Biochemical Pharmacology 114 (2016) 3–13

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Review

Metabolism meets immunity: The role of free fatty acid receptors in the immune system

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ARTICLE INFO

Article history:
Received 9 February 2016
Accepted 17 March 2016
Available online 19 March 2016

Keywords:
Free fatty acids (FFAs)
GPCR
Inflammation
Obesity
Immuno-metabolism
Macrophages

ABSTRACT

There are significant numbers of nutrient sensing G protein-coupled receptors (GPCRs) that can be found in cells of the immune system and in tissues that are involved in metabolic function, such as the pancreas or the intestinal epithelium. The family of free fatty acid receptors (FFAR1-4, GPR84), plus a few other metabolite sensing receptors (GPR109A, GPR91, GPR35) have been for this reason the focus of studies linking the effects of nutrients with immunological responses. A number of the beneficial anti-inflammatory effects credited to dietary fats such as omega-3 fatty acids are attributed to their actions on FFAR4.This might play an important protective role in the development of obesity, insulin resistance or asthma. The role of the short-chain fatty acids resulting from fermentation of fibre by the intestinal microbiota in regulating acute inflammatory responses is also discussed. Finally we assess the therapeutic potential of this family of receptors to treat pathologies where inflammation is a major factor such as type 2 diabetes, whether by the use of novel synthetic molecules or by the modulation of the individual's diet.

Contents

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http://dx.doi.org/10.1016/j.bcp.2016.03.017
0006-2952/

1. Introduction

The replacement of a regular intake of healthy oils and fibres for a diet based substantially on high fat- and high sugar-content foods has had profound consequences for public health. These changes in the way that the populations of high income, particularly Western countries, manage their dietary habits have undoubtedly triggered what is now considered an epidemic of obesity that has consequently resulted in an increase in serious, chronic conditions associated with dysfunctions of energy balance, including type 2 diabetes and cardiovascular diseases [1,2]. Furthermore, it is now widely accepted that low grade chronic inflammation associated with obesity may be directly connected to other inflammatory related pathologies such as asthma, colitis and, potentially, some forms of cancer, including colon cancer [3–9].

These effects have triggered a major increase in interest with regard to the role of metabolite sensing and how this may affect physiology in health and disease, with concepts including the interface between the metabolic and immune systems, i.e. immuno-metabolism, coming to the front of scientific discussions [9,10]. There has been particular interest in free fatty acid (FFA) sensing and its association with the mode of signalling of a number of recently de-orphanised G protein-coupled receptors (GPCRs) [11]. This is a fast moving and exciting area of research focusing the interest of pharmacologists, chemists, immunologists and physiologists in an interdisciplinary manner. FFAs, including health boosting omega-3 fatty acid containing oils, are therefore no longer considered only as metabolic intermediaries but also as critical signalling molecules due to their role as agonists for different members of the family of free fatty acid receptors (FFARs) [12–16]. Although widely expressed, their presence on key cell types regulating both metabolic and immune health acts to link the regulation of energy homoeostasis with the control of inflammatory responses [17,18].

FFARs are therefore now considered very attractive targets for the development of either novel medicines or novel strategies to treat both metabolic and inflammatory pathologies. However, as they are a relatively newly described group of receptors there remain a substantial number of open questions with regard to their function and the roles that they subserve. This review maps out the key players and connections between FFAs that are obtained through the diet or as a result of the actions of the gut commensal microbiota and the immune system and addresses how further understanding of these systems might be used to limit or treat disease.

2. Overview of the family of free fatty acid receptors

2.1. Free fatty acids

The basic structure of a 'free' fatty acid, i.e. one unbound or non-esterified within larger species such as triglycerides or phospholipids, is a carboxylic acid linked to an aliphatic chain of variable length that may be saturated or unsaturated. As such, fatty acids are widely classified based on the length of their carbon chains and grouped into short chain fatty acids (SCFAs, C2–C6), medium chain fatty acids (MCFAs, C7–C12) and long chain fatty acids (LCFAs, >C12). These may have a number of different origins. Most of the 'essential' fatty acids such as linoleic acid (18:2, n-6) or alpha-linolenic acid (18:3, n-3), which humans cannot synthesise directly, and other LCFAs and MCFAs, are generally obtained through the diet [19,20]. Some other FFAs are obtained through the breakdown of fats (triglycerides) in adipose tissue (AT) and the liver. By contrast the vast majority of SCFAs including acetate (C2) and propionate (C3) are derived from the fermentation of fibres and breakdown of dietary carbohydrates by the bacteria present in the gut [21,22]. As will be discussed further, there is mounting evidence to support a central role for the gut microbiota in the regulation of energy homoeostasis and its impact in inflammatory processes [23–25].

2.2. Free fatty acid receptors

FFARs are members of the 'rhodopsin-like' GPCR family and currently four receptors (FFAR1-4) are so classified. FFAR1, 2 and 3 are closely related in terms of sequence and are co-located (on chromosome 19q13.12 in humans) [26]. Between the coding regions for FFAR3 and FFAR1 (formerly GPR41 and GPR40 respectively) in this chromosome there is a further sequence which was considered initially as a likely pseudogene [27]. However in recent times, analysis of this allele, which is designated as GPR42, has indicated that it may be active in many individuals [28]. It has clearly arisen from a tandem duplication of the *FFAR3* gene as the sequence differs in only 6 amino acids. FFAR2 (formerly GPR43) appears as the last FFAR in the group at this genomic location. In contrast to this group of tandemly encoded sequences, FFAR4 (formerly GPR120) is located on chromosome 10 (10q23.33) in humans and displays little overall homology with the other FFARs. Nevertheless, it was identified as a receptor for LCFAs in 2005 [29]. Previous high-throughput screening and more focussed programmes had led to the de-orphanisation of FFAR1-3 [27,30–33] and these showed that FFAR2 and FFAR3 respond to SCFAs of carbon chain length C2–C6, displaying various potencies for the different ligands [27,33]. In contrast both FFAR1 and FFAR4 are activated selectively by LCFAs. Potentially of importance, although yet to be fully explored, human FFAR4 is produced as two isoforms which differ structurally by a 16 amino acid insertion into the third intracellular loop of the long isoform [34,35]. This is not present in other species investigated to date and although these two isoforms differ in their downstream signal transduction (see Section 2.3 and Table 1) the importance of this for overall function remains unclear, not least because the long isoform has only been detected in a limited number of tissues [35,36].

