# Chemokine System in the Development of NAFLD

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Chemokines (Greek—kinos, movement) are a large family of chemotactic cytokines that involve immune and inflammatory responses through the chemoattraction and activation of leukocytes. These small proteins (approximately 8–12 kilodaltons) are classified into four different subfamilies (CC, CXC, CX3C and XC) based on the presence of four cysteine residues in the conserved locations ofN-terminals that are key to forming their 3-dimensional shape. Approximately 50 chemokines expressed in various cell types and tissues have been identified in humans and mice. Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world. Sustained hepatic inflammation is a key driver of the transition from simple fatty liver to nonalcoholic steatohepatitis (NASH), the more aggressive form of NAFLD. Hepatic inflammation is orchestrated by chemokines, a family of chemoattractant cytokines that are produced by hepatocytes, Kupffer cells (liver resident macrophages), hepatic stellate cells, endothelial cells, and vascular smooth muscle cells. Over the last three decades, accumulating evidence from both clinical and experimental investigations demonstrated that chemokines and their receptors are increased in the livers of NAFLD patients and that CC chemokine ligand (CCL) 2 and CCL5 in particular play a pivotal role in inducing insulin resistance, steatosis, inflammation, and fibrosis in liver disease.

chemokine nonalcoholic fatty liver disease inflammation

## 1. An Update on the Chemokine System in the Development of NAFLD

### 1.1. CCL3-CCR1 and CCR5

CCL3 (known as macrophage inflammatory protein-1α) is expressed in macrophages and secreted to recruit macrophages themselves, various leukocyte subtypes, and T cells to inflamed sites [1][2]. Various proinflammatory stimuli, such as viral infections, lipopolysaccharide, tumor necrosis factor-α (TNF-α), interferon-γ, and interleukin-1β (IL-1β), can induce the expression of CCL3 [3][4]. CCL3 signals through its receptors CCR1 and CCR5. T cells, eosinophils, and neutrophils express CCR1 [5]. NK cells and subsets of resting memory T cells, including some but not all Th1 cells, express CCR5 [5]. Monocytes and mature macrophages express both CCR1 and CCR5 [6].

### 1.2. CCL25-CCR9

The chemokine CCL25 is selectively and constitutively expressed in the thymus and small intestine. CCR9, the sole functional receptor of CCL25 [7], is expressed on thymocytes and intestinal lymphocytes [8]. The CCL25-CCR9

axis is crucial for mucosal lymphocyte recruitment to the small intestine followed by accumulating $CCR9^+CD4^+$ tissue-infiltrating T cells in both Crohn's disease and a murine model of inflammatory bowel disease [9][10][11]. With regard to liver immunology, $CCR9^+$ macrophages play a pathogenic role in a murine acute hepatitis model and humans [12]. Peripheral blood samples from patients with acute hepatitis had more TNF-α-producing $CCR9^+$ monocytes than healthy volunteers [12]. Similarly, in concanavalin A-injected mice, bone marrow-derived $CCR9^+$ macrophages accumulate in the liver, which produces high levels of TNF-α and promotes the Th1 differentiation of naive $CD4^+$ T cells, thereby contributing to acute liver inflammation [12]. Additionally, Morikawa et al. provided multiple lines of evidence indicating that the CCL25-CCR9 axis also plays a pivotal role in NASH pathogenesis [13]: (1)Serum CCL25 and hepatic CCR9 and CCL25 levels were higher in patients with NASH compared with healthy volunteers and patients with simple fatty liver. (2) CCL25 was expressed in $CD31^+$/$LYVE1^+$ sinusoidal endothelial cells, whereas CCR9 was expressed in $CD68^+$ macrophages and $GFAP^+$/α-$SMA^+$ HSCs in the livers of patients with NASH, and the numbers of these $CCR9^+$ cells were significantly lower in the control samples. (3) CCR9-deficient mice showed alleviated diet-induced steatohepatitis associated with the decrease in the amount of $CD11b^+$ inflammatory macrophage accumulation in the liver. (4) Consistent with human NASH, CCR9 was also expressed on HSCs in NASH mice and CCR9-deficient HSCs show fewer fibrogenic phenotypes. Finally (5) A CCR9 antagonist, vercirnon (CCX282-B) ameliorated steatohepatitis and the development of diethylnitrosamine-induced hepatocellular carcinoma in a high-fat diet-fed mice. These results indicate a therapeutic potential of CCR9 blockade in NAFLD.

