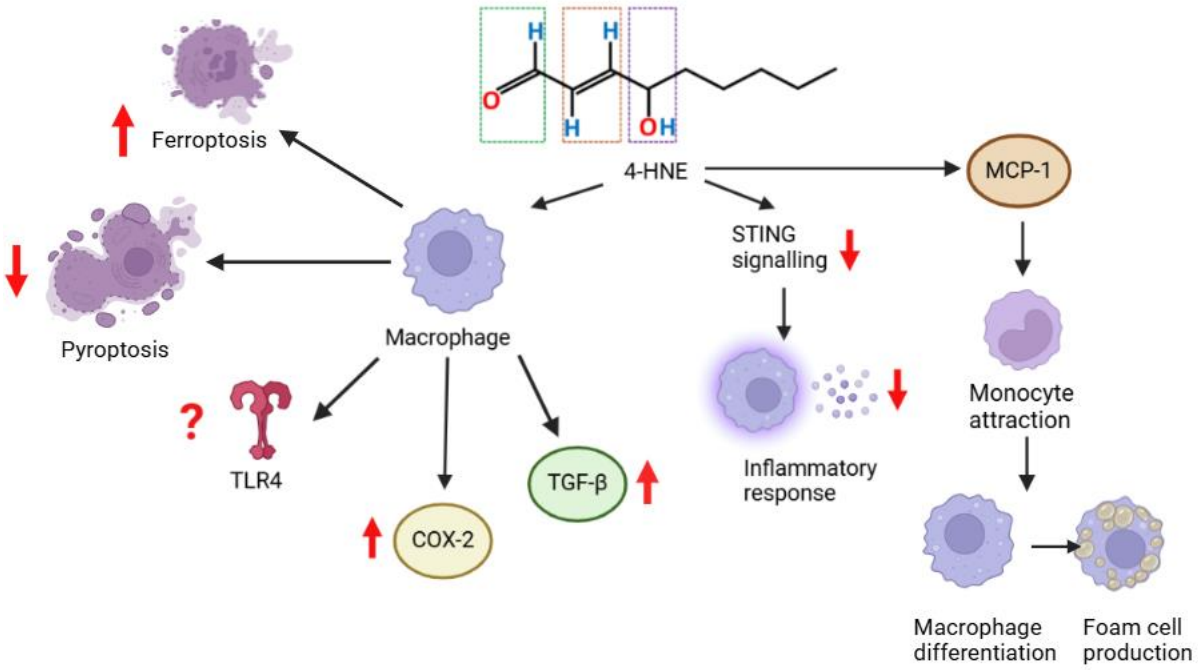
Immunomodulatory Effect of 4-Hydroxynonenal on Macrophages



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Abstract

Lipid peroxidation or the reaction of oxygen with unsaturated lipids can produce a wide variety of oxidation products. One of these products is 4-hydroxynonenal (4-HNE). 4-HNE is highly diffusible, and can therefore affect different cell types including immune cells. In this review, the immunomodulatory effect of 4-HNE on macrophages will be discussed. These effects include inhibition of pyroptosis, induction of ferroptosis, increased expression of TGF-β and COX-2 and modulating TLR4 signaling. Furthermore, 4-HNE downregulates STING signaling and increases MCP-1 secretion, leading to altered autocrine signaling that enhances macrophage recruitment and activation. Additionally, macrophages not only respond to but also produce 4-HNE upon bacterial stimuli. All of these effects of 4-HNE on macrophages, make that it plays a role in multiple diseases. Because of the effect on TLR4, COX-2 and STING signaling, 4-HNE plays a role in inflammatory diseases. Moreover, 4-HNE is involved in fibrotic diseases as well through its effect on TGF-β. Lastly, its effect on MCP-1 reveals that it is highly involved in atherosclerosis as this is also an inflammatory disease. The immunomodulatory effects discussed in this essay, give an insight into the effect of 4-HNE on different diseases. However, further investigating these immunomodulatory effects of 4-HNE on macrophages, could potentially lead to therapies for all these diseases. This could potentially relieve patient symptoms, but not fully cure these diseases as it is just a part of the immune response present in these diseases and probably not causal for these diseases.

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Introduction

Reactive oxygen species

Reactive oxygen species (ROS) are produced during normal metabolic activity. This can be done through either enzymatic or non-enzymatic pathways (B. Wang et al., 2023). There are different types of ROS, including hydrogen peroxide, superoxide, singlet oxygen, hydroxyl radical and lipid peroxyl radicals (Sharma et al., 2022). These ROS all have different reactivities and can also be converted into other highly reactive and cell-damaging radicals (Mustafa et al., 2018). ROS production is limited under healthy conditions, but excessive ROS production can be observed in pathological conditions. When the ROS production exceeds the levels that our antioxidant machinery can cope with, this is referred to as oxidative stress. Under oxidative stress, ROS can react with DNA, proteins and lipids. This may also damage the cell membrane and organellar membranes, which contain polyunsaturated fatty acids (X. Zhang et al., 2023).

Lipid peroxidation

Non-enzymatic lipid peroxidation is initiated by the attack of free radicals, such as ROS, on the carbon-carbon double bonds of (mainly) membrane polyunsaturated fatty acids (PUFAs)(Dalleau et al., 2013). Lipid peroxidation is an autocatalytic process that occurs in three phases, the initiation phase, the propagation phase and the termination phase (Figure 1). During initiation, a lipid radical is formed as a result of hydrogen abstraction by radicals. In the propagation phase, this lipid radical reacts with molecular oxygen which then produces a lipoperoxyl radical that can react with fatty acids to form lipid radicals or lipid hydroperoxides. Lastly, during termination a nonradical species is generated, here antioxidants can participate as hydrogen atom donors (Reyes-Jiménez et al., 2021). Lipid peroxidation or the reaction of oxygen with unsaturated lipids can produce a wide variety of oxidation products. Primary products are mostly lipid hydroperoxides (LOOH). However, these lipid hydroperoxides are not stable end products. Instead, they can undergo beta-scission reactions, leading to the formation of secondary lipid peroxidation products (Liu et al., 2022). These secondary products include fatty acid alcohols, ketones and aldehydes. A lot of different aldehydes are produced upon lipid peroxidation, including hexanal, propanal, malondialdehyde and 4-hydroxynonenal (4-HNE). Among these products, 4-HNE is the best-characterized (Ayala et al., 2014).

