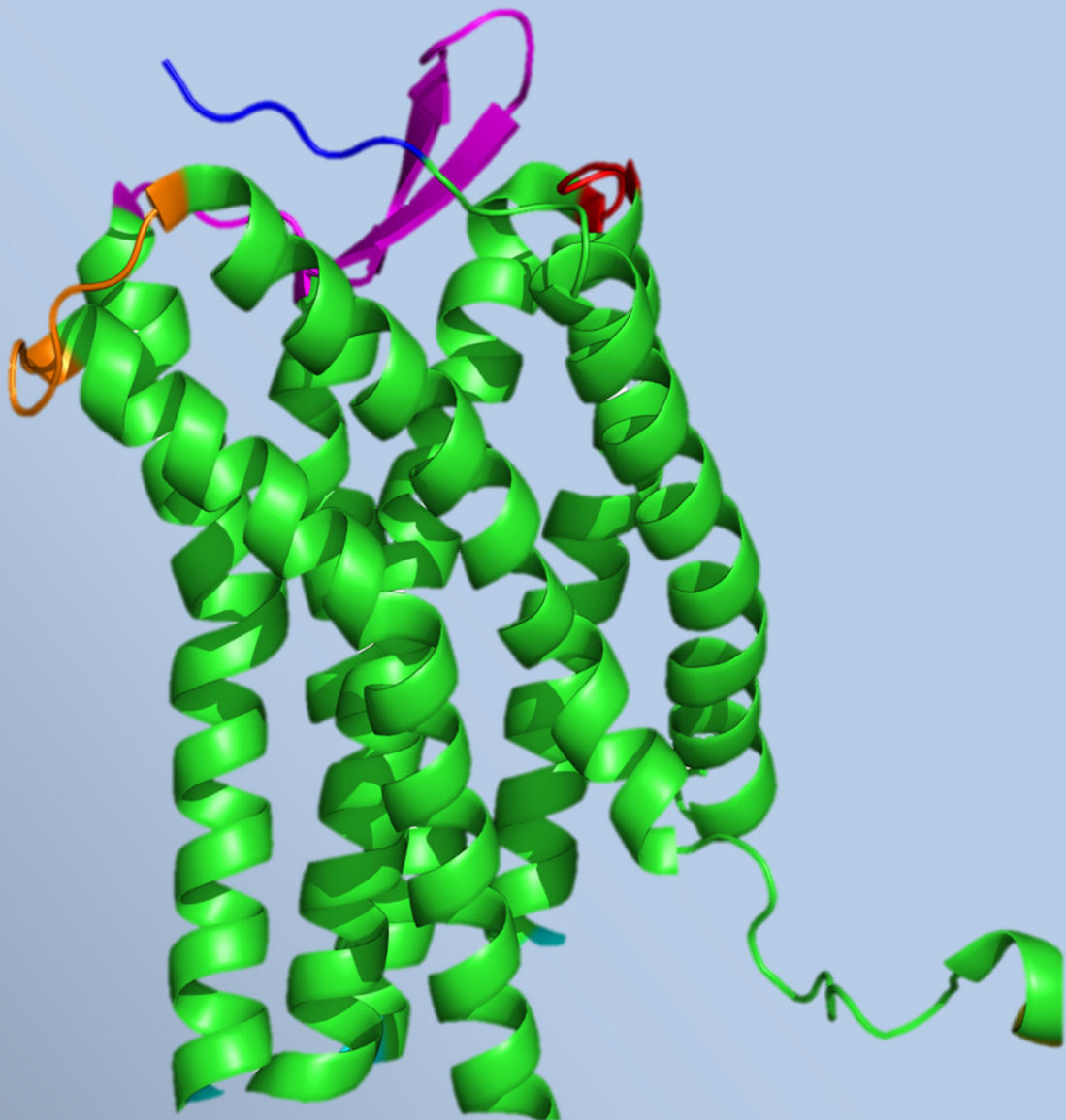


THE CXCR4 LIGAND/ RECEPTOR FAMILY AND THE DPP4 PROTEASE IN HIGH-RISK CARDIOVASCULAR PATIENTS

EDITED BY : Heidi Noels and Jürgen Bernhagen
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THE CXCR4 LIGAND/RECEPTOR FAMILY AND THE DPP4 PROTEASE IN HIGH-RISK CARDIOVASCULAR PATIENTS

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Crystal structure of the chemokine receptor CXCR4.

Image taken from: Pawig L, Klasen C, Weber C, Bernhagen J and Noels H (2015) Diversity and inter-connections in the CXCR4 chemokine receptor/ligand family: molecular perspectives. *Front. Immunol.* 6:429. doi: 10.3389/fimmu.2015.00429

Cardiovascular disease (CVD) is the most common cause of morbidity and mortality worldwide, putting a major burden on life quality and social health care systems. Type 2 diabetes mellitus (T2DM) and chronic kidney disease (CKD) have been identified as important risk factors for CVD, severely increasing the risk on e.g. myocardial infarction, and cardiovascular complications constitute the main cause of death in patients presenting with T2DM, CKD or a combination of both. As these pathologies are expected to rise alarmingly in the next decades, a better understanding of molecular and cellular mechanisms contributing to T2DM, CKD and CVD is required to improve prevention and treatment of these diseases. Furthermore, insight into the interplay between these pathologies and identification of molecular players interconnecting these comorbidities is of tremendous importance for optimal health management in the future.

This Research Topic will focus on the chemokine receptor CXCR4 and its ligands CXCL12/SDF-1 α and macrophage migration inhibitory factor (MIF) in the context of CVD and its link with T2DM and CKD, as well as address dipeptidyl peptidase-4 (DPP4) as an important protease destabilizing CXCL12. Chemokines and their receptors are important mediators of

cell mobilization, recruitment and arrest, and also more broadly induce cell activation by triggering various intracellular signalling tracks. They control homeostatic conditions, but are also critically involved in inflammatory and pathological processes. Genome-wide association studies revealed single nucleotide polymorphisms connecting CXCL12 as well as MIF with CVD, and a role for both chemokines in T2DM and CKD has also been reported. In this review collection, current knowledge on molecular aspects of the CXCR4 ligand/receptor family and associated signalling pathways will be discussed. The physiological roles of CXCR4, CXCL12, MIF and DPP4 will be summarized, and recent findings on their function in pathological conditions of CVD, T2DM and CKD will be highlighted. This is combined with an extensive introduction providing insight into the pathologies of CVD, T2DM and CKD, discussing clinical features and common pathological aspects of these comorbidities on cellular and molecular level. Also, an overview of available animal models to study these diseases will be provided. This way, this Research Topic summarizes latest knowledge on this crucial molecular axis and its relationship with cardiovascular pathologies for both specialists and interested non-specialists and aims to stimulate further initiatives to unravel the mechanistic involvement of the CXCR4 ligand/receptor family in these morbidities, potentially paving the way for new therapeutical initiatives in the future.

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Table of Contents

A. Editorial:

- 06 Editorial: The CXCR4 Ligand/Receptor Family and the DPP4 Protease in High-Risk Cardiovascular Patients**

Heidi Noels and Jürgen Bernhagen

B. Insights into the comorbidities cardiovascular disease, chronic kidney disease and diabetes:

- 09 High-Risk Cardiovascular Patients: Clinical Features, Comorbidities, and Interconnecting Mechanisms**

Katharina Andrea Schuett, Michael Lehrke, Nikolaus Marx and Mathias Burgmaier

- 18 Cellular and molecular mechanisms of chronic kidney disease with diabetes mellitus and cardiovascular diseases as its comorbidities**

Prathibha Reddy Gajjala, Maryam Sanati and Joachim Jankowski

- 33 Animal models to study links between cardiovascular disease and renal failure and their relevance to human pathology**

Tim D. Hewitson, Stephen G. Holt and Edward R. Smith

C. Introduction to the CXCR4 receptor/ligand family:

- 42 Diversity and inter-connections in the CXCR4 chemokine receptor/ligand family: molecular perspectives**

Lukas Pawig, Christina Klasen, Christian Weber, Jürgen Bernhagen and Heidi Noels

D. MIF and CXCL12 in cardiovascular disease, diabetes and kidney disease

- 65 MIF and CXCL12 in cardiovascular diseases: functional differences and similarities**

Emiel P. C. van der Vorst, Yvonne Döring and Christian Weber

- 78 The importance of the CXCL12/CXCR4 axis in therapeutic approaches to diabetes mellitus attenuation**

Melita Vidakovi, Nevena Grdovi, Svetlana Dini, Mirjana Mihailovi, Aleksandra Uskokovi and Jelena Arambaši Jovanovi

- 85 Role of macrophage migration inhibitory factor in obesity, insulin resistance, type 2 diabetes, and associated hepatic co-morbidities: a comprehensive review of human and rodent studies**

Martine C. Morrison and Robert Kleemann

- 98 Macrophage Migration Inhibitory Factor in Clinical Kidney Disease**

Annette Bruchfeld, Mårten Wendt and Edmund J. Miller

- 105 CD74 in kidney disease**

Lara Valiño-Rivas, Ciro Baeza-Bermejillo, Laura Gonzalez-Lafuente, Ana Belen Sanz, Alberto Ortiz and Maria Dolores Sanchez-Niño

E. DPP4 in cardiovascular disease, diabetes and kidney disease:

- 113** *The dipeptidyl peptidase family, prolyl oligopeptidase, and prolyl carboxypeptidase in the immune system and inflammatory disease, including atherosclerosis*

Yannick Waumans, Lesley Baerts, Kaat Kehoe, Anne-Marie Lambeir and Ingrid De Meester

- 131** *Dipeptidyl peptidase-4 regulation of SDF-1/CXCR4 axis: implications for cardiovascular disease*

Jixin Zhong and Sanjay Rajagopalan

- 139** *DPP4 in diabetes*

Diana Röhrborn, Nina Wronkowitz and Juergen Eckel

- 159** *The role of dipeptidyl peptidase – 4 inhibitors in diabetic kidney disease*

Usha Panchapakesan and Carol Pollock



Editorial: The CXCR4 Ligand/ Receptor Family and the DPP4 Protease in High-Risk Cardiovascular Patients

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The Editorial on the Research Topic

The CXCR4 Ligand/Receptor Family and the DPP4 Protease in High-Risk Cardiovascular Patients

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Cardiovascular disease (CVD) is the most common cause of morbidity and mortality worldwide and was responsible for 17.5 million deaths in 2012. This equals 31% of all deaths globally and is almost double the amount of cancer-related deaths (1). Although CVD encompasses a broad range of pathologic conditions, 80% of all CVD-related deaths are due to heart attacks and stroke (1). An important underlying pathology is atherosclerosis, a chronic inflammatory state of the arterial wall that is characterized by lipid deposition, dysfunction of the endothelium, and infiltration of inflammatory cells into the vessel wall, resulting in the development of atherosclerotic lesions (2).

