Modulation of macrophage response mechanisms against persistent pathogens by PUFA

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Infectious diseases occur when a pathogen overwhelms a host`s immune response, establishes a local niche, replicates and spreads over the whole body. Immunocompromised people are especially in danger to develop severe pathologies. An important factor influencing the immune defence is the diet. In particular polyunsaturated fatty acids (PUFA) are believed to play a key role in modulating immune response mechanisms. The present review intends to summarise actual data concerning the immune modulation by PUFA thereby focussing on macrophage defence mechanisms. This includes the impact of PUFA on macrophage membrane composition, macrophage phagocytosis, macrophage respiratory burst as well as macrophage cytokine synthesis.

Keywords PUFA; macrophages; membrane composition; phagocytosis, respiratory burst; cytokine synthesis

1. Background

Phagocytosis and killing of pathogens by macrophages is a pivotal point in human and animal health. Some infectious agents like *Rhodococcus equi* and *Pseudomonas aeruginosa*, however, are known to restrain macrophage respiratory burst and to survive in the immune cells thereby leading to chronic infections [1-4]. Immunocompromised individuals are especially in danger to develop severe diseases [1-4].

R. equi, a Gram-positive soil bacterium, is a known pulmonary pathogen of young horses and immunocompromised humans [5]. Via arresting phagosome maturation, *R. equi* is able to survive and to multiply in macrophages establishing a pathogen-specific niche inside the immune cell [4]. *R. equi* prevents the establishment of the phagolysosome via inhibiting the fusion of phagosom and lysosom hence resting in a phagosomal vesicle with neutral pH and without lysosomal enzymes [4].

P. aeruginosa is a Gram-negative nosocomial pathogen of immunocompromised individuals. The microorganism colonizes the pulmonary tract, urinary tract, burns, wounds as well as medical devices [6]. Often these infections are difficult to treat since *P. aeruginosa* demonstrates various resistance mechanisms [2, 7]. For example, *P. aeruginosa* possess a natural resistance against numerous antibiotics and is able to form biofilms thereby increasing environmental persistence [2, 7].

Immune cell activity depends on numerous external as well as internal stimuli. One important factor modulating immune function is the diet. Nutritional fatty acids, in particular polyunsaturated fatty acids (PUFA), have an impact on membrane lipid composition [8]. Basic chemical and physical properties of membranes are determined by the lipid composition [8]. Accordingly, changes in membrane fatty acid pattern may impact cell signalling pathways and membrane-associated enzymes via a modulated membrane fluidity [8]. This provides a link between dietary fatty acid uptake, inflammation and immunity. The directed supply of PUFA to immunocompromised individuals might be a useful tool in the supportive therapy of chronic infections caused by persistent pathogens as *R. equi* and *P. aeruginosa*.

2. Macrophage membrane composition due to PUFA supplementation

With the introduction of the lipid raft hypothesis [9] the relevance of the membrane lipid composition for a variety of cellular processes has come in the focus of medical research. Lipid rafts – membrane domains, which are enriched with saturated fatty acids and cholesterol – are proposed to play key roles in signal transduction as well as transport processes of cells [10-12]. It is assumed that there is an interrelation between the lipid composition of membrane domains and the physiological processes occurring at the cell membrane [8]. This gives rise to the presumption that an enrichment of cells with unsaturated fatty acids disturbs the organisation and thus the physiological function of lipid rafts [13].

The introduction of a detergent-free lipid raft isolation technique [14-16] has paved the way to reliable analyse the lipid composition of membrane domains. So far, lipid rafts have been isolated by means of detergents [17]. However, there are accumulating observations that the use of detergents may result in raft domains, which do not exist in the intact cell [17]. The newly established detergent-free isolation technique bypasses this source of error thus being a suitable method to study membrane domains.