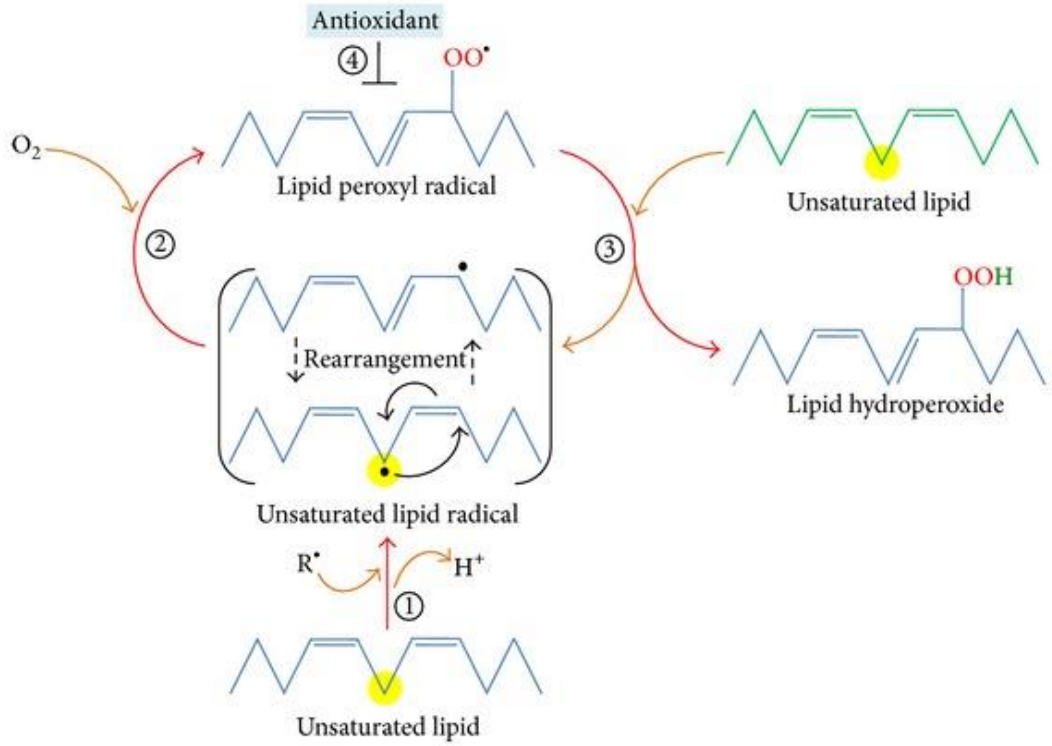

Figure 1: Lipid peroxidation (Ayala et al., 2014)
This figure shows the lipid peroxidation process. With the initiation phase (1), where a lipid radical is formed. The propagation phase (2/3), where the lipid radical reacts with oxygen forming a lipid peroxyl radical (2), which abstracts hydrogen from another lipid molecule, thereby forming a new lipid radical and lipid hydroperoxide (3). And the termination phase (4), where a hydrogen atom is donated to the lipid peroxyl radical species, thereby generating nonradical products.

4-HNE

4-HNE is a chemically reactive short-chain alkenal that was first discovered in 1963 by Herman Esterbauer and Schauenstein (Schauenstein & Esterbauer, 1963), but they first published it as 4-hydroxy-octenal. Due to its functional groups (Figure 2), 4-HNE is electrophilic and highly reactive towards nucleophilic thiol and amino groups (Reyes-Jiménez et al., 2021). 4-HNE can react with proteins containing histidine, cysteine and lysine residues, with lipids containing phosphatidylethanolamine as an amino group and with the guanosine part of the DNA (Dalleau et al., 2013). 4-HNE can influence cells, its effects include DNA damage, protein inactivation, inhibition of antioxidant mechanisms, inhibition of proliferation and cell death (Reyes-Jiménez et al., 2021). Because it is a highly diffusible molecule, it can travel throughout the body and affect all different kinds of cells including immune cells, thereby affecting the immune system (Dalleau et al., 2013). This paper will discuss the immunomodulatory effect of 4-HNE, focussing on macrophages.

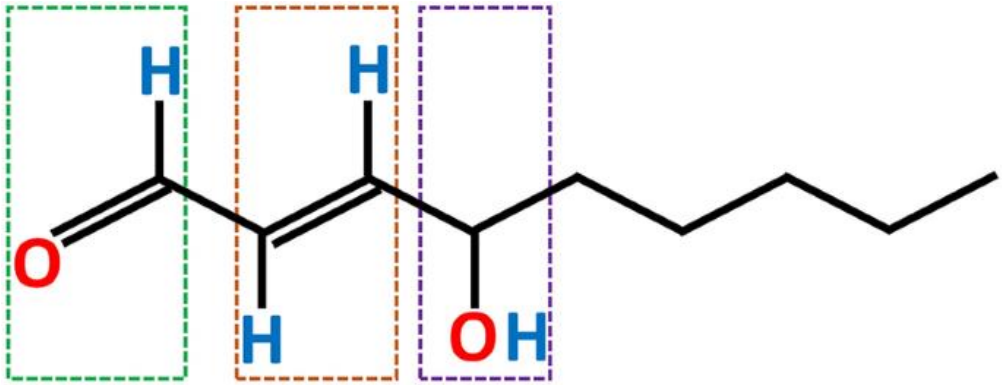

Figure 2: Structure of 4-hydroxynonenal (Reyes-Jiménez et al., 2021)
This figure shows the structure of 4-hydroxynonenal, with in green its carbonyl group, in orange its C-C double bond and in purple its hydroxy group.