## 1.3. CXCL1-CXCR2

CXCL1 is one of the major chemoattractants for neutrophils [14]. After binding to its receptor CXCR2, CXCL1 activates PI3K/Akt, MAP kinases, or phospholipase-β signaling pathways, increasing the recruitment of neutrophils into inflamed sites [15]. CXCL1 is also involved in the processes of wound healing, angiogenesis, tumorigenesis, and cell motility [16]. CXCL1 is highly expressed in the liver of NASH patients but not in the simple fatty livers in obese individuals or in high-fat diet (HFD)-fed mice [17][18]. In the choline-deficient amino acid-defined (CDAA) diet-induced mouse NASH model, the hepatic mRNA levels of CXCL1 are increased in a toll-like receptor 4 (TLR4)-MyD88-dependent manner, resulting in increased neutrophil infiltration associated with hepatic inflammation and fibrosis [19]. Additionally, adenoviral overexpression of CXCL1 in the liver is sufficient to activate progression from steatosis to steatohepatitis in HFD-fed mice by inducing hepatic neutrophile infiltration, oxidative stress, and hepatocyte apoptosis [20]. These studies indicate the importance of CXCL1-/CXCR2-mediated neutrophile recruitment during NAFLD development.

## 1.4. CXCL16-CXCR6

In conjunction with CD4, CXCR6 can serve as a co-receptor for the entry of human and most simian immunodeficiency viruses (human immunodeficiency virus type I and simian immunodeficiency virus) [21]. Similar to CCR5 and CXCR3, the expression pattern of CXCR6 is restricted to memory/effector T cells such as natural killer T (NKT) cells [22][23] and $CD8^+$ T cells [24]. In the liver, $CXCR6^+$ NKT cells patrol liver sinusoids and provide the intravascular immune surveillance of pathogens [25]. CXCL16, a membrane-bound ligand for CXCR6, is expressed

on the hepatocytes and biliary epithelial cells in the portal tracts and on sinusoidal cells in both normal and chronically inflamed liver tissue such as hepatitis C [26]. CXCL16 promotes the adhesion of $CXCR6^+$ cells to cholangiocytes and hepatocytes by triggering the conformational activation of β1 integrins and the binding to vascular cell adhesion molecule-1 (VCAM-1), thereby promoting liver inflammation [26]. Regarding NAFLD, Jing et al. demonstrated that serum levels of CXCL16 were elevated in NAFLD patients and that CXCL16 was strongly expressed around the steatotic hepatocytes in liver biopsy specimens [27]. Additionally, in the co-culture of murine hepatocytes and HSCs, lentiviral overexpression of CXCL16 increased lipid accumulation and mitochondrial stress in hepatocytes and induced the activation and proliferation of HSCs [27], suggesting that the CXCL16-CXCR6 axis mediates the crosstalk between hepatocytes and HSCs in NAFLD development.

### 1.5. CX3CL1-CX3CR1

CX3CL1, also known as fractalkine, a membrane-anchored chemokine, is expressed on epithelial cells, dendritic cells, and neurons and could be induced by inflammatory cytokines, such as TNF-α and IFN-γ [28][29][30][31][32][33]. CX3CL1 drives integrin-dependent adhesion and promotes the retention of specific CX3CR1-expressing leukocytes. The receptor is mainly expressed on circulating monocytes, tissue-resident macrophages, dendritic cells, and T cells [28][34][35]. The *N*-terminal domain of CX3CL1, containing a CX3C motif, can be cleaved by ADAM Metallopeptidase Domain 10 (ADAM10) [36] and ADAM17 [37], yielding a soluble form that also ligates CX3CR1 and exerts potent chemotactic activity [38].