Although now widely accepted, there was initially debate as to whether the low potencies observed for most FFAs at their receptors, for example propionate displays high microM to low milliM potency at FFAR2 and FFAR3, could be reconciled with them being the true endogenous ligands for the receptors [37]. However, levels of SCFAs in the gut or plasma can reach high milliM levels [31,38–41]. Moreover, LCFAs, although generally displaying low microM potency in *in vitro* assays at FFAR1 and FFAR4, are highly plasma protein bound, e.g. to serum albumin, and it remains uncertain how this affects presentation of the ligands to the relevant receptors *in vivo* or what are the true available ligand amounts.

The modest potency that most FFAs display at their target receptors, and the small chemical size of the SCFAs, has resulted in a view, although atomic level structures of these receptors are not yet available, that the ligand pocket of FFAR2 and FFAR3 must also be small [42]. This has resulted in challenges in terms of the development of novel selective and potent synthetic ligands at these receptors [43]. However, the FFAR2 antagonist GLPG0974 did enter first-in-man clinical trials, even if these were rapidly abandoned, as a potential treatment for the lower gut inflammatory condition ulcerative colitis [44] (see Section 3.1.1 for further details). By contrast, in part because of the clear potential for regulation of glucose homoeostasis and, therefore diabetes, by targeting the receptors for LCFAs, the development of ligands for these receptors is significantly more advanced [44]. For example, the FFAR1 receptor agonist TAK-875/fasiglifam progressed into phase III clinical trials and was able to reduce blood glucose levels, increase insulin levels, and to cause a significant 1.2–1.4%

reduction in HbA1c levels with no associated weight gain/hypoglycaemia in type 2 diabetics. It was, however, withdrawn from these trials due to potential liver toxicity. No selective FFAR4 ligands have yet advanced into clinical trials, but a number have also been shown to have positive effects on blood glucose and insulin levels in rodent models of diabetes and obesity [44].

Although remaining officially an orphan receptor, GPR84 is a further potential receptor for FFAs. In recent years this GPCR has been shown to be activated by a group of MCFAs [45]. As with FFAR4, this receptor is only distantly related to other FFAR family members and was originally isolated using an *in silico* EST data mining strategy [46] designed to identify GPCR sequences that were divergent in sequence from those identified previously. Although the chain length selectivity of fatty acids at GPR84 is very narrow [45], meaning that it is activated only by a small group of fatty acids, various synthetic molecules have been shown to promote activation [44] and, therefore, provide a wider pharmacology to explore the functions of this receptor.

2.3. Signal transduction and expression of FFARs

The most common feature of GPCRs is their ability to signal through the activation of heterotrimeric G proteins (Table 1). The four FFARs are no exception and show diverse coupling preferences to G proteins as well as also promoting a variety of G protein-independent pathways. Both of the SCFA receptors, FFAR2 and FFAR3, have an established preference for coupling to pertussis toxin-sensitive $G\alpha_{i/o}$ G proteins that results in inhibition of adenylyl cyclase and a reduction in cAMP production. However, FFAR2 is more pleiotropic than FFAR3 and is also able to couple to $G\alpha_{q/11}$ G-proteins with subsequent elevation of intracellular calcium [47]. As well as sharing coupling to $G\alpha_{i/o}$ G proteins FFAR2 and FFAR3 have overlapping patterns of expression as both can be found in adipocytes, pancreatic islets and in several incretin-releasing enteroendocrine cells such as K cells (gastric inhibitory polypeptide (GIP) release), I cells (cholecystokinin (CCK) release), and L cells (glucagon-like peptide-1 and peptide YY (GLP-1 and PYY) release) [27,43,48,49]. In recent years immunologists and computational biologists have joined forces to compile gene expression profiles of immune cell populations from mice in efforts to define potential inflammatory profiles and targets. Data from this consortium (www.immgen.org) indicate two key populations of immune cells where FFAR2 is heavily expressed: neutrophils and mesenteric and small intestine dendritic cells (DC). More specifically FFAR2 expression is found in $CD11b^+$ neutrophils from blood and synovial fluid and resident macrophages from adipose and lung tissues also show a relatively high level of FFAR2 expression. FFAR3 expression can be found in sympathetic ganglia [48] and the enteroendocrine cells mentioned above [27,43,48,49], although datasets from the Immunological Genome Project also indicate expression broadly but at low levels in white cells, in DCs and some subclasses of neutrophils (Table 1).