Immunomodulatory effect of 4-hydroxynonenal on macrophages

While 4-HNE is still most often described as a toxic end-product of lipid peroxidation, it is also an important signalling molecule (Dwivedi et al., 2007). One of the cell types that are influenced is macrophages.

Macrophages

Macrophages are important immune cells responsible for the defence against pathogens. They can have both pro-inflammatory or anti-inflammatory phenotypes. Pro-inflammatory functions include antigen presentation, oxidative stress, tissue remodelling and pathogen clearance (Lendeckel et al., 2022). This plays a large role in the innate immune response against pathogens. In case of pathogenic infection or tissue damage, pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) can be detected by pattern recognition receptors (PRRs). Macrophages can sense these danger signals using these PRRs such as toll-like receptors (TLRs) and C-type lectin receptors (CLRs). When PAMPs and DAMPs are recognized, usually the inflammatory response is started and cytokines such as TNF-α, IL-1β and IL-6 are released (S. Chen et al., 2023). Macrophages with pro-inflammatory functions are called classically activated or M1 macrophages. Canonical M1 macrophages are polarized with pro-inflammatory, usually IFN-γ and lipopolysaccharide (LPS), and can activate the JAK-STAT1 pathway for a more pro-inflammatory phenotype. In contrast to M1 macrophages, macrophages can also have anti-inflammatory phenotypes, which are called alternatively activated or M2 macrophages. M2 macrophages can be stimulated with IL-4 and IL-13 and induce tissue repair and immunosuppressive functions (Ohkuri et al., 2018) Anti-inflammatory functions of macrophages include wound healing, resolution of inflammation and secretion of anti-inflammatory cytokines such as IL-10 and TGF-β (Lendeckel et al., 2022). Thus, based on the type of activation (e.g. DAMPs, PAMPs or other stimuli) this can result in 2 classes, M1 or M2 macrophages. Because of these properties, macrophages can affect the development of many different diseases such as cancer, infectious diseases, chronic inflammatory diseases and atherosclerosis (Hirayama et al., 2018).

Toll-like receptors

Toll-like receptors (TLRs) are key regulators of the innate immune system. They can trigger the release of specific cytokines defining distinct immune cell compartments, this can generate different macrophage phenotypes (Zeng & Jewell, 2019). TLR4 was one of the first TLRs discovered. It can detect gram-negative bacteria by recognising lipopolysaccharide (LPS). TLR4 signalling promotes M1 polarisation and phagocytosis activity. Before LPS can bind to TLR4, TLR4 needs to form a complex with MD2. The binding of LPS with this complex causes a structural change, facilitating the dimerization of two of the LPS-MD2-TLR4 complexes. Hereafter, downstream signalling can take place (Park & Lee, 2013).

In 2009, Kim et al. investigated the effect of 4-HNE on TLR4 activation and its downstream signals (Kim et al., 2009). They pretreated RAW264.7 macrophages with 4-HNE (1,5,10,20 μM) and stimulated macrophages for 8h with LPS (10ng/ml) in the presence or absence of the 4-HNE from the pre-treatment. Results of a luciferase assay, gene expression and western blot revealed that exposure to 4-HNE downregulated TLR4-mediated pathways and gene expression. Furthermore, analysis of the phagocytic capacity was performed using yellow-green carboxylate-modified

beats. After 30 min incubation with these beats, cells containing these beats were analysed with fluorescent microscopy. This revealed that the phagocytic function of macrophages was decreased in the presence of 4-HNE. Suggesting that 4-HNE might contribute to the termination of inflammatory processes by suppressing TLR4 activation. To investigate how 4-HNE inhibits TLR4 signalling, they investigated the effect of 4-HNE on TLR4 dimerization. To do this, Ba/F3 cells stably expressing Flag- and GFP-tagged TLR4 were used. Assessing the amount of co-immunoprecipitation of GFP-tagged TLR4 and Flag-tagged TLR4 revealed that 4-HNE suppresses ligand-induced and ligand-independent receptor dimerization. This means that 4-HNE already inhibits TLR4 in the initial steps of activation (Kim et al., 2009).

After this study by Kim et al., the role of 4-HNE on TLR4 signalling was not pursued further until 2020, when Wang et al. published a paper in which they reported the opposite effect that 4-HNE activates TLR4 signalling (Y. Wang et al., 2019). However, their study design was completely different from the study design of Kim et al. In their study, Wang et al. investigated the effect of 4-HNE treatment on TLR4 signalling in inflammatory bowel disease (IBD) in mice. Mice were stimulated with DSS (2% w/v) in drinking water to induce IBD, and one week later treated with 4-HNE (5 mg/kg/day) or vehicle DMSO. Mice were sacrificed 7 days after 4-HNE treatment, and blood and colon tissue were collected for analysis. Increased concentrations of TLR4 ligands such as LPS and other bacterial products were observed in circulation. Furthermore, a TLR4 reporter assay revealed a significant increase ($p=0.002$) of the reporter after 4-HNE treatment, this means increased activation of TLR4 signalling. This effect was abolished in $TLR4^{-/-}$ mice, supporting the evidence that 4-HNE contributes to IBD by increased TLR4 signalling (Y. Wang et al., 2019).