Using the detergent-free lipid raft isolation technique in a systematic study the fatty acid profiles of non-raft as well as raft domains of the murine monocyte/macrophage cell line RAW264.7 has been investigated [16]. At this, distinct differences in the fatty acid composition of non-raft and raft domains were observed. The proportion of PUFA of the n-

3 and the n-6 family was considerably lower in rafts compared to non-rafts as expected [16]. The differences in the fatty acid profiles of the two membrane domains were also reflected by the Methylene Bridge Index (MBI) [16]. The MBI is defined as the mean number of bis-allyl-methylene positions per fatty acid contained in the membrane domains. It is calculated by multiplying the number of bis-allyl-methylene positions contained in each fatty acid methyl ester species by its respective mole fraction and summed for all fatty acids present [18]. The higher the MBI, the higher the unsaturation of the membrane lipids. Consequently, the MBI of the non-raft domains was about 3 times higher than the MBI of the rafts [16].

In a next step the macrophages were supplemented with saturated fatty acids as well as with PUFA from the n-3, the n-6 and the n-9 family [16]. Supplementation with the n-9 fatty acid oleic acid (C18:1n9) and the saturated fatty acids palmitic acid (C16:0) and stearic acid (C18:0) respectively resulted in only marginal modifications in the fatty acid profile of both non-raft and raft domains [16]. Supplementation with PUFA of the n-3 or the n-6 family, however, resulted in an increase in the content of these fatty acids in non rafts as well as rafts [16]. The supplemented PUFA were not only incorporated into the membrane but also metabolized leading to a significant increase in their desaturation and elongation products [16]. It is important to note that the increase in n-3 PUFA content was more pronounced for the raft domains than for the non-raft domains [16]. The enrichment of the macrophages with PUFA of either the n-3 or the n-6 family was connected with a significant rise of the MBI of both membrane domains [16]. Remarkably, there was a relation between the number of bis-allyl-methylene positions of the supplemented PUFA and the dimension of unsaturation of the membrane domains [16]. So, for example, supplementation with the n-3 PUFA docosahexaenoic acid (C22:6n3), which has 6 double bonds, resulted in a significant higher MBI of both non-rafts and rafts than supplementation with the n-3 PUFA alpha-linolenic acid (C18:3n3), which has 3 double bonds [16]. The same was true for the n-6 PUFA arachidonic acid (C20:4n6) with 4 double bonds and linoleic acid (C18:2n6) with 2 double bonds [16].

Taking together it was found that:

1. PUFA of both the n-3 and the n-6 family modulate the macrophage membrane lipid composition of non-raft as well as raft membrane domains.
2. There is a positive relation between the number of bis-allyl-methylene positions of an added n-3 or n-6 PUFA and the MBI of macrophage non-raft as well as raft membrane domains.

3. Phagocytosis due to PUFA supplementation

Phagocytosis is a membrane-mediated process. Hence modulation of macrophage membrane structure and fluidity is proposed as a potential method to promote the phagocytic capacity of macrophages [19-21]. In fact, studies concerning the internalisation of zymosan particles or heat-killed bacteria by macrophages demonstrate a positive relation between the dimension of unsaturation and the phagocytosis rate of the immune cells [19, 21, 22].

To gain a deeper understanding in a study using RAW264.7 macrophages the phagocytosis of viable bacteria in context of PUFA supplementation has been investigated [23]. As model organisms the persistent pathogens *R. equi* and *P. aeruginosa* were used [23]. The examination gives first insights into the impact of a PUFA supplementation of macrophages on the internalisation rate of viable and infectious microorganisms by the immune cells. Moreover, the survival rates of the persistent pathogens inside the macrophages were elucidated [23]. Ideally, internalisation of bacteria is connected with intracellular killing of the microorganisms. Persistent pathogens as *R. equi* or *P. aeruginosa*, however, are able to resist macrophage killing mechanisms thus surviving or even replicating inside the immune cells [1-4]. An increase of macrophage microbicidal properties, therefore, is of great importance in context of chronic infections.