FFAR1 is found in pancreatic islets, with particularly enriched expression in the β-cells which produce and release insulin and, therefore, its function has been linked to the modulation of glucose-stimulated insulin release [31,50,51]. This receptor is expressed together with FFAR4 in enteroendocrine cells of the gastrointestinal tract where it mediates FFA-stimulated incretin secretion [52–54]. FFAR4 is also found in the pancreas, where expression is suggested to be restricted to the δ-cells [55], in adipocytes, lung [35,56] and in immune cells, particularly $CD11c^+$ bone marrow-derived macrophages and intra-peritoneal-derived macrophages [57]. The distribution of FFAR4 expression is supported by reports available via the Immgen database, where FFAR4 expression is also noted to be present in a number of types of DCs (mesenteric, lung $CD11b^+$, small intestine) and, in particular, in lung-resident macrophages (Table 1).

Macrophages are generally found as heterogeneous mixed populations and based on gene expression patterns have been categorised into two activation/polarisation states, namely classic (M1) or alternative (M2) [58–60]. The release by Th1 helper cells of inflammatory cytokines such as interferon-gamma (INFγ) during the initial phases of acute inflammation promotes polarisation into activated M1 macrophages, whereas anti-inflammatory cytokines (IL-4, IL-13), released by Th2 helper cells, drive polarisation towards the M2 phenotype [57,61,62]. This leads towards the resolution of the inflammatory process via the activation of peroxisome proliferator-activated receptor gamma (PPARγ). Expression of FFAR4 in different types of macrophages (monocytes, Kupffer cells in the liver, osteoclasts in the bone, resident macrophages in the lung) and its potential to regulate inflammation (see Section 3.2.2) make this receptor a very exciting target for the development of novel compounds to treat metabolic syndrome and its many ramifications [14,63–65] (Table 1).

As previously alluded to, G protein-independent signalling mechanisms are often important for GPCR function and although FFAR1 and FFAR4 signal predominantly via $G\alpha_{q/11}$ G proteins [30,31,50,54] they are also able to signal via the recruitment of β-arrestin-2 [66–69]. β-Arrestins are scaffold proteins that historically have been studied largely in relation to their capacity to induce receptor desensitisation and internalisation processes that frequently follow receptor activation by agonists [70–75]. However, it is now abundantly clear that β-arrestins also function as part of alternative signalling cascades [70,73,76–78], leading to the activation of a myriad of effector enzymes, including protein kinases of the mitogen activated family such as the extracellular-signal regulated kinases 1 and 2, the c-Jun kinases and the stress-activated p38 kinases [79–81]. Of the four FFARs, FFAR3 appears to be the one with the least propensity for β-arrestin recruitment in response to ligand activation as there are currently no reports to this effect. Nonetheless, signalling through this arm seems to be important for the function of the other three receptors, especially for FFAR4 where the divergence between G protein- and β-arrestin-dependent pathways seems to be cell type dependent and critical for the regulation of metabolic or inflammatory processes [57]. It is worth noting that the long isoform of FFAR4 that can be found in human colon [36] is reported to almost exclusively engage with the β-arrestin pathway, leading to receptor internalisation when over-expressed in a recombinant system whilst it seems to be impaired in G protein coupling as evaluated in intracellular Ca^{2+} release and dynamic mass redistribution (DMR) assays [29,30,82]. This may reflect that the 16 amino acid insertion that produces the long isoform is located within the third intracellular loop, an important element in receptor function. Although the insertion introduces additional potential phosphorylation sites this does not to have a substantial effect on the overall capacity for β-arrestin recruitment [34,82]. Agonist-induced phosphorylation and subsequent recruitment of β-arrestin largely reflects post-translational modification of amino acids within the C-terminal tail of FFAR4 [83,84] which is identical between the isoforms (Table 1).

3. The role of free fatty acid receptors in metabolism and immune responses

3.1. Short-chain free fatty acid receptors in immune cell signalling and regulation

Although both SCFA receptors, FFAR2 and FFAR3, are co-expressed in certain cells and tissues, such as pancreatic α and β cells and some enteroendocrine cells, as noted earlier, FFAR2 in

Table 1
Summary of FFA receptors, signalling properties and functions.

Receptor	Agonists	Signalling	Immune cells	Function Immune system	Other functions tissues/cells
FFAR1	LCFA (>C12) Natural: palmitic acid, oleic acid, pinolenic acid, αLA, DHA Synthetic: GW9508, TAK-875/Fasiglifam, AMG-837, AM-5262, TUG-424, TUG-770	$G\alpha_{q/11}$[#] $G\alpha_{i/o}$ β-arrestin-2	–	–	Improved GSIS Increased incretin release Enteroendocrine L, K and I-cells Pancreatic β-cells Taste buds
FFAR2	SCFA (C2–C6) Natural: formate, acetate[#], propionate, butyrate Synthetic: 4-CMTB	$G\alpha_{i/o}$[#] $G\alpha_{q/11}$	Neutrophils, intestinal Treg cells, eosinophils, macrophages, dendritic cells	Regulation of inflammatory processes in the gut Control of epithelial integrity Neutrophil chemotaxis	Epithelial colonic cells Adipocytes Enteroendocrine L, K and I-cells Pancreatic α and β-cells
FFAR3	SCFA (C3–C7) Natural: acetate, propionate#, butyrate Synthetic: AR420626	$G\alpha_{i/o}$	–	Regulation of inflammatory processes in airways	Enteroendocrine cells Sympathetic ganglia
FFAR4 (short)	LCFA (>C12) Natural: αLA, DHA, EPA Synthetic: GW9508, TUG-891, Compound A, NCG 21	$G\alpha_{q/11}$ β-arrestin-2	Dendritic mesenteric, lung $CD11b^+$ cells, small intestine cells, bone marrow-derived and intra-peritoneal derived macrophages, Kupffer cells, eosinophils	Anti-inflammatory effects in the gut and adipose tissue	Incretin mediated regulation of insulin secretion (increased GLP-1 release) Adipocytes Enteroendocrine L, K and I-cells Pancreatic δ cells Clara/Club lung cells Taste buds
FFAR4 (long)	LCFA (>C12) Natural: αLA,DHA, EPA Synthetic: GW9508, TUG-891	β-arrestin-2	–	–	Increased incretin release (GLP-1) Enteroendocrine L-cells
GPR84	MCFA (C7-C12) Natural: capric/decanoic acid, undecanoic acid Synthetic: DIM, 6-OAU	$G\alpha_{i/o}$	Macrophages, neutrophils, T-cells, glial cells	–	Adipocytes Sciatic nerve Microglia