That the results of Kim et al. and Wang et al. are so different, is interesting. However, their experimental designs are also completely different. First of all, Kim et al. performed their experiment on macrophages, while the study of Wang et al. was performed in mice. Of course, mice have a complete immune system which could impact TLR signalling, and maybe other cells are activated instead of macrophages increasing TLR signalling this way. Furthermore, it could be a dose-dependent effect, because Kim et al. used a lower concentration of below 20µM which could explain decreased TLR4 activation. Compared to Wang et al. who used 32µmol/kg/day which might be high enough to stimulate TLR4 activation. As TLR4 activation promotes phagocytosis, it would make sense that it is activated with a high dose of 4-HNE, as high doses of 4-HNE have previously been proven to be toxic. Overall, comparing these studies suggests a biphasic effect of 4-HNE where in low concentrations it can inhibit TLR4 signalling and therefore inhibit phagocytosis by macrophages while in high doses it can activate TLR4 signalling and increase phagocytosis by macrophages.

STING signalling

Another important player in the innate immune system is the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway, this pathway senses cytosolic DNA for activation of interferon genes and regulation of T-lymphocytes. When activated, STING can translocate to the nucleus and recruit TBK1 for the activation of IRF3. This induces expression of the interferon and production of specific cytokines, eventually causing inflammation (Miao et al., 2020). Gluthathione peroxidase 4 (GPX4) is an antioxidant enzyme. It can remove accumulated intracellular lipid peroxides for the maintenance of redox homeostasis. By maintaining this redox homeostasis, GPX4 also has an effect on STING signalling (Jia et al., 2020).

In 2020, Jia et al. (Jia et al., 2020) revealed that 4-HNE induced STING carbonylation, preventing the palmitoylation and translocation of STING from the endoplasmic reticulum (ER) to the Golgi. This resulted in the down-regulation of immune responses. This study also revealed that GPX4 inhibited STING carbonylation, thereby activating the STING pathway. This means that GPX4 has the opposite effect of 4-HNE (Jia et al., 2020). This was later confirmed by multiple studies, under which in 2023 when Chen et al. published a paper where they did a immunoblot analysis of STING, TBK1 and IRF3 phosphorylation in GPX4-knockdown COAD cells. Results revealed that a GPX4 knockdown resulted in increased 4-HNE levels and subsequently decreased STING signalling (B. Chen et al., 2023). In 2024 Chen et al. further investigated STING interference. They used AAV-sh-STING to knockdown STING in mice. Immunohistochemistry revealed decreased 4-HNE levels and increased GPX-4 levels in the STING knockdown mice (J. Chen et al., 2024). Since activation of the STING pathway in macrophages leads to activation of the inflammatory response, decreased activation of the STING pathway by 4-HNE could cause macrophages to cause less inflammation (Q. Wang et al., 2022).

Cyclooxygenase 2

As previously mentioned, macrophages play a large role in the inflammatory response. For example by upregulating cyclooxygenase 2 (COX-2), which produces prostaglandins. COX-2 is the enzyme responsible for the rate-limiting steps in arachidonic acid conversion to eicosanoid lipid signalling molecules. One of the best-known lipid eicosanoid lipid signalling molecules is prostaglandin E_2 (PGE_2), which has a key role in many early inflammatory events. PGE_2 itself is secreted in large quantities by macrophages and acts as an autocrine regulator of macrophage activation (Giroux & Descoteaux, 2000). However, PGE_2 does not only cause pro-inflammatory reactions, but it can also have anti-inflammatory effects, among which the differentiation of macrophages to the M2 (anti-inflammatory) phenotype (Tang et al., 2017).

In 2004, kumagai et al. (Kumagai et al., 2004) revealed that 4-HNE could potentially induce COX-2 in macrophages. In their study, they treated macrophages with 4-HNE for 1-24 hours. This experiment revealed that 4-HNE induces COX-2 expression in RAW264.7 macrophages, with a peak at the 6-hour timepoint. To confirm these findings *in vivo*, mice were treated intraperitoneally with 10µmol 4-HNE for 48 hours. Here they found that the amount of recruited immune cells, under which macrophages significantly increased in the peritoneal cavity of treated mice. This was associated with increased expression of COX-2 in peritoneal macrophages. Furthermore, significantly higher levels of COX-2 were found in the liver, lung, kidneys and spleen of 4-HNE-treated mice. Additionally, they investigated whether the increase in COX-2 in macrophages can be explained by increased NF-κβ signalling. This was done by measuring Iκβ and nuclear vs. cytosolic NF-κβ on an immunoblot. Here, they found that NF-κβ signalling was not increased after 4-HNE treatment in macrophages. Thereby suggesting that HNE significantly increases COX-2 expression through an NF-κβ-independent mechanism. This sparked the question of which mechanism could be responsible for this. Therefore, they investigated the p38 MAPK pathway, which was previously proven to be induced by 4-HNE in rat liver epithelial cells (Kumagai et al., 2002). These findings were confirmed in macrophages, where rapid phosphorylation of p38 MAPK was observed after 4-HNE stimulation, which lasted for 60 minutes. Suggesting the involvement of the p38 MAPK pathway in increasing COX-2 expression in macrophages (Kumagai et al., 2004).

