynthesis pathway

Target enzyme	Experimental model (in vivo/ in vitro)	Ceramide species & Tissues	Intervention	Ceramide concentration	Change in OIIM	Change in pathology	Ref.
SPT (serine palmitoyltransferase)	C57BL/6J mice on HFD 12-20 weeks	Total ceramide including C24:0,C24:1, C22:0 in Adipose tissue, liver, muscle and serum	Pharmacological inhibition via Myriocin and Sptic knockout model	Reduced	Reduced pro-inflammatory cytokines IL-6, MCP-1, and TNFα with increased anti-inflammatory cytokines (IL-10) More anti-inflammatory M2 macrophages Less macrophage infiltration to periphery	Improvemened insulin sensitivity and glucose homeostasis	Chaurasia et al. 2016
	In Vitro- Human 3T3-L1 adipocytes and RAW264.7 macrophages	Total ceramide and C2:0	Genetic Knockdown of SPT	Reduced	Reduced pro-inflammatory cytokines; IL-6, IFN-γ,TNFα and MCP-1	Restoring the homeostasis of the OIIM	Hamada et al. 2014
Ceramide synthases (CerS 1-6)	C57BL/6 mice on 55.2% HFD for 17 weeks	C16:0, C18:0- adipose tissue, liver	Cers6 genetic knockout	Reduced	No macrophage inflatration and reduced pro-inflammatory cytokines IL-6 and IL-1b	Protected from diet-induced obesity, glucose intolerance and type II diabetes	Turpin et al. 2014
	C57BL/6J mice on 60% HFD for 18 weeks	C16:0- liver and plasma	Cers6 knockdown via antisense oligonucleotides	Reduced (50%)	NA	Improved insulin sensitivity, glucose tolerance with reduced body fat content	Raichur et al. 2019
	In Vitro- Human 3T3-L1 adipocytes and RAW264.7 macrophages	Total ceramide and C2:0	Pharmacological inhibitior of CERs via Fumonisin B1	Reduced	Reduced pro-inflammatory cytokines; IL-6, IFN-γ,TNFα and MCP-1	Restoring the homeostasis of the OIIM	Hamada et al. 2014
	C57BL/6JCrl Cers5 KO mice on 60% HFD for 24	C16:0 and C18:0- lung, spleen, muscle, liver and white adipose tissue	CerS5 genetic knockout	Reduced	Reduction in pro-inflammatory gene expression such as NF-kB, TNFα and IL-1β	Reduced weight gain, ameliorate OIIM	Gosejacob et al. (2016)
	*Global knockdown of Cers1 in C57BL/6 mice on 55.2% HFD for 17 weeks	C18:0	Cers1 genetic knockout	Reduced (95%)	NA	Decreased body weight and fat content with improved insulin sensitivity and glucose tolerance	Turpin et al. 2019
Dihydroceramide Desaturase (DES)	C57BL/6 mice on 60% HFD for 12 weeks	Total ceramide- Serum, liver, white adipose tissue	Genetic Degs1 knockout	Reduced	Reduced pro-inflammatory cytokines; IL-1 and TNFα	Reduced fat density and improved insulin sensitivity	Chaurasia et al. 2019

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