[#] Preferred; αLA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DIM, diindolylmethane; 6-OAU: 6-n-octylaminouracil; GSIS: glucose stimulated insulin secretion. See text for details on references.

particular is also highly expressed by a number of white cell types, including neutrophils, eosinophils, monocytes and, quite importantly, epithelial colonic cells, where it may play a role in maintaining epithelial integrity and in intestinal Treg cells [85–90].

3.1.1. Roles of FFAR2 receptor in inflammatory processes related to the gut and other tissues

There is a growing belief that a healthy gut microfloral population can positively influence immune responses such that the individual might be protected from the development of inflammatory pathologies such as ulcerative colitis, arthritis or even asthma, with a growing number of studies linking general wellbeing and healthy gut function with highly diverse intestinal microbiota and intake of fibre in the diet [1,91–93]. Bacteria belonging to the *Bacteroidetes* and *Firmicutes* phyla have been found to produce high levels of the SCFAs C2–C4 which are the main agonists for FFAR2. Levels of these SCFAs in the intestinal tract can vary depending on the bacterial composition within individuals and dietary habits but they reach the systemic blood circulation in sufficiently high levels to activate these receptors. However, the molecular link between microbiota SCFA production, FFAR2 function and immune regulation had not been clear until recently. In studies carried out using FFAR2 knock-out and germ-free mice, Maslowski and colleagues [94] showed that FFAR2 was necessary for the resolution of a number of inflammatory responses in models of colitis, asthma and arthritis. Chemically-induced colitis in germ-free mice that lack the ability to produce any SCFAs due to the absence of the gut microbiota resulted in an exacerbated inflammatory phenotype that could be all but reverted to that of the wild type by supplementing the drinking water with 150 mM acetate. This was accompanied by a reduction in levels of inflammatory mediators such as tumour necrosis factor alpha (TNFα), myeloperoxidase (MPO) and the chemokine CCL3. On the other hand, inflammation was not resolved in the FFAR2 knock-out mice, providing further evidence that the effect observed in the germ-free mice treated with acetate was FFAR2 mediated [94]. This report and others also showed that neutrophil chemotaxis to SCFA agonists in a transwell-based Boyden chamber was lacking in cells produced from FFAR2 deficient mice [87,94]. At the molecular level this chemotactic effect is likely to reflect the activation of the p38 MAP kinase cascade, a pathway known to mediate chemotaxis in cells of the immune system [87,95]. Despite the elegant studies of Maslowski et al. [94] conflicting results have been reported. For example, [87] noted a detrimental effect of FFAR2 on polymorphonuclear leucocyte (PMN) infiltration that could lead to more severe tissue damage and, therefore, less receptor-mediated beneficial effects. This clearly poses challenges for the potential development of FFAR2 selective medicines for the treatment of intestinal inflammatory pathologies such as irritable bowel disease (IBD) because it is unclear from the above whether an FFAR2 antagonist might be beneficial or contra-indicated. One potential scenario is that if inflammatory processes induced by infiltration of PMNs and other immune cells in response to cytokines are driven by the activation of FFAR2 then inhibition of this receptor would prevent this from happening. As noted earlier this hypothesis was assessed recently by the development and clinical trial of the FFAR2 antagonist GLPG0974. However, no improvements of clinical symptoms were reported during a 4 week treatment period, resulting in the termination of the study. Nevertheless, in pre-clinical studies this molecule was able to efficiently inhibit acetate-induced chemotaxis of human neutrophils *in vitro*, to block acetate-mediated elevation of intracellular calcium in HEK293 cells transfected to express FFAR2 with potency in the nanoM range and to regulate expression of CD11b, an activation specific marker (CD11b[AE]) used as an indicator of neutrophil adhesion to the endothelium and extravasion towards inflamed tissues [96]. It may be, therefore, that current understanding of the biology of FFAR2 is incomplete or over-simplistic. Moreover, there may well be significant species variability in disease pathology and progression and because GLPG0974, although a high affinity antagonist of human FFAR2, does not show significant affinity at mouse or rat FFAR2 [97], no pre-clinical effectiveness in these species could be assessed prior to the first-in-man trials.

FFAR2 function has also been linked to non-gut related inflammatory processes such as gouty arthritis. Vieira and co-workers have suggested that FFAR2 is responsible for the amplification of acute inflammation in a rodent model, through the regulation of NLRP3 inflammasome assembly [98]. FFAR2 expression was upregulated in the knee tissue of mice injected with monosodium urate monohydrate (MSU). Furthermore FFAR2 knockout mice showed significantly less IL-1β production and subsequent neutrophil migration to the MSU injected joints. These authors proposed the idea that the gut microbiota and its products might shape the ability of the host to respond to inflammasome-mediated processes [98]. However, most of the data were only indirectly related to signalling from FFAR2 and hence caution is required in interpreting these data. Despite this, the fact that FFAR2 expression can be readily detected in the neutrophils of synovial fluid may indicate a role for the receptor in the joints. Nonetheless, this highlights ideas that may lead to new investigations in NLPR3 inflammasome biology and also of FFAR2-mediated disorders related to diet-induced pathologies [99].