Type 2 diabetes mellitus (T2DM) and chronic kidney disease (CKD) have been identified as important risk factors for CVD. Fifty percent of patients with CKD stages 4–5 suffer from CVD (3), and cardiovascular mortality accounts for ~40–50% of all deaths in these patients, compared with 26% in controls with normal kidney function (4, 5). Also in patients with diabetes, CVD accounts for at least 50% of deaths (6). With currently around 10–13% of people presenting with CKD and more than 8% of adults suffering from diabetes (6–8), the social and economic burden of diabetes, CKD, and CVD is extremely high. Thus, a better understanding of the mechanisms contributing to and mediating the interplay between T2DM, CKD, and CVD is required to improve the prevention and treatment of these diseases.

In this *Research Topic*, we focus on the classical CXC chemokine receptor CXCR4 (9), its cognate ligand CXCL12 (10), and the chemokine-like cytokine macrophage migration inhibitory factor (MIF), which functions as a non-cognate ligand of CXCR4 (11), in the context of CVD. An emphasis is made on links with T2DM and CKD. Also, we discuss dipeptidyl peptidase-4 (DPP4) as an important protease known to destabilize CXCL12 and thus to influence signaling through the CXCL12/CXCR4 axis. Chemokines and their receptors are important mediators of cell mobilization, recruitment and arrest, and additionally more broadly induce cell activation by triggering various intracellular signaling tracks. Chemokines control basic homeostatic conditions but are also critically involved in inflammatory processes, e.g., in atherosclerosis (12, 13). Genome-wide association studies revealed single nucleotide polymorphisms connecting CXCL12 as well as MIF with CVD (2, 14–19), and a role for both of these mediators in T2DM and CKD has been reported. In this *Research Topic*, we introduce the reader to the comorbidities T2DM

and CKD and their connection with CVD, and provide up-to-date information on the involvement of CXCL12/MIF/CXCR4 and DPP4 in each of these pathologies. One focus is laid upon providing insight on mechanistic level.

T2DM, CKD, AND CVD: COMORBIDITIES INTERLINKED ON MECHANISTIC LEVEL

There is clear-cut epidemiologic evidence linking the comorbidities T2DM and CKD with CVD. Progress has also been made to explain the comorbid status of these diseases on molecular level; however, the interaction seems highly complex and insight into the connecting mechanisms is mostly still in its infancy. Here, Schuett et al. (Germany) present an overview of the inflammatory processes underlying atherosclerosis and highlight the role of inflammation in T2DM and chronic inflammatory diseases in relation to CVD. The current prevalence and incidence of T2DM, CKD, and CVD are summarized by Gajjala et al. (Germany). They also provide insight into molecular mechanisms interconnecting these comorbidities.

Animal models allow us to investigate these interconnecting mechanisms in more detail and enable us to evaluate potential therapeutic strategies. Hewitson et al. (Australia) summarize available animal models for investigating mutual interactions between cardiac and renal injury and critically discuss how representative they are for human disease. This also includes an overview of animal models for hypertension, diabetes, and obesity, which are linked with kidney and cardiac injury through systemic alterations.

THE CXCR4 CHEMOKINE RECEPTOR AND ITS LIGANDS CXCL12 AND MIF: MOLECULAR ASPECTS AND INVOLVEMENT IN CVD, T2DM, AND CKD

Pawig et al. (Germany) present the diversity and interconnections in the CXCR4 receptor/ligand family. Signaling pathways initiated by binding of CXCL12 vs. MIF to CXCR4 are discussed, and it is elaborated on how ACKR3 (previously called CXCR7) affects CXCR4 signaling. Finally, authors summarize the (patho)biological functions of CXCR4 signaling mediated by CXCL12 or MIF that are likely to be important in devising potential future therapies targeting this signaling axis.

van der Vorst et al. (Germany) present an overview of the role of CXCL12 vs. MIF in CVD, highlighting the differences and similarities. Vidakovic et al. (Serbia) discuss the controversial role of the CXCL12/CXCR4 axis in diabetes, whereas Morrison and Kleemann (The Netherlands) summarize the role of MIF in obesity, insulin resistance, T2DM, and associated hepatic comorbidities as revealed by both human and animal studies. Bruchfeld (Sweden) discusses MIF in the context of kidney disease, while

complementarily, Valiño-Rivas et al. (Spain) elaborate on the expression and role of CD74, an additional receptor for MIF and the MIF homolog MIF-2 (or D-DT) in kidney injury.

DPP4 AS REGULATOR OF CXCL12 AND ITS INVOLVEMENT IN CVD, T2DM, AND CKD

DPP4 (also known as CD26) is known to destabilize CXCL12. Therefore, our Research Topic also addresses molecular aspects and functions of this protease as well as its role in CVD, T2DM, and CKD. Waumans et al. (Belgium) provide a comprehensive insight into DPP4 and its role in the immune system and inflammatory diseases including atherosclerosis. This also includes an overview of other family members of the dipeptidyl peptidase family as well as of prolyl oligopeptidases and prolyl carboxypeptidases. Circulating DPP4 is increased in patients with T2DM, and DPP4 inhibition is used for treatment of T2DM. Thus, Röhrborn et al. (Germany) provide detailed insight into the expression, enzymatic activity, and function of DPP4 in the context of diabetes, based on *in vitro*, animal, and human studies. Also, mechanistic insight into the role of DPP4 in the T2DM-associated morbidities CVD and liver disease is provided. The role of DPP4 and its regulation of the CXCL12/CXCR4 axis in CVD are then discussed in more detail by Zhong and Rajagopalan (USA). Finally, Panchapakesan and Pollock (Australia) summarize potential renoprotective effects of DPP4 inhibitors in diabetic kidney disease and also discuss the cardiovascular safety profile of DPP4 inhibitors.

Altogether, this *Research Topic* aims to assist both specialists and interested non-specialists and to stimulate further initiatives to unravel the mechanistic involvement of the CXCR4 ligand/receptor family in the detrimental interplay between the comorbidities T2DM, CKD, and CVD, potentially paving the way for new therapeutic initiatives in the future.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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High-Risk Cardiovascular Patients: Clinical Features, Comorbidities, and Interconnecting Mechanisms

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Cardiovascular disease is the leading cause of death in the Western world with an increase over the last few decades. Atherosclerosis with its different manifestations in the coronary artery tree, the cerebral, as well as peripheral arteries is the basis for cardiovascular events, such as myocardial infarction, stroke, and cardiovascular death. The pathophysiological understanding of the mechanisms that promote the development of vascular disease has changed over the last few decades, leading to the recognition that inflammation and inflammatory processes in the vessel wall are major contributors in atherogenesis. In addition, a subclinical inflammatory status, e.g., in patients with diabetes or the presence of a chronic inflammatory disease, such as rheumatoid arthritis, have been recognized as strong risk factors for cardiovascular disease. The present review will summarize the different inflammatory processes in the vessel wall leading to atherosclerosis and highlight the role of inflammation in diabetes and chronic inflammatory diseases for cardiovascular morbidity and mortality.

Keywords: atherosclerosis, type 2 diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis, coagulation, inflammation, cardiovascular disease

INTRODUCTION

Cardiovascular disease is the leading cause of death in the western world with an increase over the last few decades (1–4).

Atherosclerosis may result in myocardial infarction (MI), stroke, or peripheral artery disease according to its manifestations in the coronary artery tree, in cerebral arteries as well as peripheral arteries. The pathophysiological understanding of the mechanisms that promote the development of vascular disease has changed over the last few decades, leading to the recognition that inflammation and inflammatory processes in the vessel wall are major contributors in atherogenesis (5).