## 2. Chemokine-Chemokine Receptor Axis as a Therapeutic Target of NAFLD (Small Molecules and Food Factors)

### 2.1. Cenicriviroc (CVC)

Since CCR2 and CCR5 play an important role in the infiltration of myeloid cells and the activation of HSCs, CVC, a once-daily, orally available CCR2/CCR5 dual antagonist, has been expected to improve NASH by suppressing both inflammation and fibrosis, as shown in animal models of steatohepatitis [39][40]. In the Phase 2b CENTAUR (NCT02217475) of adults with NASH and liver fibrosis (NAFLD activity score ≥ 4 and NASH Clinical Research Network stage 1–3 fibrosis), CVC treatment showed a favorable safety and tolerability profile and improved liver fibrosis without worsening steatohepatitis compared with the placebo [41]. However, and unfortunately, the Phase 3 AURORA (NCT03028740), which enrolled 1778 participants, of whom 1293 participated in Part 1 [42], was terminated early due to lack of efficacy based on the results of the planned interim analysis of the Part 1 data. The disappointing results of the Phase III trial likely reflect the complexity of the pathogenesis of NAFLD, which involves diverse immune and metabolic pathways. Currently, for CVC, a Phase IIb has been planned testing the combination therapy with a farnesoid X receptor agonist candidate involving bile acid, cholesterol, and lipid and glucose metabolism [43][44], for treating NASH.

### 2.2. Dietary Carotenoids and Sulforaphane

Many epidemiological studies have demonstrated that the development of NAFLD is closely linked to lifestyle factors (e.g., nutrition, physical activity) [45]. A nutritional intervention with fruits and vegetables could be effective in preventing NAFLD since dietary factors, including antioxidant carotenoids, are useful for decreasing the risk of inflammation-related diseases, including cancer, cardiovascular diseases, and obesity [46][47][48]. β-Cryptoxanthin and lycopene, carotenoids that specifically exist in *Citrus unshiu* (Satsuma mandarin orange) and *Solanum lycopersicum* (tomato), respectively, are relatively abundant in human blood [49][50][51] and have been reported to provide beneficial effects in a murine model of NAFLD. The supplementation of these carotenoids attenuated hepatic lipid accumulation and fibrosis in CL diet- or HFD-fed mice along with the decreased accumulation of T cells in the liver and enhanced anti-inflammatory M2-dominant liver macrophages [52][53][54]. The mechanism of this action was mediated, at least partly, through the downregulation of chemokines, including CCL2, CCL3, CCL5, and CXCL10 [52][53].

Sulforaphane, an isothiocyanate derived from cruciferous vegetables, such as broccoli, is a potent inducer of nuclear factor (erythroid-derived 2)–like 2 (Nrf2), a master transcription factor that regulates oxidative stress responses [54][55]. In addition to its antioxidative effects, sulforaphane has anti-inflammatory properties, suppressing pro-inflammatory IL-8 and CCL2 synthesis by inhibiting NF-κB, STAT6, and MAP kinase pathways [56][57]. It also reported that broccoli extract supplementation mitigated HFD-induced insulin resistance, hepatic steatosis, and the upregulation of CCL2-CCR2 axis [58]. Improved NAFLD by the broccoli extract supplementation was associated with decreased hepatic macrophage accumulation and the M2-dominant polarization of hepatic and adipose macrophages [58]. Additionally, the randomized, placebo-controlled, double-blind trial conducted by Kikuchi et al. demonstrated that supplementation with a dietary dose of broccoli extract for 2 months significantly decreased plasma liver enzymes, ALT, and AST in male participants, suggesting improved fatty liver by sulforaphane [59]. Further nutritional intervention studies, including large, long-term randomized clinical trials with histological assessment of NAFLD, are warranted.

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