Since the paper of Kumagai et al., multiple studies confirmed the correlation between 4-HNE and COX-2 expression, for example in 2022 in a study by Wang et al. (Z. Wang et al., 2022). In their study, they investigated the anti-inflammatory effect of L-methionine on rats with 4-HNE accumulation. To do this, these rats were fed a diet containing different levels of L-methionine

(200-400 mg/kg bodyweight) for 2 weeks. After 2 weeks, they found that hepatic mRNA levels of COX-2 went down. Furthermore, a significantly positive correlation was observed between hepatic 4-HNE content and COX-2 expression. Additionally, analysing hepatic protein levels of Nf-κβ revealed that L-methionine depresses NF-κβ activation. The authors suggest that this decrease in NF-κβ activation could be because of decreased 4-HNE accumulation. This is opposite to what Kumagai et al. (Kumagai et al., 2004).previously described, since they observed that 4-HNE treatment did not activate the NF-κβ pathway. This difference could potentially be because of the different dose that they use, or because they use a different timeline for their experiment. However, since the study of Kamagai et al., multiple studies have come out, describing that 4-HNE does activate NF-κβ (Jang et al., 2016)(H. Zhang & Forman, 2017) (Nègre-Salvayre et al., 2017). Therefore, the findings of Kamagai et al. could also be specific to the type of macrophages they used in their study. Or it could be because of the assay that they used. Western blot can give different results, depending on the sensitivity of the antibody on NF-κβ in different cell types. Overall, all of the above mentioned studies reveal a strong correlation between 4-HNE and COX-2 expression. Furthermore, the study of Wang et al. reveals a positive effect of L-methionine against the negative effects caused by 4-HNE. L-methionine is thought to do this through the activation of Nrf2-antioxidant responsive element pathway, which in term can increase glutathione which is an antioxidant (Z. Wang et al., 2022).

MCP-1 and PKC

MCP-1 is involved in the pro-inflammatory response of macrophages. MCP-1 stimulates classical activation of M1 macrophages and therefore results in an inflammatory response (Singh et al., 2021). MCP-1 attracts monocytes and drives the differentiation of these monocytes to macrophages. Depending on the site of attraction these macrophages can contribute to different diseases (Singh et al., 2021). For example, in vascular walls MCP-1 production can be increased in the presence of oxLDL (Hashizume & Mihara, 2012). Oxidation of LDLs by ROS in vascular walls leads to the formation of 4-HNE and other aldehydes (Dalleau et al., 2013). When MCP-1 is produced, monocytes are attracted to the site of production. When more MCP is produced by endothelial cells in blood vessels, macrophages can form foam cells. Foam cells play a role in plaque formation in atherosclerosis (Leonarduzzi et al., 2005). Besides MCP-1, protein kinase C (PKC) also plays a role in foamcell formation, as it promotes processes that lead to the accumulation of cholesterol and other lipids in macrophages. Furthermore, PKC activation plays a role in pro-inflammatory cytokine production in LPS induced inflammation. MCP-1 can drive this reaction, as it is known to activate PKC (Yadav et al., 2010).

In 2002, Nitti et al. reported a stimulative effect of 1μM 4-HNE on the release of MCP-1 (Nitti et al., 2002). In their study, J774.A1 mouse macrophages were treated with only 0.1, 1 and 10 μM 4-HNE in medium without serum supplementation. 4-HNE levels were kept constant by supplying 4-HNE every 5 or 10 minutes within the first hour of exposure. Hereafter, MCP-1 concentrations were determined in total lysate using an ELISA. When the macrophages were exposed to 1μM 4-HNE, their MCP-1 excretion increased with 63% compared to untreated macrophages. Next, they wanted to investigate whether MCP-1 release was PKC dependent. To do this, macrophages were pre-treated with a PKC inhibitor (0.5μM Go6976 or 15μM rottlerin), whereafter 4-HNE treatment was performed in the same way as before. Go6976, which is a classic PKC inhibitor, was able to prevent 4-HNE induced MCP-1 excretion. Rottlerin, which is selective for novel isoenzymes, did not reveal a significant difference. Subsequent, they performed immunoprecipitation with isoform-specific antibodies to investigate which isoform was activated.

This revealed that PKC-β was significantly increased but PKC-α was not. Overall, this means that MCP-1 release after 4-HNE treatment is PKC dependent. They concluded that activation of PKC-β mediates the MCP-1 release after 4-HNE stimulation, but the exact mechanism is not known yet (Nitti et al., 2002)

This role of 4-HNE on PKCs was further investigated in 2012 by Harry et al. (Harry et al., 2012). They explored the PKC modifying ability of 4-HNE. To do this, they cultured RAW 264.7 cells and incubated them with 0-50μM 4-HNE for 3 hours. They analysed PKC by western blot after 35μM HNE treatment, this revealed time-dependent sensitivity towards 4-HNE. Hereby, they identified PKC as a target for 4-HNE.

Overall, these studies reveal a effect of 4-HNE on MCP-1 though PKC, which eventually increases foam cell formation.

TGF-β

The secretion of TGF-β is one of the anti-inflammatory properties of macrophages. It downregulates the production of cytokines such as TNF-α, IL-1β and IL-6 and suppresses the response of M1 macrophages. Additionally, it stimulates alternative activation of M2 macrophages (Gauthier et al., 2023). TGF-β is a major player in tissue repair, but overproduction of TGF-β can lead to fibrosis (Budi et al., 2021). TGF-β is the key activator of the SMAD pathway. This pathway is involved in production of matrix proteins under which collagen, fibronectin, actin and elastin, which are also overexpressed in fibrosis.

In 1997 already, Leonarduzzi et al. (Leonarduzzi et al., 1997) discovered that 4-HNE can upregulate TGF-β expression by the murine J774-A1 macrophages and human U937 monocytes. This revealed a link between lipid peroxidation and excessive TGF-β function. But the exact mechanism in which this works had not been investigated yet. In 2019, Tsubouchi et al. (Tsubouchi et al., 2019) investigated this mechanism in relation to GPX4. They started of their research by proving that 4-HNE was increased in fibrotic tissue, they did this by means of an immunohistological staining on idiopathic pulmonary fibrosis (IPF) tissue and by western blot on lung fibroblasts from IPF patients. To assess if this increase in 4-HNE in fibrotic tissue was dependent on GPX4, they used heterozygous GPX4-deficient mice, as total GPX4 knockdown would result in the death of the mice. GPX4-deficient mice showed increased lipid peroxidation and increased 4-HNE. Overall, this study revealed that the increase of TGF-B production by macrophages after 4-HNE treatment is related to decreased GPX4. This affects macrophages, because increased TGF-β expression signals macrophages to activate the SMAD pathway, subsequently increasing the production of collagen.