ATHEROGENESIS

For decades, atherogenesis has been seen as a degenerative process in the vessel wall leading to the progressive occlusion of the artery up to a certain point where a few activated platelets are sufficient to occlude the vessel and cause cardiac events, such as acute MI. In the 1990s, we learned from our pathology colleagues that a majority of lesions causing an acute coronary syndrome show a stenosis <50%, and further pathological analysis revealed that most of these lesions are the so-called unstable lesions (6). These lesions are characterized by a high inflammatory burden of cells, such as monocytes and T cells, and a large necrotic lipid core as well as a very thin fibrous cap

making the atherosclerotic lesion prone to rupture. At the same time, experimental data have shown that atherogenesis is an inflammatory process in the vessel wall in different phases and stages (7). Also not strictly defined endothelial dysfunction, fatty streak formation and the formation of advanced and potentially complicated lesions have been described as the main phases in lesion development (8). Over the last decade, our understanding of the inflammatory cells involved in these processes has grown. The characteristic cell type of an atherosclerotic lesion is the lipid-laden macrophage which is attracted by certain chemokines to the vessel wall and then can contribute to the local inflammatory response by the expression and release of inflammatory mediators. The uptake of LDL in these mononuclear cells by scavenger receptors results in the characteristic foam cell. In addition, the accumulation of cholesterol within macrophages leads to crystal formation, and these intracellular microcrystals can activate the inflammasome of the cells subsequently resulting in the release of cytokines (9). Interestingly, experimental work from various groups around the world has shown that different subtypes of macrophages can be found in atherosclerotic lesions with the predominance of the M1-subpopulation which release proinflammatory cytokines (10). Additional cellular components of the innate immune response in the vessel wall are mast cells and to a certain extent polymorphonuclear leukocytes, but the latter have mainly been found in murine models, and the relevance for human atherosclerosis remains unclear (11). In addition to the innate immune response, clinical and experimental data support the recognition that the adaptive immune system is of critical importance in atherogenesis. As such, dendritic cells have been identified as a critical “link” between the innate and adaptive immune responses by initiating T-cell response. T cells in the atherosclerotic lesion are mainly CD4 cells which to a large extent release proinflammatory Th1 cytokines. These cytokines, such as interferon (IFN)- γ and tumor necrosis factor (TNF)- α , do not only induce the expression of certain chemokines thus favoring the recruitment of additional leukocytes but also lead to the activation of other vascular cells [reviewed in Ref. (12)].

In addition, recent work has shown that so-called regulatory T cells are an important atheroprotective cell population in the vascular wall through their capacity to control effector T cells (13).

The recognition that inflammation plays an important role in atherogenesis has been paralleled by the clinical observation that elevated levels of high-sensitive C-reactive protein (hsCRP) is a useful biomarker to predict cardiovascular events in patients (14). Elevated levels of hsCRP are associated with an increased risk for MI and major cardiovascular events in a primary prevention population. In addition, a clinical trial with stratification of patients according to their CRP levels has shown that the implementation of a lipid lowering therapy by statins is of greatest benefit in those subjects that exhibited elevated levels of the inflammatory marker hsCRP (15).

COAGULATION AND INFLAMMATION

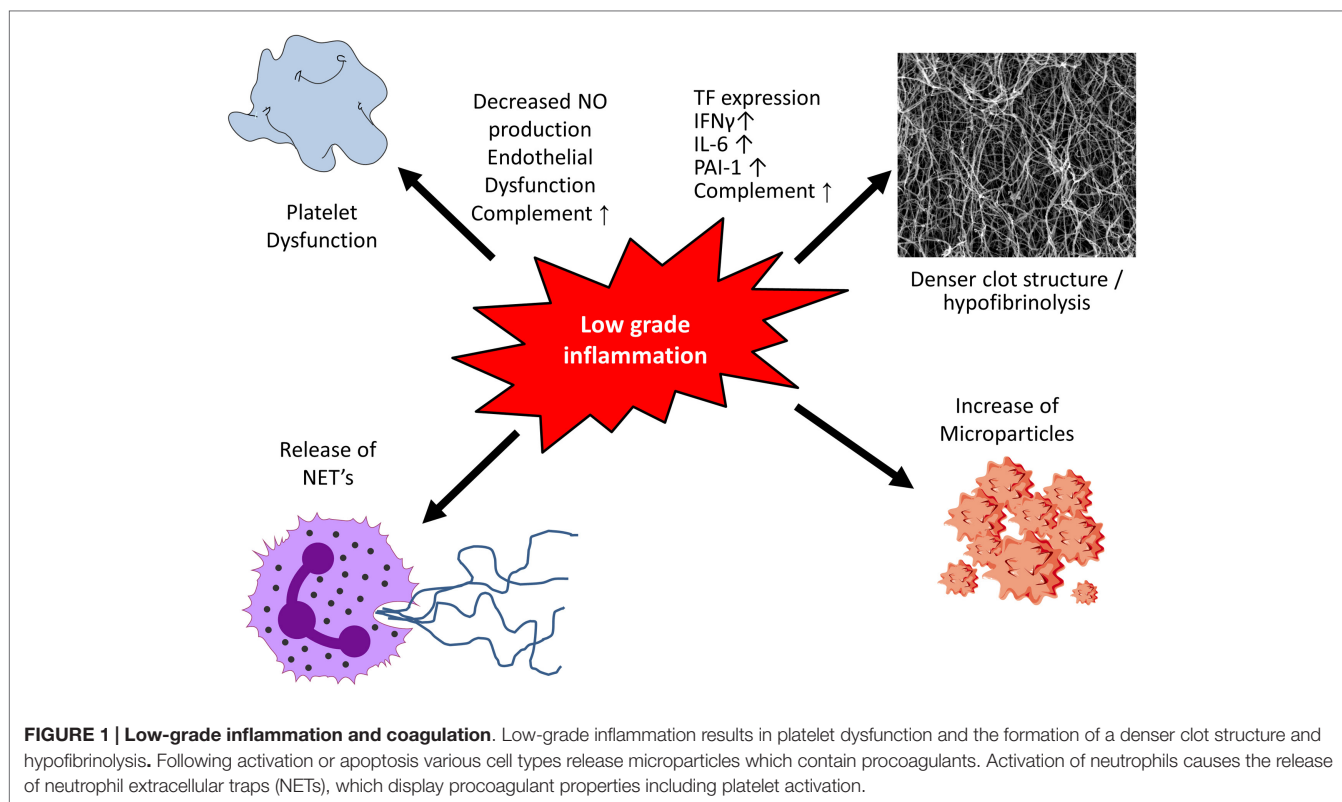
Over the last two decades, experimental data have shown an interaction of inflammation with procoagulatory mechanisms

in the vessel wall: inflammatory cells, such as macrophages and T lymphocytes, produce cytokines leading to the expression of tissue factor (TF) by endothelial cells, macrophages, and smooth muscle cells (SMCs), thus enhancing prothrombotic properties of the plaque (16). However, the interaction of inflammation and coagulation is not limited to the vessel wall. In obesity, which is frequently associated with cardiovascular disease and diabetes, adipose tissue is a major source for elevated inflammatory cytokines (17, 18). Macrophages and T lymphocytes produce IFN- γ , interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1, TNF- α , and PAI-1 (19), which are all able to impact on the coagulation system (**Figure 1**). Not only inflammatory cytokines but also the complement system interacts with coagulation. The complement system is an important immune surveillance system with over 30 components involved. It can be activated by three different pathways, the classical, the lectin, and the alternative pathway (20). Activation of any of these consequently results in activation of complement C3, the central component of the complement system. Subsequently, complement C5 is activated leading to the formation of the terminal complement complex (C5b, C6, C7, C8, and C9). The biological functions of the complement system are broad including, among others, innate immune responses and coagulation (21). In coagulation, complement affects both platelet function and fibrin clot structure and lysis.

Primary Hemostasis

Following plaque rupture, thrombosis is initiated when thrombotic components of the plaque are exposed to circulating blood. Platelets are the first to respond and following their adhesion, platelets are activated leading to additional recruitment and aggregation with stabilization of the clot (22). In addition, inflammation can alter platelet function (**Figure 1**). Early changes include endothelial dysfunction with decreased nitric oxide (NO) production. NO is a key player in negative regulation of platelet activity, and decreased levels result in hyper-reactivity of platelets (23).

In addition to their primary role in hemostasis, platelets actively participate in inflammatory and immune processes. They carry a multiplicity of chemokines and cytokines, which are mostly found within the various platelet granules. Platelet activation and consequent degranulation result in the release of chemokines, including CXC-chemokine ligand 1 (CXCL1), platelet factor 4 (also known as CXCL4), CXCL5 and CXCL7, CC-chemokine ligand 3 [CCL3; also known as macrophage inflammatory protein 1 alpha (MIP-1 α)], CCL5 [also known as regulated on activation, normal T Cell expressed and secreted (RANTES)], and CCL7, ILs (IL-1 β , IL-7, and IL-8), prostaglandins, and the transmembrane protein CD154 (24, 25). CD154 interacts with CD40 on endothelial cells leading to an upregulation of cell adhesion molecules on endothelial cells, including intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1), and the release of CC-chemokine ligand-2 (CCL2), thereby facilitating leukocyte recruitment to the inflammatory site (26). In addition, activated platelets also release soluble CD154 likewise leading to an upregulation of vascular cell adhesion molecules and the release of IL-6. Through the expression of these chemokines, cytokines, CD154, and cell adhesion molecules, activated platelets



promote neutrophil tethering and activation. Furthermore, they also activate monocytes and dendritic cells, particularly through the CD154–CD40 interaction (24). For an extended overview about this topic, see the review article of Semple et al. (24).

Complement and platelets interact on different levels. Platelets contain several complement receptors as well as complement regulatory molecules, and several components of the complement cascade have been recognized on the platelet surface. Complement activation can be initiated by several platelet agonists, such as adenosine diphosphate (ADP), epinephrine, arachidonic acid, thrombin, and exposure to shear stress (27). However, a study using complement C3 knock-out mice revealed C5 convertase activity of thrombin suggesting a platelet-independent mechanism of complement activation (28). The other way round, several complement components are known to activate platelets including the mannose-binding lectin (MBL)/MBL-associated serine protease (MASP) complex, binding of C1q to its receptor as well as the complement C3 cleavage products (iC3b, C3d, and C3dg) to their receptors CR2 and CR3 (only for iC3b) (27, 29–31). *In vitro* studies further suggest that activated platelets bind the anaphylatoxins C3a and C5a resulting in platelet aggregation (32, 33). In addition to classical complement receptors, P-selectin binds C3b resulting in the generation of C3a and the terminal complement complex C5b–9, thereby enhancing platelet response (34, 35). Furthermore, the anaphylatoxins C3a and C5a are generated which are known to have cytokine-like properties with enhanced leukocyte recruitment and support of the general inflammatory response (27). Under normal circumstances, complement activation is tightly regulated due to expression of

complement regulatory molecules. On the platelet surface, factor H inactivates C3b and destabilizes the C3 convertase (36). Similarly, membrane cofactor (MCP or CD46) inactivates C3b, and decay-accelerating factor (DAF or CD55) interferes with C3 convertase. Furthermore, CD59 is known to destabilize the terminal complement complex (35).