PUFA-supplemented RAW264.7 macrophages, which are characterized by an enhancement of membrane unsaturation, showed a marked increased in phagocytosis rates [23]. A PUFA-mediated improvement of pathogen internalisation was observed in context of an enrichment of the immune cells with fatty acids of both the n-3 (alpha-linolenic acid (C18:3n3), eicosapentaenoic acid (C20:5n3), docosahexaenoic acid (C22:6n3)) and the n-6 family (linoleic acid (C18:2n6), arachidonic acid (C20:4n6)) [23]. The boost in internalisation rate was independent from the surface structure or the virulence of the bacterial strains tested. Both the Gram-positive *R. equi* (virulent and non-virulent strain tested) and the Gram-negative *P. aeruginosa* were internalised more efficiently by PUFA supplemented macrophages [23]. In addition, PUFA enrichment of the macrophages was found to enhance the killing of the microorganisms within the first 30 min after internalisation and to impede the replication of the pathogens inside the macrophages [23]. Interestingly, the inhibiting effect of the PUFA on intracellular survival rates was evident for virulent and persistent pathogens only [23].

Taking together it was found that:

1. Enrichment of macrophages with PUFA of the n-3 and the n-6 family modulates both the number of internalised bacteria and the intracellular surviving of persistent microorganisms.

2. PUFA supplementation increases the phagocytosis rates of macrophages against viable bacteria of the species *R. equi* and *P. aeruginosa*.
3. PUFA supplementation impedes the intracellular survival of virulent *R. equi* and *P. aeruginosa*.

4. Respiratory burst due to PUFA supplementation

Reactive oxygen and nitrogen intermediates (ROI and RNI), which are produced during the respiratory burst, are of special importance for the killing of pathogens inside of macrophages [24, 25]. However, the radicals also have cytotoxic effects [26]. In particular in the course of chronic inflammations cell death and tissue damages are proposed to be at least in part due to an overproduction of ROI and RNI [27-31]. For that reason the attenuation of the radical-mediated cytotoxicity is a declared goal in the development of new therapeutic approaches to cure chronic inflammatory processes [28]. In this regard the modulation of a host`s respiratory burst by the diet has come into the focus of research [31]. PUFA, which are easily available from the food, are already described to influence the immune system [32] thus making the fatty acids suitable candidates for therapeutic purposes.

To elucidate the impact of a PUFA supplementation of macrophages on the respiratory burst of the immune cells a study has been performed in which the radical production of RAW264.7 cells was measured in context of the unsaturation index and the activation status of the macrophages [33]. At this it emerged that, in fact, the enrichment of macrophages with PUFA of both the n-3 and the n-6 family impacts the synthesis rate of ROI and RNI by the immune cells [33]. Moreover, the effects of a fatty acid supplementation on the respiratory burst were found to depend on macrophage activation status [33]. For unstimulated macrophages a significant increase in radical production was to observe due to PUFA supplementation [33]. Interestingly, significant differences in the amounts of detectable intracellular ROI and RNI were seen depending on the fatty acid tested. The higher the number of bis-allyl-methylene positions of an added PUFA, the higher the respiratory burst of the supplemented macrophages [33]. Indeed there was a positive relation between the MBI of the macrophages and the radical production of the immune cells [33]. For stimulated macrophages situation was completely different. Stimulation of RAW264.6 induced a significant increase in intracellular ROI and RNI synthesis [33]. This stimulator-induced enhancement of macrophage respiratory burst could be diminished by enrichment of the immune cells with PUFA [33]. The diminution of macrophage radical production was evident in the context of macrophage activation using both chemical stimulators as lipopolysaccharide (LPS) or phorbol-12-myristate-13-actetate (PMA) and viable bacteria as *R. equi* or *P. aeruginosa* [33].

Taking together it was found that:

1. There is a relation between the macrophage membrane lipid composition and the respiratory burst of the immune cells.
2. The consequences of a PUFA enrichment of macrophages on respiratory burst depend on the activation status of the immune cells. For unstimulated macrophages there is an increase in intracellular radical production due to PUFA supplementation. For stimulated macrophages instead there is an impeding action of added unsaturated fatty acids on the stimulator-induced respiratory burst.