From a molecular perspective, implications of interactions between FFAR2 and β-arrestin-2 are poorly understood [100,101]. However, FFAR2 can clearly interact with β-arrestin-2 (there is less information on interactions with β-arrestin-1) and in so doing can modulate the activity of nuclear factor κB (NF-κB) [101]. Although a full description of how the receptor might regulate this pathway is uncertain it may provide a novel avenue to study further potential anti-inflammatory effects of FFAR2.

3.1.2. FFAR3 is a key player in airway inflammation

FFAR3 may also be a key player in inflammatory processes. In airway inflammation the intestinal microbiota composition, fibre content of the diet and subsequent SCFA production can have a profound impact on the immunological makeup of the lungs and has been suggested to regulate inflammation during induced allergic asthma [102]. A relation between dietary fibre, FFAR3 function and susceptibility to allergic airway inflammation was found in studies in which mice were provided with either a regular content of fibre in their diets (~4%) or fed low-fibre diets (<0.3%). Animals maintained on low-fibre diets displayed an increase in white cell infiltration in the lungs as well as the production of a variety of cytokines (IL-4, IL-13, IL-5, IL-17A) following airway inflammation induced by the administration of house dust mite (HDM). These effects were reversed by changing diet to contain the highly fermentable fibre pectin [102]. Further experiments to elucidate the mechanisms by which SCFAs derived from the dietary fibre control airway inflammation involved treatment of wild type and FFAR2- or FFAR3-deficient mice with C3, (which can activate either receptor), prior to exposure to HDM. A reduction in inflammation markers (e.g. eosinophil infiltration, goblet cell hyperplasia) was observed in the case of the wild type and FFAR2-deficient mice treated with C3 but not the FFAR3 knock-out animals, where there were no differences between the SCFA-treated and un-treated groups. Notably, C3 changed the balance between resident DCs found in the lung and those that migrate to it after the onset of inflammation. It is possible that precursors of macrophages and DCs in the bone marrow migrate to mitigate the Th2 pro-inflammatory allergic response driven by the presence of SCFAs acting on FFAR3.

The role of FFAR3 in patients suffering from cystic fibrosis (CF) has also been examined as this group is reported to have large populations of anaerobic bacteria present in the lungs due to the hypoxic environment developed under this condition [103,104]. These studies confirmed that the lungs are far from sterile and contain sufficient anaerobic bacteria, able to produce SCFAs at milliM concentrations, as detected in the broncho-alveolar fluid from CF patients. It remains unclear, however, how these bacteria come in contact with potential substrates to produce the SCFAs. These studies also demonstrated that CF patients display elevated levels of FFAR3 expression [103] and that bronchial epithelial cells from this group were able to release more of the pro-inflammatory cytokine IL-8 upon stimulation with SCFA compared to non-CF patients. siRNA knock-down of FFAR3 reduced production of the cytokine [103].

Although this is a rapidly developing area it is already clear that FFAR2 and FFAR3 provide the molecular targets within an emerging gut-lung axis that regulates the balance between pro- and anti-inflammatory processes between the two mucosal tissues and this may be influenced greatly by the diet and composition of the intestinal microflora.

3.2. Medium and long-chain free fatty acid receptors in immune cell signalling and regulation

3.2.1. Roles of GPR84, a newly described MCFA receptor, in immunity

The MCFA receptor GPR84 displays an interesting tissue distribution, found mostly on cells of the immune system such as neutrophils, T-cells, macrophages, including glial cells [12,105–107]. GPR84 expression is also found in adipocytes [105,108] and although still poorly defined, its function has been linked to pro-inflammatory phenotypes [108–111]. Expression of GPR84 has also been found by PCR studies in the spinal cord and sciatic nerve of mice, which has triggered some investigations into a putative role in pro-nociceptive pathways [112]. According to some the role GPR84 is directly connected to immune function and metabolic dysregulation [45,108,110]. GPR84 expression is upregulated in macrophages by the presence of lipopolysaccharide (LPS) [45,106] where it also seems to mediate the release of the pro-inflammatory cytokine IL-12. IL-12 and its regulation of the balance (Th1/Th2) of T helper responses play an important role in inflammatory diseases such as rheumatoid arthritis and IBD, and a potential role of GPR84 in this context has been supported by evidence that shows an increase in Th2 (IL-4) cytokine production in T cells derived from GPR84 knock-out mice [111]. Furthermore, LPS-stimulated macrophages derived from GPR84 knock-out mice show markedly reduced expression of several pro-inflammatory mediators [112].

Whilst GPR84 is, to date, the least studied of the FFA-regulated GPCRs, members of the family that respond to LCFAs have been more widely studied and their links to immune function are becoming better established. FFAR1 and FFAR4 can be activated by a broad range of both saturated and unsaturated LCFAs [113] but there has been a special focus on FFAR4 and the actions that both omega-3 and omega-6 PUFAs may exert via this receptor. Omega-3 fatty acids, such as docosahexaenoic acid (DHA) present in high levels in fish oils and α-linolenic acid present in some vegetable and nut oils, have increasingly been promoted as healthy supplements and dietary sources rich in these fatty acids have become fashionable "super-foods" in recent years [113–116]. This has been supported by a number of studies that suggest a link between inflammation and saturated fats, as these promote inflammation via Toll-like receptors [117], and the protective, anti-inflammatory effects of omega-3 fatty acids such as DHA on adipoctyes and macrophages [57,62,118–120]. Although FFAR4 responds in very similar ways to saturated, omega-6 and omega-3 fatty acids when expressed in recombinant systems, in cells expressing endogenous levels of the receptor, such as the murine enteroendocrine cell line STC-1, it is reported that only the omega-3 LCFAs are able to produce convincing evidence of FFAR4-mediated beneficial effects, such as release of the incretin GLP-1 [29]. Moreover, evidence supporting an anti-inflammatory role for the receptor has been shown in the model macrophage-like cell line RAW264.7 where relatively high levels of FFAR4 are expressed. Here, stimulation with the synthetic agonist GW9508, which activates FFAR4, is able to inhibit LPS-mediated release of the inflammatory cytokines TNF-α and IL-6 [57].