Secondary Hemostasis

Fibrin clot structure plays a crucial role in determining predisposition to atherothrombotic events. Clots with compact structure and impaired fibrinolysis are associated with premature and more severe cardiovascular disease.

Inflammatory cytokines produced by macrophages and T lymphocytes, including IFN- γ , IL-6, MCP-1, and TNF- α , are able to influence both clot structure and lysis (Figure 1). By leading to increased production of acute phase products, such as fibrinogen and PAI-1, they alter clot structure and prolong fibrinolysis, thereby enhancing the prothrombotic milieu (19, 37). TF, the key initiator of the coagulation cascade, is produced by various cell types with monocytes being a major source. TF expression is very low under basal conditions (38) but increases upon stimulation with cytokines (i.e., TNF- α and IL-1 β) resulting in activation of the coagulation system (39–41).

Complement interacts with the fluid phase of coagulation at different levels. Mannose-associated serine protease 1 (MASP-1) exhibits a thrombin-like profile by activating fibrinogen and FXIII. It thereby leads to the formation of a crosslinked fibrin clot although the catalytic efficiency compared with thrombin is greatly reduced (42). Furthermore, MASP-1 is able to induce

prothrombin and thrombin activatable fibrinolysis inhibitor (TAFI), altogether leading to the formation of thinner fibrin fibers and longer lysis time (43). Similarly, MASP-2 cleaves prothrombin to thrombin (42, 44). The C1 inhibitor not only inhibits C1r/s, MASP-1, and MASP-2 but also inhibits FXIIa and FXIa (45). Shats-Tseytina demonstrated already in 1994 that the activation of the complement cascade leads to the formation of a prothrombotic clot with thinner fibers. Using proteomics analysis, complements C1, C3, C4, C5a, and factor B were demonstrated in plasma clots (46). Of these, C3 binds with high affinity to fibrinogen leading to a prothrombotic clot structure and prolongation of clot lysis *in vitro* (47, 48). In addition to complement C3, C5a is known to induce TF and PAI-1 expressions (49, 50). Activation of the complement system and generation of the terminal C5b-9 complex further induce endothelial cells to produce TF and vWF thereby generating a prothrombotic phenotype (51).

Microparticles

Microparticles (MPs) are cell membrane-derived particles that can promote both coagulation and inflammation (Figure 1). Following activation or apoptosis, they are released from various cell types, including platelets, endothelial cells, red blood cells, and leukocytes. Depending on their origin they vary in size (0.2–1 μm) and their membrane composition of phospholipids and proteins. MPs can be detected in the circulation of healthy control individuals and are elevated in inflammatory situations, such as sepsis (52) and diabetes mellitus (53). MPs are directly able to modulate nitric oxide production from endothelial cells, induce cytokine release and prostacycline production as well as adherence of monocytes to the endothelium (54). The two major procoagulants found on the surface of MPs are phosphatidylserine and TF (55) thereby contributing to a prothrombotic state in various diseases. Furthermore, MPs can harbor and transport microRNA thereby impacting protein expression of target cells (56, 57).

Neutrophil Extracellular Traps

Activation of neutrophils causes the release of web-like structures of DNA, so-called neutrophil extracellular traps (NETs). Studies demonstrated NETs to display procoagulatory properties, including the induction of platelet adhesion, aggregation, and fibrin deposition on their surface. Histones, cationic proteins that are associated with DNA, can be actively secreted by activated inflammatory cells, such as neutrophils, contributing to the formation of NETs. In this context, they have been shown to promote platelet aggregation and thrombin formation through platelet-dependent mechanisms, including platelet toll-like receptor (TLR) 2 and TLR4 (58). Bosmann and colleagues demonstrated complement C5a to be able to trigger the appearance of histones by binding to its receptors C5aR and C5L2 (59), thereby contributing to an increased inflammatory and prothrombotic milieu. A more recent study in acute STEMI demonstrated that the interaction of thrombin-activated platelets with polymorphonuclear neutrophils at the site of plaque rupture results in local NET formation and delivery of active TF (60).

ATHEROSCLEROSIS IN PATIENTS WITH CHRONIC INFLAMMATORY DISEASES

As highlighted above, atherosclerosis is characterized by an inflammatory process in the vessel wall (61). Thus, inflammatory biomarkers and cytokines, such as hsCRP, have been related with progressive cardiovascular disease as well as an increased risk for cardiovascular events (14, 62–65). On the other hand, patients with a chronic high state of inflammation, such as autoimmune diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), are at a high risk for cardiovascular morbidity and mortality, and the increased cardiovascular risk of these patients is related to the extent of inflammation (66) (Figure 2). However, the underlying pattern, pathophysiology, and phenotype of this increased cardiovascular risk in patients with chronic inflammation vary between diseases and are discussed below for RA and SLE.

Rheumatoid Arthritis

Patients with RA exhibit an increased cardiovascular risk with higher rates of sudden cardiac death and unrecognized MI compared with age- and sex-matched patients without RA (67). Furthermore, atherosclerosis starts early and is more diffuse over multiple vascular beds in this high-risk group (68). It could be demonstrated that patients with RA and diabetes share similar frequency and severity regarding preclinical atherosclerosis (69). An autopsy study of 41 patients with RA and 82 age- and sex-matched controls revealed a higher incidence of vulnerable plaques in the left anterior descending coronary artery and more plaque inflammation in patients with RA (70). Recently, a study by Karpouzas et al. analyzed plaque morphology using CT angiography and found a higher atherosclerotic burden in patients with RA. Interestingly, disease activity was associated with the presence of non-calcified and mixed, but not calcified plaques in this study, suggesting that disease activity is associated with plaque vulnerability (71). In light of these data, it is tempting to speculate that a more vulnerable plaque phenotype is responsible for the increased cardiovascular risk in patients with RA. Furthermore, these findings are in line with an increased prevalence of traditional cardiovascular risk factors, such as diabetes mellitus, hypertension, smoking, and obesity, in patients with RA [for review, see Ref. (72)]. However, several studies demonstrated that the cardiovascular risk in patients with RA following adjustment for classical cardiovascular risk factors remained high, suggesting that the increased risk in these patients cannot be fully explained by classical cardiovascular risk factors (67, 73). Vice versa, traditional cardiovascular risk assessment models are of limited value in patients with RA and should thus be used with caution in this high-risk group (74). Taken together, these data emphasize the link between systemic inflammation on the one hand and atherothrombosis on the other hand. However, patients with RA are not solely characterized by increased atherothrombosis but also by high rates of valvular heart disease, myocarditis, and non-ischemic cardiac failure, which all contribute to the increased cardiovascular risk in this high-risk group of patients (75).

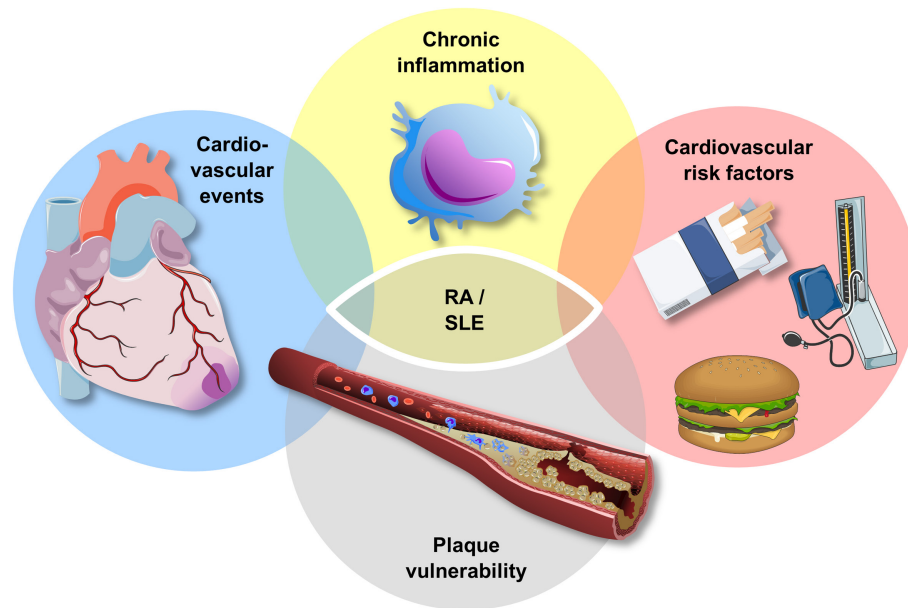


FIGURE 2 | Atherosclerosis and chronic inflammatory disease. Patients with chronic inflammatory disease, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), exhibit an increased risk for traditional cardiovascular risk factors, plaque vulnerability, and cardiovascular events. The increase in cardiovascular events in this high-risk group cannot be explained entirely by the increase in cardiovascular risk factors.

The recognition of progressive vascular disease by the use of circulating biomarkers is a challenge in patients with a chronic high state of inflammation as conventional, inflammatory biomarkers may lack specificity in this high-risk group. Recently, we have been able to associate C-peptide, which is mainly regulated by insulin secretion and kidney function (76), to the presence of coronary artery calcification in patients with RA (77). Interestingly, the inflammatory biomarker YKL-40, which is thought to play a role in both atherogenesis and RA (62, 78–80), was a predictor for the presence of coronary artery calcification in patients with RA in this study, supporting the link between inflammation and atherosclerosis in patients with RA (**Figure 2**).