5. Cytokine synthesis due to PUFA supplementation

A precise control of a host`s immune response is of high relevance for animal and human health. In this regard macrophages play a key role. The immune cells not only engulf and intracellularly destroy microbial pathogens but also participate in the regulation and activation of the innate and the adaptive immune response by the production and synthesis of pro-inflammatory as well as anti-inflammatory cytokines. Pro-inflammatory cytokines synthesized by macrophages include IL-1β, IL-6 and TNF-α [34]. The predominant anti-inflammatory cytokine produced by macrophages is IL-10 [34].

A number of cellular functions depend on the membrane lipid composition and hence the dynamic properties of the cell membrane [35, 36]. With respect to the fact that dietary fatty acids are able to modulate the lipid composition of cells including macrophages [16, 37-39] it is standing to reason that macrophage cytokine synthesis may be modulated by PUFA supplementation. In a systematic study investigating the production of the cytokines IL-1β, IL-6, IL-10 and TNF-α by PUFA enriched RAW264.7 macrophages, in fact, an interaction of PUFA of both the n-3 and the n-6 family and the cytokine synthesis by activated macrophages was shown [40]. The data gained revealed the unsaturated fatty acids to arrest the secretion of the pro-inflammatory mediators IL-1β, IL-6 and TNF-α [40]. In addition, for the n-3 PUFA docosahexaenoic acid a promoting effect on the secretion of the anti-inflammatory cytokine IL-10 was observed [40]. It is important to note that the modulation capacity of the PUFA could be seen for adequately stimulated macrophages only (LPS, viable *R. equi* or *P. aeruginosa*) [40].

Taking together it was found that:

1. PUFA of both the n-3 and the n-6 family suppress the synthesis and secretion of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α by stimulated macrophages.
2. The anti-inflammatory effects of the unsaturated fatty acids are evident in context of stimulation using both chemical agents as well as complex biological stimuli.

6. Concluding remarks

In the previous chapters the consequences of an enrichment of macrophages with PUFA on the defence reactions of the immune cells were elucidated. Using the monocyte/macrophage cell line RAW264.7 PUFA supplementation was found to:

1) Modulate the lipid composition of both rafts and non-raft membrane domains.
2) Promote the phagocytosis rate as well as the bactericidal capacity of macrophages.
3) Impact the respiratory burst in an activation-dependent manner: unstimulated macrophages – increase in radical synthesis; stimulated macrophages – impeding action of PUFA on the stimulator-induced radical synthesis.
4) Down-regulate the synthesis of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α.

Of note, the data show that mainly the carbon chain length and the number of double bonds, to a lesser extent the fatty acid family, are critical for the potency of a PUFA`s action. The particular value of the findings is based on the fact that the mentioned results were gained in context of a stimulation of the macrophages by means of viable bacteria (*R. equi* and *P. aeruginosa*). The data underline the potential of unsaturated fatty acids in the supportive therapy of chronic infections caused by persistent pathogens. On the one hand the PUFA improve the engulfment and intracellular killing of the pathogens by macrophages. On the other hand the PUFA attenuate pro-inflammatory cytokine synthesis and respiratory burst thus counteracting tissue damage due to an excessive immune reaction.

There are two different functional phenotypes of macrophages: M1 macrophages and M2 macrophages [41]. The binding of ligands, e. g. *R. equi* or *P. aeruginosa*, to macrophage Toll-like receptors (TLR) induces the polarisation of the immune cells to the M1 type. M1 macrophages preferably synthesize and release pro-inflammatory cytokines and are characterised by the ability to develop a severe respiratory burst [41]. They ideally mediate the killing of pathogens [41]. However, excessive immune reactions by M1 macrophages, which are typical of chronic infections with persistent pathogens as *R. equi* or *P. aeruginosa*, bring about tissue damages, lead to functional restrictions and favour secondary infections [28, 30]. M2 macrophages are characterised by a high phagocytic activity and preferably synthesize and release anti-inflammatory cytokines [41]. They are, thus, able to prevent destructive immune reactions linked to M1 activation. Taking into consideration the data mentioned in the previous chapters it is apparent that PUFA supplementation of macrophages leads to a polarisation of the immune cells from the M1 type to the M2 type hence contributing to the protection against an excessive immune defence.