3.2.2. FFAR4 receptor: the paradigm of an anti-inflammatory mediator with important metabolic consequences

The role of FFAR4 in metabolic function has been analysed in detail using FFAR4 deficient mouse models [121,122]. When FFAR4 knock-out mice were subjected to a high fat diet, they developed obesity, glucose intolerance and fatty liver to a higher degree than wild type animals. These mice displayed exacerbated insulin resistance and, furthermore, they had a higher proportion of pro-inflammatory infiltrated macrophages in the AT [122]. In humans it has been shown that FFAR4 expression is upregulated in the AT of obese individuals. Furthermore, in a French population, those carrying a single amino acid variation (reported as Arg270His, which corresponds to Arg254His in the more common short isoform) in the FFAR4 gene are reportedly at a higher risk of developing obesity [122]. Although these findings suggest a potentially causative link between obesity, the development of insulin resistance and a fundamental role for the nutrient-sensing FFAR4 as a protective element [122], this polymorphism is uncommon in other populations, being virtually absent in a Japanese study and a more recent study noted a lack of linkage to the risk of type 2 diabetes or variation of fasting insulin levels [123]. Despite this, FFAR4 expression is found in isolated AT stromal vascular fractions, that contains macrophages that can account for as much as 40% of the total number of cells in obese AT [124], and it is also found in the resident hepatic macrophages, known as Kupffer cells, in high fat diet-induced obese mice [57,62,125]. In summary, the presence of FFAR4 in macrophages and its role in regulating their ability to migrate to other tissues, coupled with the balance of macrophage phenotypes have resulted in a number of proposals that link these phenomena to the function of FFAR4 and the pathological ramifications of obesity.

Other evidence supports that underlying chronic inflammation experienced by the adipose and hepatic tissues of obese individuals may lie at the core of the development of type 2 diabetes [126–128]. Inflammatory responses should be considered as multistep processes. In general, the onset of an inflammatory response comprises an increase of tissue vascularisation, followed by enhanced permeability of the vascular wall that allows the endothelial crossing of large molecules (chemo-attractants, cytokines, chemokines). Subsequently, the migration of white cells to the site of inflammation in response to released mediators resumes and there is the possibility of either resolution of the inflammation through the production of resolvins or maintenance of the inflammatory signals (prostaglandins, leucotrienes, cytokines, chemokines) depending on the tissue and cells involved in the process [8,129,130]. The infiltration of cells of the immune system, including macrophages, towards the hypertrophic AT is prompted by the release of cytokines and chemokines (e.g. MCP-1, LTB4) [131]. The infiltrated activated macrophages release more cytokines (e.g. TNF-α) [128,132,133] that may have paracrine effects on neighbouring cells and endocrine actions on insulin-target cells (e.g. adipocytes, hepatocytes and muscle cells). The maintenance of this cycle of pro-inflammatory signals in the longer term promotes the development of insulin resistance [134]. From a molecular perspective,