In addition, others found striking similarities between the pathogenesis of both RA and atherosclerosis, including endothelial activation, inflammatory cell infiltration and activation, neovascularization, and collagen degradation via matrix metalloproteinases [for review, see Ref. (72)].

Systemic Lupus Erythematosus

Patients with SLE exhibit an increased risk for mortality (81, 82), which is in part attributed to disease activity and in part to increased cardiovascular deaths. This used to result in a bimodal mortality pattern and was described almost four decades ago (83). Presently, the mortality associated with disease activity has declined with advances in disease treatment, which results in increased life expectancy and a constant mortality rate over time (84). With these improvements in treating patients with SLE, cardiovascular and infectious complications are now the main direct cause of death among both early and late fatalities (84).

Recently, a large retrospective cohort study in Taiwan compared the incidence of acute MI between patients with SLE compared to age- and sex-matched controls. The investigators found an adjusted hazard ratio of 5.11 for acute MI associated with SLE, which was even larger in the female population (hazard ratio 6.28) (85). Interestingly, patients with SLE were not only associated with a higher risk of acute MI but also with an increased mortality post-MI (85).

Similar to what is described above for patients with RA, patients with SLE exhibit an increased prevalence of traditional cardiovascular risk factors, including smoking, dyslipidemia, hypertension, and diabetes (86). However, adjustment for Framingham risk factors could not fully account for the increased cardiovascular risk in patients with SLE (87). These findings are in line with a recent meta-analysis including 17,187 patients, which found that both traditional and disease specific risk factors contribute to the risk in patients with SLE. Disease-specific risk factors included the presence of autoantibodies, neurological disorders, and to a lesser extent organ damage and SLE activity (88). Furthermore, antiphospholipid antibodies were related to mitral valve nodules and significant mitral regurgitation (89), suggesting that certain SLE-specific risk factors may also be associated with cardiovascular disease but not related to atherothrombosis.

Taken together, there appears to be a link between patients with a chronic high state of inflammation and progressive cardiovascular disease. Whereas RA and SLE demonstrate distinct immunological profiles and unique cytokine patterns [for review, see Ref. (90, 91, 75)], both share an increased cardiovascular mortality. Further studies are needed to evaluate the molecular

mechanisms involved in the increased cardiovascular risk in patients with chronic inflammation and the interplay between chronic inflammation, cardiovascular risk factors, and progressive cardiovascular disease.

INFLAMMATION IN PATIENTS WITH DIABETES MELLITUS

The cardiovascular vulnerability of diabetic and prediabetic patients is predictable by a variety of inflammatory markers. These are indicative of an activated innate immune system featuring elevated circulating levels of IL-1, TNF- α , IL-6, and hsCRP (92). Among these, hsCRP has gained the most attention as a predictor of diabetes, cardiovascular disease, and mortality (14, 93). In addition, low-grade inflammation is linked to activation of the coagulation system featuring increased levels of fibrinogen and PAI-1 as predisposing factor for thrombus formation (94).

A variety of mechanisms have been proposed for the initiation of subclinical inflammation in metabolic disease. Among these, obesity leads to hypertrophic adipocytes, which are unable to cope with an overwhelming supply of fatty acids (95). The consequential release of free fatty acids into the circulation causes steatosis of various organs including liver and muscle (95). Limited lipid storage capacity of their organelles results in metabolic disturbance and oxidative stress, which has been termed lipid toxicity. The consequential cellular damage and apoptosis requires debris-clearing recruitment of macrophages. These eliminate cell fragments as a prerequisite for tissue regeneration. Low-grade inflammation accompanying this cellular turnover is beneficial and part of a healing process. Persistence of pathogenic stimuli with ongoing cellular damage, however, leads to chronic inflammation and disturbed tissue integrity (96). Initiation of fibrosis may lead to an irreversible scarring process as found for steatohepatitis with transition to liver cirrhosis.

Saturated fatty acids further serve as ligands for pattern recognition receptors including TLR2 and 4 (97, 98). These provide a primitive line of defense of the innate immune system able to recognize a variety of microbial fragments and cell debris. TLRs signal downstream to various inflammatory pathways, including NF- κ B and MAP-kinase, with secretion of inflammatory cytokines, including TNF- α , IL-1, and IL-6. In addition, activation of NF- κ B causes direct inhibition of the insulin receptor cascade as an alternative mechanism of inflammation-mediated insulin resistance (99). Genetic or pharmaceutical inhibition of NF- κ B improves glucose metabolism in mice and men. Indeed treatment with salsalate – a prodrug of salicylic acid, which inhibits NF- κ B at high doses – lowers fasting glucose in non-diabetic, obese individuals (99, 100). Salsalate, however, also slightly increases plasma LDL-cholesterol levels and was ineffective in improving flow-mediated dilatation in patients with diabetes, questioning its clinical utility (100). Another anti-inflammatory strategy in metabolic disease, which has advanced to the clinics, is blockade of the IL-1 receptor system. This improved glucose metabolism and insulin sensitivity in small clinical studies and was associated with a reduction of inflammatory serum parameters (101). Blockade of the IL-1 system is currently evaluated in a large cardiovascular

endpoint study (CANTOS) in which the IL-1-directed antibody canakinumab is tested versus placebo in a randomized trial in 17,200 stable, post-MI patients with persistent high CRP levels (102). A dedicated sub-study further investigates the effect of IL-1 system blockade on glucose metabolism and insulin resistance with trial termination being expected in 2017. The efficacy of inflammation targeting therapies in metabolic disease is further investigated in the Cardiovascular Inflammation Reduction Trial (CIRT) using low-dose methotrexate (target dose 15–20 mg/week) treatment. This study randomizes 7000 prior MI patients with either type 2 diabetes or the metabolic syndrome with an average follow-up period of 3–5 years expecting termination in 2018 (103).

More recently, metabolic inflammation has further been attributed to a disturbed intestinal barrier function (104). Increased circulating concentrations of endotoxin are detected in patients with obesity and diabetes with direct associations to cardiovascular disease (105). A disturbed intestinal barrier enables the passive diffusion of bacterial fragments and lipopolysaccharides across the intestinal mucosa. Interestingly, this seems to be modulated by dietary factors with high nutritional fat intake promoting endotoxin absorption (106). Circulating endotoxin serves as a ligand for TLR2 and 4 with activation of the innate immune system. Chronic low-dose endotoxin application was consequently found to cause obesity and insulin resistance in rodent models while knockout of TLR4 prevented diet-induced obesity, glucose intolerance, and atherosclerosis (107–109). Furthermore, mice lacking the functional LPS receptor CD14 were resistant to diet-induced obesity and related disorders (110) while antibiotic eradication of the gut flora led to reduced metabolic inflammation, insulin resistance, and fat mass development (111). In consequence, germ-free mice were protected from high-fat diet-induced inflammation (112). Modulation of the intestinal microbiome as a source for endotoxin generation might therefore provide a new treatment strategy for diabetes and cardiovascular disease prevention.

These observations demonstrate a close interaction between the immune system and metabolism which is particularly present in patients with diabetes. Activation of the immune system does thereby not depend on an external stressor but is the consequence of overnutrition as an endogenous stressor. Concepts of anti-inflammatory therapies might prove effective in improving diabetes and cardiovascular outcome.

CONCLUSION

Over the last 30 years, inflammation has increasingly been recognized as an important pathophysiological link between vascular disease, atherothrombosis, metabolic disorders, and chronic autoimmune diseases. A plethora of mediators, such as cytokines, chemokines, and adipokines, released from various cells in different organs contribute to both local and remote stimulations of inflammatory cells, thus creating a network of interactions at different levels in the organism. Future therapeutic strategies need to be developed to examine how and by which tools this interaction can be targeted to modulate cardiovascular disease in high-risk patients.

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Cellular and molecular mechanisms of chronic kidney disease with diabetes mellitus and cardiovascular diseases as its comorbidities

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Chronic kidney disease (CKD), diabetes mellitus (DM), and cardiovascular diseases (CVD) are complex disorders of partly unknown genesis and mostly known progression factors. CVD and DM are the risk factors of CKD and are strongly intertwined since DM can lead to both CKD and/or CVD, and CVD can lead to kidney disease. In recent years, our knowledge of CKD, DM, and CVD has been expanded and several important experimental, clinical, and epidemiological associations have been reported. The tight cellular and molecular interactions between the renal, diabetic, and cardiovascular systems in acute or chronic disease settings are becoming increasingly evident. However, the (patho-) physiological basis of the interactions of CKD, DM, and CVD with involvement of multiple endogenous and environmental factors is highly complex and our knowledge is still at its infancy. Not only single pathways and mediators of progression of these diseases have to be considered in these processes but also the mutual interactions of these factors are essential. The recent advances in proteomics and integrative analysis technologies have allowed rapid progress in analyzing complex disorders and clearly show the opportunity for new efficient and specific therapies. More than a dozen pathways have been identified so far, including hyperactivity of the renin–angiotensin (RAS)–aldosterone system, osmotic sodium retention, endothelial dysfunction, dyslipidemia, RAS/RAF/extracellular-signal-regulated kinase pathway, modification of the purinergic system, phosphatidylinositol 3-kinase (PI 3-kinase)-dependent signaling pathways, and inflammation, all leading to histomorphological alterations of the kidney and vessels of diabetic and non-diabetic patients. Since a better understanding of the common cellular and molecular mechanisms of these diseases may be a key to successful identification of new therapeutic targets, we review in this paper the current literature about cellular and molecular mechanisms of CKD.