Cell death

One of the most important function of macrophages is the role they play in cell death. One of the best known examples of this is the phagocytosis of apoptotic cells called efferocytosis. But macrophages also play a role in pyroptosis and ferroptosis, which are different from apoptosis since they have pro-inflammatory and immunogenic roles (Makuch et al., 2024).

Pyroptosis is a form of cell death occurring through the lytic pathway. During pyroptosis, osmotic pressure changes lead to an influx of water making the cell swell and eventually rupture, releasing inflammatory factors such as IL-1β, IL-18 and DAMPs (Peng et al., 2024). Initiation of pyroptosis in macrophages starts with the formation of the NOD-like receptor protein 3 (NLRP-3)

inflammasome. This inflammasome can than trigger the caspase pathway, which can cause pore formation in the cell membrane (Ni et al., 2024).

In 2022, Hsu et al. investigated the activation of the NLRP-3 inflammasome in presence of 4-HNE (Hsu et al., 2022). To do this, they used different experimental groups. They used a mouse group, in which they treated female and male C57BL/6J mice with 2mg/kg bodyweight LPS from *E.coli* with or without 6μM 4-HNE by pipetting this in the oral cavity. The control group only received standard saline solution. They also used a second mouse group in which they induced sepsis in C57BL/6J mice. To do this, they gave an intraperitoneal injection with 10mg/kg LPS followed by 100mM/100μl ATP. Half an hour before LPS, mice were intraperitoneally injected with 2mg/kg bodyweight 4-HNE , DMSO or 2mg/kg RSL3 (a GPX4 inhibitor). From these mice, blood was collected. Furthermore, peritoneal macrophages were isolated from C57BL/6J mice. Besides mouse experiments, they also isolated human peripheral blood mononuclear cells (PBMCs) from 20ml whole blood. These human monocytes were differentiated into macrophages, which were then transfected for ASC-GFP overexpression. Mouse, human and THP-1 macrophages were stimulated with LPS for 3h with 0.3-3μM 4-HNE or ethanol. To activate the NLRP3 inflammasome, cells were primed for 3h with LPS and then treated with 2-6μM ATP or 50μM R837 for 30-60 minutes depending on the cell type (Hsu et al., 2022). In one of their experiments, they revealed that 4-HNE inhibits pyroptotic death in human and mouse macrophages. To do this, they used nigericin which is a K+ ionophore that can activate the NLRP3 inflammasome. They added this to the cells after the LPS and additional 4-HNE treatment. 4-HNE itself did not affect cell death, but it significantly decreased LPS-nigericin-stimulated cell death. Meaning that 4-HNE protects macrophages from LPS-nigericin-mediated pyroptosis. Furthermore, they investigated Nrf2 which is a transcription factor that has been proposed to downregulate NLRP3 inflammasome activation. Here, they found that 4-HNE induces Nrf2 activation and stimulates its nuclear translocation. However, after Nrf2 knockdown, 4-HNE still inhibited the pyroptosis of macrophages. To investigate if this inhibition was NF-κβ dependent, they assessed p65 phosphorylation, p65 nuclear translocation and Iκβ-a degradation and the expression of TNF-α and NLRP3 in macrophages. LPS did significantly affect all of these factors, however additional treatment with 4-HNE revealed no significant effect. From these results, they concluded that 4-HNE inhibits pyroptosis independent of the NF-κβ pathway (Hsu et al., 2022). Furthermore, investigation of the mouse model and sepsis mouse model also revealed decreased NLRP3 inflammasome activation with 4-HNE treatment. LPS treatment did significantly increase IL-1β and TNF-α, additional 4-HNE treatment decreased these markers again. Overall, from this study we can conclude that 4-HNE inhibits pyroptosis in both human and mouse macrophages.

Ferroptosis is an iron-dependent form of cell death which is characterized by uncontrolled membrane lipid peroxidation. Because of this, the link between ferroptosis and 4-HNE is more straightforward, as 4-HNE production is one of the end products of lipid peroxidation. This means that the process of ferroptosis, increases 4-HNE production, which can then affect macrophages (X. Zhang et al., 2023). But besides this 4-HNE can also directly affect ferroptosis.

In 2022, Chen et al. performed a study in which they identified 4-HNE as both a product and mediator of ferroptosis. One of their research questions was to assess whether increased 4-HNE could affect feedback loops, thereby reducing the sensitivity of cells to ferroptosis. To do this, they studied aldehyde dehydrogenase 1 family member B1 (ALDH1B1), which was previously reported as a 4-HNE metabolizer. Eukaryotic initiation factor 4E (EIF4E) can interact with ALDH1B1 in membranes, limiting the clearance of 4-HNE. They found that overexpression of ALDH1B1 limited 4-HNE accumulation in EIF4E-overexpressed Calu-1 cells, this suggests that ALDH1B1 plays a

role in limiting 4-HNE production during ferroptosis. At the same time, administration of an ferroptosis inhibitor (ferrostatin-1), inhibited cytotoxic effects of 4-HNE in ALDH1B1 knockdown Calu-1 and HT-108 cells, further confirming the role of the EIF4E-ALDH1B1complex in regulating 4HNE accumulation and toxic effects during ferroptosis. Next, they wanted to investigate whether a low (subtoxic) dosis of 4-HNE (12.5μM), affected the susceptibility to ferroptosis. Here, they found that this low dosis enhanced RSL3 induced cell death and lipid peroxidation, indicating that even low doses of 4-HNE can accelerate ferroptosis. Together, these results reveal that 4-HNE is not only a product of ferroptosis, but it also can induce ferroptosis (X. Chen et al., 2022).