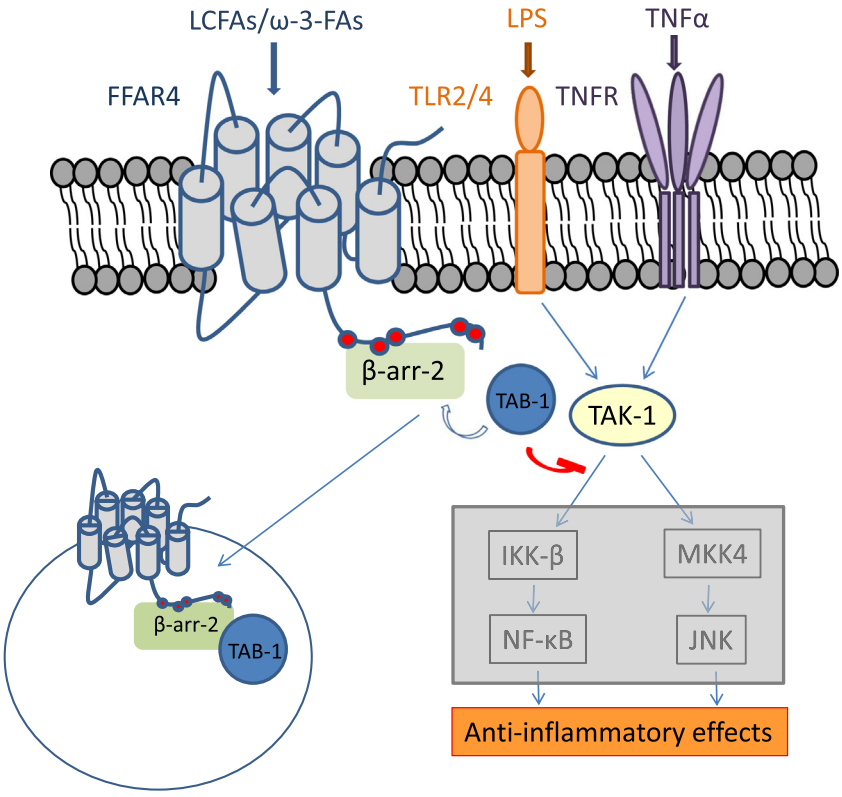

Fig. 1. FFAR4-mediated anti-inflammatory effects in macrophages. Macrophages expressing FFAR4 respond to omega-3 fatty acids that activate the receptor eliciting phosphorylation of the carboxyl-terminal tail (represented by red dots), subsequent β-arrestin-2 recruitment and receptor internalisation. At this point the receptor-β-arrestin-2 complex interacts with the TAB-1 protein, that is taken into the endocytic vesicle, making it unavailable to activate the TAK-1 protein responsible for the transduction downstream of signalling coming from activated Toll-like and TNFα receptors. This further blocks the NF-κB/JNK cascades, therefore inhibiting inflammatory responses in these cells. (LCFA, long chain fatty acid; ω-3-FAs, omega-3 fatty acids, TLR2/4, Toll-like receptors 2 and 4, TNFR, TNFα receptor).

the signalling pathways leading to inflammatory cytokine release are regulated via the actions of LPS and TNF-α on Toll-like receptors (TLR2 and TLR4) and tumour necrosis factor receptor (TNFR) respectively and subsequent activation of the I kappa B kinase-β (IKK β)/NF-κB and JNK cascades [135,136]. The activation of IKK β-dependent pathways in macrophages has been shown to be sufficient for the development of insulin resistance in obesity due to a central role of this kinase as controller of many inflammatory responses. Studies using mice that lacked functional IKK β protein in either hepatic cells or in myeloid cells where NF-κB activation induces inflammatory mediators that cause insulin resistance, showed that IKK β plays a central role in the development of insulin resistance at a systemic level, as mice that had lost IKK β function in bone marrow-derived myeloid cells (Ikbkb[Δmye]) retain insulin sensitivity and are less prone to develop obesity [137].

Agonist stimulation of FFAR4 mediates signalling through $G_{q/11}$ activation or through its interaction with β-arrestin proteins but it is the G protein-independent signalling pathway that is believed to be responsible for the anti-inflammatory properties of the receptor in macrophages (Fig. 1). Relatively recent studies show that inflammatory processes in macrophages are regulated via the activation of FFAR4 [57,62]. This is a β-arrestin-2 dependent process and places FFAR4 upstream from a common node of both IKK β /NF-κB and JNK pathways [57,62]. Mechanistically it has been proposed that FFAR4 activation and concomitant recruitment of β-arrestin-2 promotes further interactions between β-arrestin-2 and TAB-1, the transforming growth factor kinase protein (TAK-1) binding protein, which acts as activator of TAK-1. This receptor mediated β-arrestin-2-TAB-1 interaction is suggested to prevent the formation of a TAB-1/TAK-1 complex and halts the subsequent relay of signalling that results in the inflammatory response (Fig. 1) [57]. If either FFAR4 or β-arrestin-2 were reduced in amount, produced via introduction of siRNA against these proteins, there was an increase in LPS-mediated TNF-α release after stimulation with the omega-3 fatty acid and FFAR4 agonist DHA in RAW264.7 cells [57]. β-Arrestin-2 pull-down experiments demonstrated the physical interaction between β-arrestin-2 and the FFA4 receptor and also with TAB-1 in both cells heterologously expressing these proteins, and in RAW264.7 cells, after stimulation with DHA, or DHA plus LPS respectively [57]. Similar results have been reported using a synthetic agonist of FFAR4 to replace the fatty acid [57]. Compound A (cpdA), is bioavailable and has reasonable potency at FFAR4 but lacks activity at FFAR1 [62]. This molecule produced anti-inflammatory effects in macrophages in both *in vitro* and *in vivo* models [62]. Treatment of primary macrophages with cpdA in conjunction with LPS inhibited phosphorylation of many of the previously discussed phospho-regulated kinases that are typically activated in inflammatory processes (e.g. p-IKK, p-JNK, pTAK-1) [62]. Furthermore, treatment with this compound blocked chemotaxis of macrophages towards adipocytes in co-culture experiments employing adipocyte-derived conditioned medium and this was lacking if the macrophages were derived from an FFAR4 knock-out mouse [62]. This is clearly consistent with FFAR4 activation regulating macrophage infiltration into AT [62]. Although, as discussed earlier, FFAR4 does not respond only to omega-3 fatty acids [66,113] studies with mice sustained on omega-3 fatty acid-enriched diets also showed decreased macrophage chemotaxis and infiltration into AT in general and a decrease in inflammatory markers typically found in M1 polarised macrophages [57,138]. Equivalent experiments performed *in vivo*, where isolated wild type circulating monocytes were labelled with a fluorescent dye and were re-injected into either wild type or FFAR4

knock-out mice which were subsequently maintained on a high-fat diet, and omega-3-enriched diet or treated with cpdA showed a marked decrease in the AT infiltrated macrophages in wild type animals that were treated with either omega-3-enriched diet or with cpdA, whilst there were no differences in the FFAR4-deficient mice [62]. This once more highlights the role of FFAR4 in mediating macrophage infiltration of AT.