Keywords: cardiovascular diseases, diabetes mellitus, inflammation, fibrosis, chronic kidney diseases

Introduction

A healthy person could be defined as “a man with highly/tightly regulated and coordinated complex biological networks.” The crosstalk between the organs and the systems via molecular, cellular, paracrine, endocrine, and neuronal factors are essential in regulating these networks (1). However, this tight–knit relationship is deregulated at some point when your body goes through a disease or

disorder, which in turn influences the function of other organs and the challenge is to find the right target for that specific pathway (2). One example is the crosstalk between the pancreas, heart, and kidney organs, leading to vascular and renal diseases as well as diabetic mellitus (DM), which are strongly entwined with each other. Although, many mediators and pathways have been reported in the literature in regard to these diseases, their genesis is still a puzzle (3, 4). In this paper, we reviewed the current incidence, pathways, and the mediators involved in these intricate diseases and made an attempt to compel the common risk factors from the current literature.

Current Status on Incidence and Prevalence of CKD, DM, and CVD

Since two decades, the shift in the mortality and morbidity is being increased from infectious diseases to non-communicable disease worldwide (5). This rise in the number of patients mostly reflects to vascular and renal disease as well as DM, which are the major public health problem both in developed and developing countries, imposing burden on economy (6).

Chronic Kidney Disease

It is defined as a change in the kidney function or structure for more than 3 months that affects the health of an individual irrespective of the cause (7). Based on the glomerular filtration rate (GFR) and albuminuria content it is set into five stages. According to KDIGO guidelines people with $\text{GFR} \geq 90 \text{ ml/min/1.73 m}^2$ are categorized into first stage where the kidney functions normally, $60\text{--}89 \text{ ml/min/1.73 m}^2$ falls in the second stage with mildly decreased function. The third stage is sub divided into two; people with $45\text{--}59 \text{ ml/min/1.73 m}^2$ and $30\text{--}44 \text{ ml/min/1.73 m}^2$, where the GFR is mildly to severely decreased. In stage 4, the GFR decreases to $15\text{--}29 \text{ ml/min/1.73 m}^2$ and in the fifth stage ($<15 \text{ ml/min/1.73 m}^2$), kidney fails to function. The early stages are asymptomatic and the end-stage is treated by dialysis or transplantation (8). Diabetes, hypertension, cardiovascular diseases (CVD), and cigarette smoking are the common risk factors of chronic kidney disease (CKD) (9, 10). CKD arises due to many pathological insults that affects the renal function and destroys some of the nephrons. As a result, the other nephrons compensate the function of injured nephrons by hyper filtration. Over a period of time, it develops glomerular hypertension, proteinuria, and eventually loss of renal function (11). An increase in the glomerular capillary pressure leads to the destruction of glomerular capillary wall leading to the dysfunction of podocytes that covers the capillaries and allow the permeability of macromolecules (12). These series of insults result in the release of inflammatory mediators and stimulate the proliferation of cells involved in fibrosis. Proteinuria impairs the reparative mechanisms resulting in the scar formation due to the accumulation of extracellular matrix (ECM) molecules finally leading to renal failure.

Incidence and Prevalence

The incidence of CKD is not clearly known since a large number of patients die due to cardiovascular disease before they progress

to end-stage renal disease. In Europe and North America, the prevalence was found to be $\sim 10\%$ and in the US, the prevalence was found to be 13.1% (13). Zhang et al. (14) reviewed 26 population-based studies conducted on the prevalence of CKD and reported that in the elderly, the prevalence was between 23.4 and 35.8% . A meta-analysis by Nitsch et al. studied the association of GFR, albuminuria with mortality, and renal failure by sex, and observed that the increased risk of CKD is equal between both sexes (15).

Diabetes Mellitus

Diabetes mellitus is a group of metabolic disorders resulting from hyperglycemia caused by genetic, molecular, or biochemical factors and activation of renin-angiotensin system (RAS), which eventually leads to the damage of end-organs like kidney (16). Diabetic nephropathy (DN), caused by DM, is one of the progressive kidney diseases characterized by damaged vessels (angiopathy) due to type 1 or type 2 diabetes, hypertension, or dyslipidemia affecting kidney filtering system that might progress to end-stage chronic kidney disease (CKD) (17). DN is also associated with CVD increasing mortality of DM patients (18). The hallmarks of DN are the abnormalities in the glomerulus that alter the structure of podocytes, the decrease in nephrin expression, and the thickening of basement, tubular, and glomerular membranes by the extracellular deposition causing tubulointerstitial and glomerular fibrosis (18).

Incidence and Prevalence

According to 2014 statistics, 387 million people are suffering from DM and the prevalence is around 8.3% worldwide, North America being on top (11.4%). 4.9 million deaths were reported in the year 2014 and 46.3% people are undiagnosed (19). DM accounts for 4% of global deaths below the age of 70 years (20). About $25\text{--}40\%$ of diabetic patients suffer from DN and/or some degree of CKD (17, 21).

Cardiovascular Disease

Cardiovascular diseases is a group of disorders that is associated with the circulatory system including coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, cardiomyopathies, cardiac arrhythmias, and deep vein thrombosis and pulmonary embolism (22). The major risk factors associated with CVD are hypertension, hypercholesterolemia, dyslipidemia, diabetes, smoking, physical inactivity, and obesity (23). Although, few studies have shown that the decrease in the kidney function and higher albuminuria are independent risk factors and not related to diabetes and hypertension (24, 25).

Incidence and Prevalence

It is estimated that more than 17 million deaths were reported in the year 2012 globally, which accounts for 31% of all deaths and might increase to 23.3 million by the year 2030. Among these deaths, 7.4 million deaths were due to CHD and the rest were due to stroke, mostly observed in low-income and middle-income countries (22).

Key Cellular and Molecular Events

RAAS System

One of the key players that have been implicated in the pathogenesis of cardiorenal disease is renin–angiotensin–aldosterone system (RAAS). In response to the decrease in the renal perfusion pressure, renin (protease) is produced by the juxtaglomerular cells of the kidney. It acts on an inactive peptide called angiotensinogen produced by the liver, converting it to angiotensin-I, which is a rate limiting and initial step of RAAS (26). Apart from juxtaglomerular cells, the highest expression of renin (Prorenin) is observed in the connecting tubules and collecting ducts in diabetes patients (27). Angiotensin-I is catalyzed into octapeptides by angiotensin converting enzyme (ACE-dipeptidyl carboxypeptidase), produced in the lungs and lymphocytes (28), to form angiotensin II, which is an active peptide (29). Angiotensin II is also produced by chymase and cathepsin G, which are independent of ACE activity (30). Furthermore, angiotensin II is cleaved to different peptides by the endopeptidases like ACE2. Recently, many new components have been identified in the RAAS system like angiotensin, Ang III, IV, V, Ang-(1–7), Angiotensin-A, Alamandine, and their co-factors like vasodilation inducing factor (VIF) (31–34). They are mainly formed by the action of several endopeptidase that act via Mas receptors or Mas-related G-protein-coupled receptor membrane D (MrgD) receptors and they have shown to have vasodilatory effects (33, 35). The effects of Angiotensin II is mediated by the G-protein coupled receptors, i.e., angiotensin type 1 (AT1), angiotensin type 2 (AT2) receptors (30), or the MAS receptor (28). The former being expressed in several tissues like cardiovascular system, kidney, and the sympathetic nervous system (35), and the latter is expressed during fetal life and in adults restricted to the adrenals, ovary, brain, heart, and uterus (36). Angiotensin receptors share overall 30% homology and as a result they have different functions and are adapted to different signal transduction pathways (37). Ang II-AT1 axis has a role in vasoconstriction, cell proliferation, and oxidative stress in the kidney and is counteracted by AT2 receptor, which possesses vasodilatory, antiproliferative, and apoptotic properties (38, 39). Activation of AT1 receptors by angiotensin II in the kidney constricts the efferent arteriole, which results in a decrease of the blood flow that affects the glomerular filtration by raising the glomerular capillary pressure. This in turn results in glomerular injury and an increase in the production of nephrotoxic reactive oxygen species (ROS), profibrotic cytokines, and growth factors. The production of these components stimulates mitogenesis of fibroblast cells that deposit the ECM (renal fibrosis) and inhibits the turnover, leading to CKD (40, 41). Angiotensin II antagonized the contractile actions of Ang II, mediated by the Mas receptor, and Ang A has the same affinity to the AT1 receptor as Ang II, but has higher affinity toward AT2 receptor thus may modulate the harmful effects of Ang II (31, 32). Alamandine acts through the Mrg receptor and shows its vasodilatory effects (33). Furthermore, the recently identified factor vasodilation inducing factor (VIF), a chromogranin peptide, reduces the direct vasoconstrictive effects of Ang II, independent of NO in human plasma (34).

In adrenal cortex, Ang II stimulates the release of aldosterone from zona glomerulosa via AT1, which sequentially regulates the

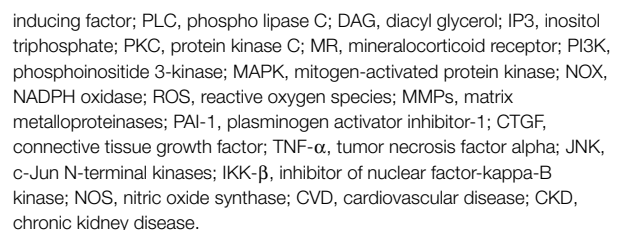
blood pressure, fluid, and electrolyte balance through mineralocorticoid receptors of the distal tubule and collecting duct (42). Aldosterone binds to the mineralocorticoid receptor that regulates the sodium–potassium pumps (43) and since it has cell proliferative and profibrotic properties, it directly increases the expression and production of the profibrotic cytokine “transforming growth factor β ” (TGF- β). In kidney disease models, aldosterone synthesis is increased and implicated in the proliferation of fibroblast cells, renal fibrosis, and induction of hypertension due to the sodium overload (44). All these effects synergistically provoke renal damage.