What is the mechanism behind? So far, a final assessment cannot be given. Nevertheless, there are some remarkable knowledge building blocks that might shed some light on this issue. An increase in the proportion of unsaturated fatty acids in the membrane leads to an attenuation of the hydrophobic interactions within the lipid bilayer [35]. Basal membrane properties influenced are the fluidity, the compressibility, the permeability, melting and flip-flop mechanisms as well as the function of membrane proteins [35, 36]. It is important to bear in mind that the phospholipids are heterogeneously distributed within the membrane at this forming microdomains of specific characteristics and functionalities, the lipid rafts [17]. A number of membrane proteins, including several membrane receptors, are associated to the raft domains [17]. The rafts, therefore, play a central role as signalling platforms of cells, including macrophages [17]. The accumulation of receptor proteins in the raft domains leads to a high efficiency of signal transduction processes [17].

There are three categories of membrane proteins: *(i)* lipid raft-associated proteins, *(ii)* non-raft domain-associated proteins and *(iii)* mobile proteins, which alter their localisation between the membrane domains [42]. The lipid raft-mediated selection of membrane proteins improves the rates of specific protein-protein collisions thus resulting in an optimisation of signal transduction rates [43]. Based on mathematic models and computer simulations it was found that the collision rate of two raft-associated proteins is maximal at a lipid raft size ≤ 14 nm [42]. The incorporation of PUFA into the rafts, however, is assumed to go along with an enlargement of the membrane domains [43]. Consequently, the enrichment of the raft domains with unsaturated fatty acids might lead to a reduction in the efficiency of cellular signal transduction. Moreover, due to their high flexibility and their low affinity to cholesterol, PUFA enrichment of rafts may result in a disruption of the molecular order of the membrane domains. This might be accompanied by a modulation of membrane protein affinity to either raft or non-raft membrane domains. Membrane proteins might be displaced into or out of the lipid rafts, with the effect that protein-protein interactions, which are essential for immune cell activation, are interrupted.

Just take the activation of macrophages by *R. equi* or *P. aeruginosa* as an example. Macrophage activation follows the binding of microorganisms to membrane receptors. At this, the Gram-positive *R. equi* is known to induce macrophage stimulation predominately via the TLR2 [44], whereas the Gram-negative *P. aeruginosa* predominately

activates the TLR4 [45]. The TLR are mobile membrane receptors, which alter their localisation depending on stimulation. In the absence of stimulation, TLR2 as well as TLR4 are localised in non-raft membrane domains [46]. Upon stimulation both receptors are recruited into the lipid rafts [46]. In addition, the interaction of the TLR with other membrane-associated co-receptors is a precondition for signal transduction. TLR4 essentially forms heterodimers with CD14, which in general is localised in the lipid rafts [46]. TLR2 signals via either TLR2/TLR1 or TLR2/TLR6 heterodimers [46]. In unstimulated cells TLR1 and TLR6 are co-localised with TLR2 in the non-raft membrane domains [46]. Upon stimulation both TLR1 and TLR6 are recruited into the lipid rafts [46]. It can be speculated that the modification of the fatty acid pattern of rafts due to PUFA supplementation influences both the membrane domain localisation of the receptors and their adaptor proteins as well as the targeted interaction of the receptors with the corresponding co-receptor(s). In fact, there are indications that the supplementation of cells with the n-3 PUFA docosahexaenoic acid modulates a LPS- or LTA-induced recruitment of TLR4 and TLR2 respectively into the lipid rafts [47, 48].

Taken together, there are a multitude of hints that PUFA impact macrophage signal transduction and, hence, the defence mechanisms of the immune cells at the membrane level.

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