This is of significance as the majority of the infiltrated macrophages found in obese AT tend to be $CD11c^{+}$, M1-like, macrophages [124], due to the nature of the adipokines and cytokines released in this tissue, whereas alternatively activated M2-macrophages tend to reside in healthy AT [58,139,140]. Moreover, a new subtype of macrophage, that differs from the M1 phenotype, has been recently described that develops under metabolic activation conditions (high FFA concentrations, high glucose and insulin) and is associated with a pro-inflammatory phenotype [59]. This metabolically-activated macrophage has been shown to express distinct surface marker proteins and is able to secrete pro-inflammatory cytokines including IL-1β, in a PPARγ-dependent manner [59]. However, to date, expression patterns of the FFAR receptors, and more specifically of FFAR4, has not been shown for this subtype.

Based on the foregoing, selective small molecule agonists of FFAR4 might be useful tool compounds to assess effects of limiting macrophage migration into the AT and to regulate the phenotypical switch between M1/M2 macrophages that has been linked to the development of further inflammation and insulin resistance [139], as well as having an insulin sensitising effect.

Further supporting a central role of FFAR4, and of FFAR1, in anti-inflammatory processes there have been a number of studies describing the mechanisms by which omega-3 fatty acids suppress inflammation by inhibiting the activation of the NLRP3 inflammasome acting predominantly via an arrestin-FFAR4 dependent pathway [140]. This provides a novel anti-inflammatory mechanism of action of FFAs, in particular omega-3 fatty acids. Activation of the NLPR3 inflammasome in response to either pathogens or danger associated molecular patterns triggers a caspase-1-dependent cascade that leads to the release of pro-inflammatory cytokines such as IL-1β, IL-33 and IL-18. This increase in levels of pro-inflammatory cytokines has been linked to the development of insulin resistance in chronic hyperglycaemic conditions, and elevated levels of IL-1β have been found at the onset of type 2 diabetes in obese individuals [141]. Moreover, the NLRP3 inflammasome is not solely involved in host defence but also in auto-inflammatory disorders, therefore further insights into how these processes are regulated will be key to developing novel therapeutics. The studies described in [140] show that both the natural ligand DHA and the small synthetic agonist molecule GW9508, acting at either FFAR1 or FFAR4, are able to suppress caspase-1 activation and IL-1β and IL-18 secretion in LPS-primed bone marrow-derived macrophages, where expression of FFAR4 in particular is high. Equivalent experiments performed in human monocytic THP-1 cells confirmed these findings and additional studies in this cell line when either FFAR1 or FFAR4 expression was knocked-down using shRNA technology demonstrated an additive effect of both receptors in the inhibition of inflammasome activation and suppression of the release of IL-1β. This has led to suggestions that the high level of inflammation found in AT reported as part of the phenotype of FFAR4 knock-out mice [122] reflects failure to inhibit the NLRP3 inflammasome actions in this context [140]. It was also noted in this work that silencing of β-arrestin-2 expression, a critical component of the downstream FFAR4 anti-inflammatory pathway, in THP-1 cells cancelled the inhibitory effect of DHA on NLRP3 and NLRP1b (another subtype of inflammasome complex) activation. Furthermore β-arrestin-2 forms a complex with either NLRP3 or NLRP1b after DHA treatment but not with some of the other members of the inflammasome proteins (NLRC4 or AIM2). Finally, it was shown in wild type mice fed a high-fat diet that there were improvements in insulin resistance when the diet was supplemented with DHA but this was without effect in $Nlrp3^{-/-}$ mice. On this basis, the authors proposed that omega-3 fatty acids are able to block metabolic stress and inflammation derived from the activation of the NLRP3 inflammasome and potentially have beneficial effects on high-fat-induced insulin resistance [140].

Expression of FFAR4 is not restricted exclusively to macrophages/monocytes as recently expression of this receptor has been described in human eosinophils isolated from peripheral venous blood and visceral AT [142,143]. This class of granulocyte is normally found at low levels in the circulation but it can accumulate in certain tissues, such as the gastro-intestinal tract and the lungs. Eosinophils can play a positive role in clearing infection, but in pathologies such as asthma, they have typically been associated with Th2-mediated airway inflammation [144,145]. Eosinophils are also present in visceral AT under normal conditions, where they help maintain glucose homoeostasis through the stimulation of alternative polarisation of macrophages in a Th2-dependent manner through the release of IL-4 [143]. Konno and co-workers reported a strong down-regulation of FFAR4 expression on the surface of isolated human blood eosinophils after treatment with the FFAR4 active agonist GW9508. This is anticipated as rapid agonist-induced internalisation of FFAR4 has been reported in many settings [66,82,146]. The role of FFAR4 in eosinophil chemotaxis remains uncertain, however, but the receptor may play a role in suppressing eosinophil apoptosis [142], which may be relevant to control of the lifespan of eosinophils in adipose and gastrointestinal tissues.

4. Conclusions and final remarks

It was already clear from early studies on the expression profiles in various immune cell populations of each of the SCFA receptor FFAR2, the MCFA receptor GPR84, and the LCFA receptor FFAR4, that these receptors would likely play important roles in immune cell function. However, in recent times a broad swathe of studies have confirmed and built on these predictions. In concert with improving understanding of the roles that these, and the other FFARs, play in other cell types and tissues in providing a means to sense metabolite availability then an emerging view is that they act to regulate the metabolic-inflammatory axis. Given that aspects of inflammation underlie many chronic and co-morbid diseases, including type II diabetes, and obesity, there is considerable anticipation that agonist and/or antagonists of these receptors may play important roles in further understanding this interface and potential in the therapeutic treatment of such diseases.

Conflict of interest

The authors declare that there are no conflicts of interest regarding this work.

Acknowledgement

These studies were supported in part by the Biotechnology and Biosciences Research Council (grant numbers BB/L027887/1 and BB/K019864/1).

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