The notion of RAAS acting systemically has been changed by the discovery of local RAAS with paracrine or autocrine action during pathogenesis (32, 45), e.g., during hyperglycemia and proteinuria, each component of RAAS was observed in the proximal tubular cells that synthesize Ang II from angiotensinogen into interstitial and luminal side, leading to the activation of sodium pumps besides aldosterone (46, 47). In β cells of pancreas, the deleterious axis of RAAS, i.e., Ang II-ACE-AT1R-aldosterone increases the oxidative stress, promotes apoptosis, decreases the uptake of glucose (by suppressing the GLUT2 through AT1R), and increases the production of ROS through NADPH oxidase (NOX), thereby decreasing the production of insulin leading to hyperglycemia (4). **Figure 1** gives the overview of the mediators in RAAS leading to CKD, DM, and CVD.

Wnt/ β -catenin signaling pathway plays an essential role in the organogenesis and tissue homeostasis, which upon activation translocate β -catenin into nucleus, that binds to the T-cell factor (TCF) or lymphoid enhancing factor (LEF) along with other co-factors like CREB-binding protein (CBP), which eventually transcribes the target genes. In healthy subjects, this pathway is silent in kidneys, but in CKD, it is reactivated (48). By bioinformatics approach, Zhou et al. (49) demonstrated that all RAS genes have putative TCF/LEF binding sites at their promoter regions. This is a likely link between the activation of RAS by Wnt/ β -catenin during CKD.

Inflammation

Chronic inflammation is observed in patients with CKD and it contributes to the CVD morbidity and mortality by accelerating the vascular inflammation (50). Release of cytokines at the site of injury recruits the activated immune cells, which further enhances the inflammatory state by producing additional mediators (51). CXCL12 is a chemokine that binds to the CXCR4/7 and has a role in homing of stem and progenitor cells in the bone marrow to the site of injury or into the circulation. CXCL12/CXCR4 axis shows atheroprotective properties by mobilizing the endothelial progenitor cells to the site of injury (52). Local production of Ang II has been implicated in the pathophysiology of inflammation (53). Ang II stimulates the production of proinflammatory molecules like NF- κ B by Toll-like receptor 4 in addition to Ang III and Ang IV via AT1 and AT2 receptors (54, 55). Ang II also upregulates the “vascular cellular adhesion molecule-1” (VCAM), “intracellular adhesion molecule-1” (ICAM), and NF- κ B, and thereby mediates the production of chemokines like monocyte chemoattractant protein-1 (MCP-1), which recruit the immune cells (56). In mesangial cells, the expression of ETS-1



decrease in the transportation of GLUT4 to the plasma membrane and therefore the buildup of glucose concentration in the blood (65). Constant exposure to glucose leads to dysfunctioning of β cells and endothelial cells (66). In hyperglycemic conditions, the endothelial cell metabolism is modified and implicated in endothelial dysfunction, e.g., presence of high glucose is activating the NADPH oxidase resulting in the increased production of ROS and an increase in oxidative stress by reducing the entry of glucose-6-phosphate into the pentose phosphate pathway thereby reducing the NADPH formation (67, 68). Excess of glucose activates the arginase uncoupling the endothelial nitric oxide synthase (eNOS) resulting in the increase of superoxide anion production. Increased levels of glucose also diverts to polyol pathway where it is converted to sorbitol by utilizing the NADPH, increasing the ROS production, and activating NF- κ B signaling pathway (69–71). Due to changes in the endothelial metabolism, advanced glycation products (AGEs) are formed, which crosslink the extracellular molecules, causing increased vessel stiffness, resulting in vascular complications (72). The AGE products bind to their receptors (RAGE) that are expressed on the monocytes, endothelial cells, and smooth muscle cells. They are involved in the increased expression of scavenger receptor class A in macrophages, which results in the uptake of the oxidized LDL leading to the formation of foam cells, a characteristic feature of atherosclerosis (69). Cholesteryl fatty esters are the main component of foam cells that induce cytotoxicity and are involved in lesion development in atherosclerosis (73). The late stages of macrophages release the lipid content and tissue factors leading to the formation of pro-thrombotic core, resulting in rupture of plaque, leading to blood clot and contributing to myocardial infarction and stroke (73). AGE–RAGE interaction with vascular smooth muscle cells (VSMCs) is involved in cell proliferation calcification process and also activates the NF- κ B signaling pathway where it controls the expression of cytokines (71). RAGE also acts as an adhesive receptor in the endothelial cells, attracting the immune cells at the site of injury (74). Nitric oxide (NO) is formed from the arginine by eNOS in endothelium and is involved in vasodilation. Asymmetrical dimethyl arginine (ADMA), an endogenous analog of arginine, which is elevated in atherosclerotic patients competes to bind at the active site of eNOS thereby decreasing the NO (75). ROS produced due to eNOS uncoupling and NADPH oxidase activity triggers the expression of adhesion molecules, transmigration of immune cells, VSMCs proliferation and migration, endothelial apoptosis, and oxidation of lipids (69). Oxidized LDL results in the mitochondrial DNA damage and dysfunction in the endothelial cells, leading to high production of ROS (76). Xu et al. (77) analyzed cohorts of elderly adults and reported that a proinflammatory diet is associated with the systemic inflammation and reduced kidney function.

Fibrosis

At the site of cell injury or tissue damage, the cells are replaced by the same cell type or with fibrous tissue after the clearance of the inflammatory response. The kidneys have an intrinsic capacity to repair cell death by the de-differentiation and proliferation of tubular epithelial cells. Failure of these processes results in fibrosis during infarction/ischemia or toxic insult (78, 79).

Renal fibrosis is a prominent feature of every stage of CKD where an excessive accumulation and deposition of ECM are observed. At the beginning of inflammatory response in interstitium, infiltrates of macrophage population can be observed, which links inversely with the kidney function (80), that could be either deleterious (M1 macrophages) or advantageous (M2 macrophages). This is followed by transdifferentiation of interstitial cell population to myofibroblasts. These myofibroblasts have characteristics of both smooth muscle cells and fibroblast cells, which produce ECM proteins, like collagen or fibronectin, that eventually results in scar formation (81, 82). Myofibroblasts are the primary effector cells involved in both tissue remodeling and fibrosis (83). They are mainly derived from the de-differentiation of resident pericytes and fibroblast cells in the presence of fibrogenic factors that promote cell to cell interaction. These fibrogenic factors are secreted by endothelial cells, epithelial cells, and myeloid leukocytes especially the monocyte-derived cells and they are also dependent on the environmental stimuli like hyperglycemia and hypoxia (84, 85). The M1 and M2 macrophages are derived from monocytes based on the local stimuli. For example, interferon gamma (IFN γ), TLR, tumor necrosis factor, and granulocyte-macrophage colony-stimulating factor stimulate M1 macrophages. M1 macrophages are involved in tissue injury that secretes proinflammatory cytokines such as IL-12, IL-23, IL-1, IL-6, type-I interferon, and also produce reactive oxygen intermediates and nitric oxide. M2 macrophages, on the other hand, play a role in tissue repair and are stimulated by IL-4, IL-10, IL-13, corticosteroids, vitamin D, macrophage colony-stimulating factor, and TGF β (82, 86). Tubular epithelium is involved in the production of ROS and inflammatory mediators that evade the interstitium via basolateral secretion or paracrine pathways (82). They secrete mediators like monocyte chemoattractant protein-1 (MCP-1), IL-8, fractalkine, TGF β , endothelin by triggering megalin receptor-mediated protein endocytosis during protein overload. They also activate specific pathways with other co-receptors, cubilin and amnionless, leading to interstitial inflammation, fibrosis, and loss of nephron (87).

TGF- β 1

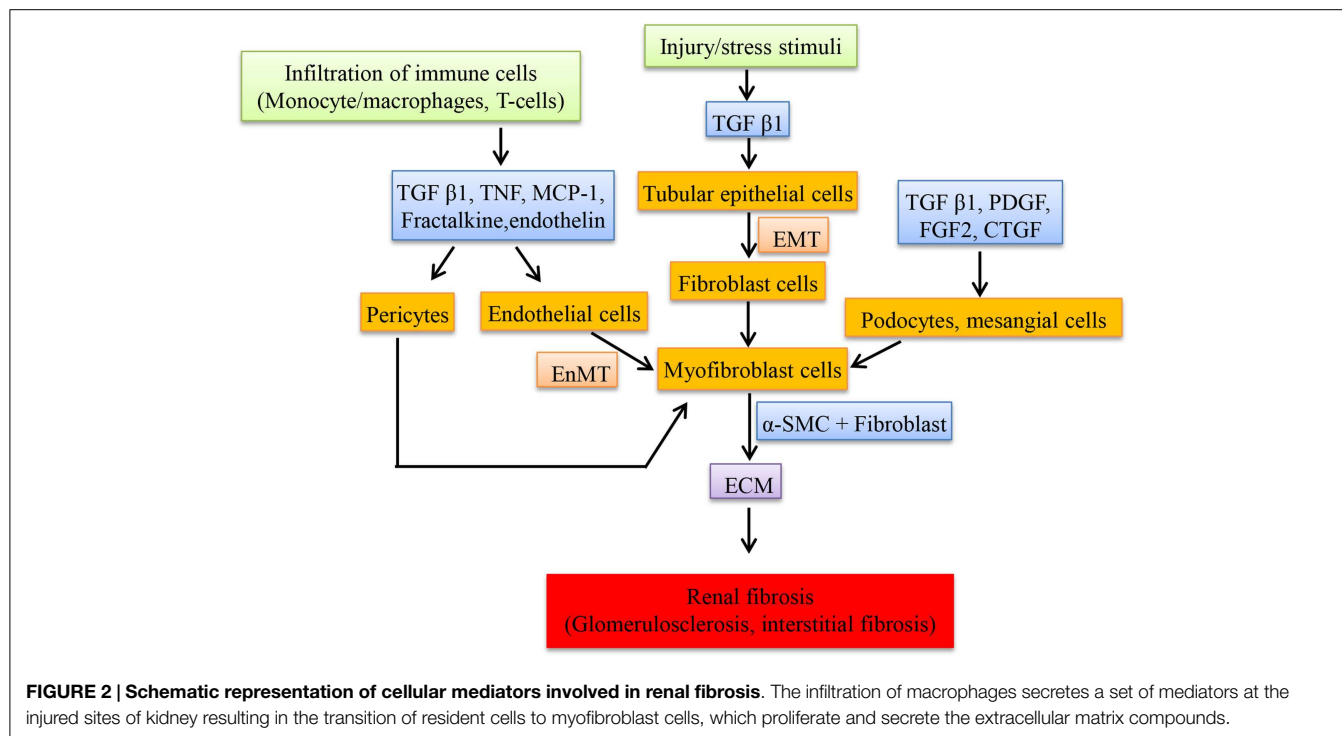
The cytokine TGF- β 1 is a prominent and powerful factor mediating myofibroblast activation, which integrates the effects of other fibrogenic factors (88). TGF- β 1 is synthesized by all types of cells in the kidney and released in association with the latency-associated peptide (LAP) binding to latent TGF- β -binding protein (LTBP). Upon stress stimuli like hypoxia (89), RAAS (90), oxidative stress (91), and TGF- β 1 are activated, binding to the Type-II TGF- β receptor. Type II TGF- β receptor is a kinase that recruits Type-I TGF- β and phosphorylates the downstream molecules like Smad2/3, which in turn forms a complex with Smad4 and then translocate to the nucleus to transcribe the target genes along with other factors (88, 92). TGF- β 1 shows profibrotic effects on kidney through different mechanisms where it induces the production of ECM through Smad3 by binding to the promoter region of collagen or Smad-independent pathways. This leads to an inhibition of its degradation by inducing tissue inhibitor of metalloproteinase (TIMPs) and inhibiting matrix metalloproteinases (MMPs). TGF- β 1 is also involved in