Overall, these studies make it clear that 4-HNE is involved in cell death, with an inhibiting effect on pyroptosis and induction of ferroptosis. That cells would favor ferroptosis when 4-HNE is present could be because of the lipid peroxidation by which ferroptosis is characterized. Furthermore, ferroptosis itself is not directly an inflammatory form of cell death, whilst pyroptosis is an inflammatory form of cell death. This could possibly mean that cells favor non-inflammatory cell death in presence of 4-HNE, which could be an anti-inflammatory effect of 4-HNE.

4-HNE production by macrophages

Besides responding to 4-HNE, macrophages themselves can also produce 4-HNE when they are stimulated with gut microbiota (Dalleau et al., 2013).

In 2012, Wang et al. (X. Wang et al., 2012). investigated this production of 4-HNE by macrophages. Herefore, they infected macrophages at a multiplicity of infection (MOI) of 1000 cfu of *E. faecalis* for 2h. Assaying these macrophages for 4-HNE production confirmed their capability to do this. Furthermore, they confirmed the structure of 4-HNE by 2-D NMR. They observed an 2-fold increase in 4-HNE production in supernatant between infected and non-infected macrophages. Besides *E. faecalis*, they also tested this for *E.coli* (MOI of 100) and here they also found increased 4-HNE production in infected compared to non-infected macrophages. This production of 4-HNE could potentially indicate ferroptosis, as this was not researched very well yet in 2012 (Dennis et al., 2012). However, it could also be a way for macrophages to regulate the immune response, as we already discussed the various effects of 4-HNE on the immune system.

Conclusion and Discussion

This essay discussed the immunomodulatory effects of 4-HNE on macrophages. The several studies that we discussed, reveal a clear immunomodulatory effect of 4-HNE on macrophages. These effects include inhibition of pyroptosis, induction of ferroptosis, increased expression of TGF-β and COX-2 and having an effect on TLR4. Furthermore, 4-HNE downregulates STING signaling and increases MCP-1 secretion (Figure 3).

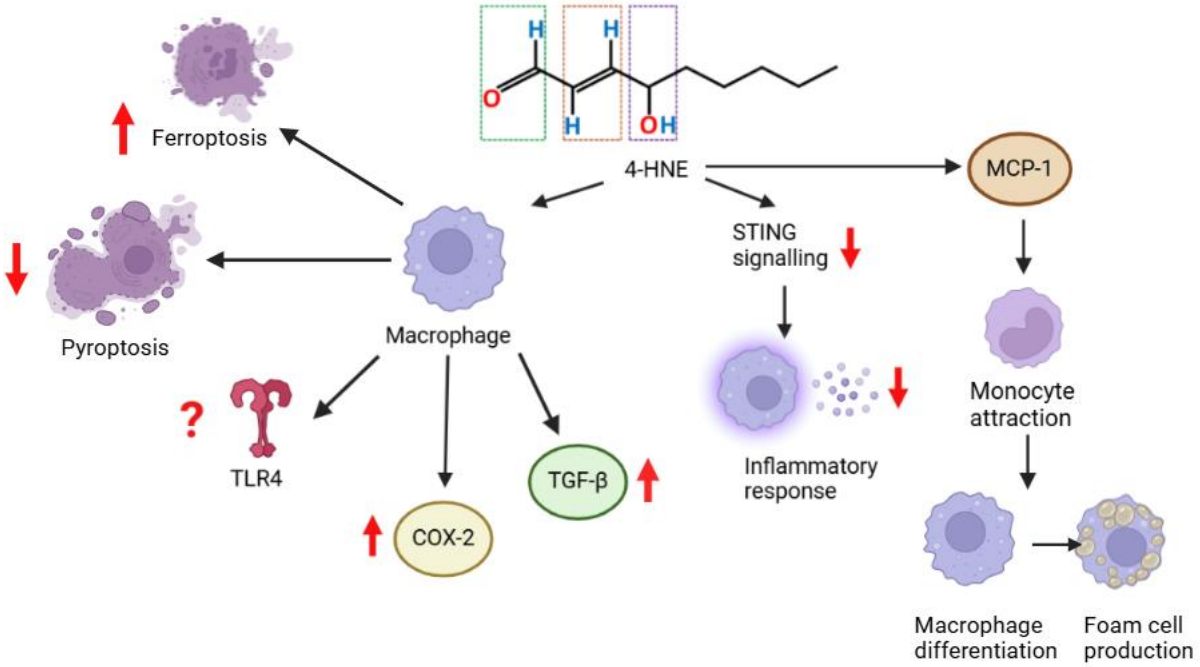

Figure 3: Immunomodulatory effect of 4-HNE on macrophages.

These effects of 4-HNE on macrophages, potentially affect different diseases. It affects multiple inflammatory diseases, as STING signaling, COX-2 and TLR4 all play roles in inflammation. Examples of these diseases are IBD, COPD, rheumatoid arthritis and atherosclerosis (Shoeb et al., 2013). In these diseases increased levels of 4-HNE have been found previously. STING signaling, COX-2 and TLR4 all play roles in the pathogenesis of these disease. This means that 4-HNE could also play a role in the pathogenesis of these diseases, and might contribute to their severity (Shoeb et al., 2013) However, whether 4-HNE has an increasing or decreasing effect on inflammation could potentially be different depending on disease type or 4-HNE doses. Its effect on TLR4 signaling is not completely clear as, the studies of Kim et al. and Wang et al. revealed different outcomes. 4-HNE decreases STING signaling, which would then also decrease inflammation caused by the STING pathway. Furthermore, when 4-HNE is present this induces ferroptosis and inhibits pyroptosis. This could also be an anti-inflammatory effect of 4-HNE as pyroptosis is an pro-inflammatory form of cell death since during pyroptosis inflammatory cytokines are released, and ferroptosis is a non-inflammatory form of cell death. However, 4-HNE was also revealed to induce COX-2, meaning it would have a pro-inflammatory effect here.

These differences in inflammatory response could thus potentially be dose-dependent. Low doses of 4-HNE might have an beneficial effect on inflammation, whereas high doses might contribute to the inflammation. To investigate whether this is actually the case, a dose-response curve of 4-HNE could be made. Firstly, this could be done for TLR4 activation, as there is a gap of knowledge here, with the studies of Kim et al. and Wang et al. being the most recent studies performed on this particular subject. This dose-response curve could first be made in macrophages, using similar methods as Kim et al. This would reveal whether high doses of 4-HNE can actually induce TLR4 activation in macrophages. Next, this should be confirmed *in vivo*, where treatment of mice with 4-HNE could reveal the effect of 4-HNE on TLR4 activation.

Another possibility is that 4-HNE treatment in mice could cause different cell types to have different reactions. Maybe the direct effect of 4-HNE on macrophages is anti-inflammatory, but intermediate products produced by different cell types could still cause a pro-inflammatory reaction in macrophages. Some intermediate products that are produced by cells upon 4-HNE exposure are 4-HNE protein adducts, among others produced by endothelial cells, smooth muscle cells and epithelial cells. These might impair the signaling pathways of macrophages, involved in the immune response and inflammation. Furthermore, inflammatory signals produced by other cell types upon 4-HNE exposure might also affect the macrophage response (Schaur et al., 2015).

To investigate whether these intermediate products affect the macrophage response, researchers could use models that are in between only using macrophages and using an animal model. For example, organoid models could be used, or coculturing macrophages with other cell types, or culturing macrophages in conditioned medium. These models could help reveal the response of other cell types to 4-HNE, and reveal whether products produced by these cells could affect the response of macrophages. Before this can be done, more literature research should be done into the effect of 4-HNE on different cell types. This could help identify cell types that would be interesting to use in such a model.

Furthermore, the effect of 4-HNE on MCP-1 further contributes to atherosclerosis, as the production of foam cells largely contributes to plaque formation. This role of 4-HNE in atherosclerosis pathogenesis is already broadly researched. However, foam cells also play roles in several disease types under which, neurodegenerative diseases, COPD, chronic kidney disease and Rheumatoid Arthritis indicating a role for 4-HNE in the pathogenesis of these diseases (Eom et al., 2015) (Guerrini & Gennaro, 2019).

Besides possibly contributing to inflammatory diseases, it could also contribute to fibrotic diseases such as cardiac, liver, kidney and pulmonary fibrosis (Rosenbloom et al., 2017). This is largely attributed to its increasing effect on TGF-β. TGF-β is a key factor in collagen production, excessive collagen production is present in fibrosis. Therefore 4-HNE might play a role in the disease pathogenesis. However, the pathogenesis of these diseases is often very complicated, and for most of these diseases not completely understood. Therefore, it is probable that 4-HNE is not causal to the disease, but does play a role in the pathogenesis. Possibly contributing to disease severity.

In most of these diseases, the concentration of 4-HNE is most likely already increased because of oxidative stress. Targeting 4-HNE production might therefore potentially have positive effects in these diseases, as it could potentially reduce inflammation, decrease TGF-β, induce pyroptosis thereby clearing damaged cells and reduce foam cell production. This targeting of 4-HNE could be achieved by antioxidant treatments, which neutralize 4-HNE. One way to do this would be by

finding drugs that boost the expression of pathways of antioxidants, such as the Nrf2 pathway (Song & Long, 2020). Additionally, for treatment of diseases that involve foam cell production, more specific approaches can be used. For example targeting CD36 might be an option. This could prevent the uptake of oxidized lipids by macrophages and thereby prevent foam cell formation (Geloen et al., 2012).

However, before 4-HNE can be used as a target in medical settings, more research should be done into its mechanism. Although it is the most investigated byproduct of lipid peroxidation, still not enough is known about its effect on macrophages. Additional research could focus on the more long term effects of 4-HNE exposure to macrophages, as the studies that have performed so far usually only give 4-HNE for short periods of time. While in diseases cells are often chronically exposed to 4-HNE due to the constant oxidative stress in the body. When 4-HNE is present over longer periods of time, its toxic effects could accumulate. Therefore, to simulate the effect of 4-HNE on macrophages in diseases, it needs to be studied after a longer incubation period. Furthermore, the effects of 4-HNE on the different macrophage subsets is something that still needs to be investigated further. As most studies right now, use the same macrophage cell lines, and do not mention anything about macrophage differentiation.

In conclusion, this review addresses the immunomodulatory effect of 4-HNE on macrophages, with a focus on how 4-HNE affects key signaling routes such as TLR4, COX-2 and STING. Investigating the role of 4-HNE on TLR4, revealed a possible dose-dependent effect of 4-HNE. This reveals the necessity for more research into the dose-dependent effects of 4-HNE. Furthermore, cross-talk between different cell types upon 4-HNE exposure can be researched further. Overall, addressing these open questions could lead to possibilities in using 4-HNE in the treatment of inflammatory and fibrotic diseases where 4-HNE seems to play a role in disease pathogenesis.

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During the writing of this essay, ChatGPT was used for brainstorming, and Grammarly was used for grammar and spelling.