transdifferentiation of different types of kidney cells to myofibroblast cells (88, 93, 94). It has a role in podocytopenia where the podocytes undergo apoptosis and detach from the glomerular basement membrane resulting in the loss of integrity of microvasculature (95). In hyperglycemic conditions, the AGEs and Ang II induce transdifferentiation of epithelial to mesenchymal transition (EMT) mediated through Smad3 phosphorylation (96, 97). TGF- β 1 stimulates the differentiation of epithelial, endothelial, and macrophage cells to mesenchymal transition synthesizing ECM (81, 98). Stimulation of myofibroblast with TGF- β 1 increases the expression of cannabinoid receptor 1 that increases collagen expression (99). The latent form of TGF- β 1 is protective against inflammation and fibrosis by phosphorylating Smad7 (inhibitory Smad) (100). TGF- β 1 shows effects via Smad-independent pathways like p38, JNK, extracellular-signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), integrin-like kinase (ILK) PI3K/Akt (101–103). TGF- β activates PI3K that phosphorylates Akt and is involved in fibroblast proliferation and ECM deposition (92). Inhibition of PI3K activity results in a decrease in TGF- β -Smad2 phosphorylation and has an effect on EMT and cell migration (104). In mesangial cells, TGF- β 1 activates phosphatidylinositol 3-kinase PI3K/Akt signaling, resulting in the mesangial cell hypertrophy and fibrosis in diabetes (105, 106). High glucose levels, protein glycation end-products, Ang II, endothelin-1, and growth factors like PDGF and EGF, induce cell proliferation through PI3K/Akt signal transduction (92).

Upregulation of renin and angiotensinogen was observed in glomeruli and involved in the renal scarring or repair by the action of Ang II (107). Transfection of glomeruli with renin and angiotensinogen stimulates the proliferation of fibroblast cells,

increases the production of ECM (by inducing the mRNA of proteins like type I procollagen and fibronectin in cultured mesangial cells) and also transcribes collagen type 1 (IV) and 3 (IV) but not type I in cultured proximal tubular cells by Ang II which in turn depends on TGF- β expression (107). Renin alone is able to stimulate the expression of TGF- β in mesangial cells and this could contribute to renal fibrosis despite of Ang II blockade (108). Expression of “connective tissue growth factor” (CTGF), a fibrotic mediator is increased in the kidney induced by TGF- β and Ang II (109). Ang II induces the “plasminogen activator inhibitor-1” (PAI-1) and “tissue inhibitor of matrix metalloproteinases-1” (TIMP-1) via AT1R, which inhibits metalloproteinase affecting the matrix turnover, resulting in accumulation of ECM. PAI-1 is also stimulated by Ang IV via AT4R in proximal tubules thereby playing a role in renal fibrosis (110). In DN, Ang IV is formed by the degradation of Ang II when in high concentrations by different enzymes and this induces PAI-I (111). Most of the fibroblast cells in the interstitium originate from tubular epithelial cells through a process called EMT. This process is mediated by TGF- β and the fibroblast cells are involved in interstitial fibrosis and tubular atrophy (112). **Figure 2** shows the cells and the mediators involved in the fibrosis.

All these factors line up, and as a result the kidney loses its function and faces oxidative stress, subsequently leading to the accumulation of toxic substrates. Some of these substrates are involved in the post-translational modification of proteins like carbamylation and oxidation of LDL and HDL, which aggravate the progression of atherosclerosis thus leading to CVD (113, 114). RAAS blockade has been shown to be effective for the treatment of CKD, CVD, DM, hypertension with proteinuria. With an increase in the plasma aldosterone concentrations, the left ventricular



mass has shown to be larger in early CKD stages in hypertensive patients (115).

Uremic Toxins

Uremic toxins are in general the waste products that accumulate in the body fluid due to dysfunction of the kidney. They are divided into small water soluble compounds, middle molecules, and protein-bound uremic compounds (116). Among protein-bound uremic toxins, para-cresol sulfate (PCS) and indoxyl sulfate (IS) are linked to the cardiovascular comorbidities (117). p-cresol, a metabolite of p-cresol sulfate showed reduced contraction rates of cardiomyocytes, resulting in the irregular beating mediated by protein kinase C (PKC) by increasing the intracellular calcium levels (118). Furthermore, p-cresol is involved in the increased endothelial micro-particle shedding mediated by Rho-kinase in hemodialysis patients, leading to endothelial dysfunction, which is also observed in acute coronary syndromes, acute ischemic stroke, and venous thromboembolism (119–122).

Indoxyl sulfate also contributes to atherosclerosis and peripheral artery diseases. Indoxyl sulfate stimulates NADPH oxidase in endothelial cells, increasing the ROS formation and decreasing the levels of glutathione, thus elevating oxidative stress (123). The uptake of IS through organic anion transporter 3 (OAT 3), further stimulates the expression of “prorenin receptors” (PRR) in the aorta of a CKD mouse model, thereby activating aryl hydrocarbon receptors (AhR) and NF- κ B in VSMCs. Activation of PRR by IS promotes cell proliferation and expression of tissue factors like platelet-derived growth factor and receptor (PDGF/R) in VSMCs. It also activates MAPK pathways, thus linking the uremia-induced cardiovascular events (124, 125). Moreover, IS stimulates the production of IL-1, IL-6, and TNF- α in THP-1 cells. Lekawanvijit et al. (126) demonstrated that IS plays a critical role in cardiac remodeling by acting as profibrotic, prohypertrophic, proinflammatory factor, mediated by the activation of MAPK (p38, p42/44) and NF- κ B pathway. IS also upregulates the expression of ICAM and MCP-1 through ROS production, activating NF- κ B pathway in endothelial cells. PCS and IS stabilize the active form of epidermal growth factor (EGFR) and help in dimerization and phosphorylation, leading to an increase in the MMP2/9 expression either directly or by its downstream molecules. Therefore, they are both involved in renal tissue remodeling (127). Under oxidative stress, the protein-bound uremic solutes have an impact on the cardiac tissue architecture as well as on the kidney tissue. For example, uridine adenosine tetraphosphate (Up₄A), which is synthesized by the endothelial cells acts via purinergic receptors P2Y that activates MEK, ERK1/2 by phosphorylation, and regulates the expression of vascular calcification (VC) mediators like Cbfa, Msx2, Osx, OCN, osteopontin (OPN) leading to the transdifferentiation of vascular smooth vessel cells to osteochondrogenic cells (128, 129). In VSMCs, Up₄A stimulates the production of ROS by Nox1 dependent way and increases the expression of MCP-1 through phosphorylation of MAPK via P2Y (2) receptor (130). The uremic toxin phenyl acetic acid is associated with arterial vascular properties in patients with CKD that are undergoing hemodialysis (131). It also inhibits the inducible nitric oxide synthase (iNOS) and impairs the macrophage function, thus increasing the incidence of infection in uremia (132, 133).

Vascular Calcification: A Link Between CKD, DM, and CVD

The mechanism of VC is multifactorial and is poorly understood. The unusual deposition of calcium is observed in media and intima of the vessel, which changes the elasticity and the hemodynamics of the vessel wall that result in stroke and ischemic heart disease (134). This ectopic calcification is noticed in atherosclerosis, diabetes, hypertension, dyslipidemia, and CKD (134). The main presentations of calcification are atherosclerotic lesions, medial calcification, aortic stenosis, and calciphylaxis, which are the most dramatic and often fatal entity. Atherosclerosis, CKD, hyperphosphatemia, and vitamin K-targeting oral anticoagulants have emerged as key contributors to VC.

Vascular calcification is an active and highly regulated process, closely resembling osteogenesis. The factors contributing to the VC are the high levels of calcium and phosphorous due to abnormal bone metabolism, transition of VSMCs to chondrocyte or osteoblast-like cells, imbalance in the levels of inhibitors of calcification, low levels of klotho expression, high levels of parathyroid hormone (PTH), and fibroblast growth factor-23 (FGF-23) expression, Vitamin D, and ECM remodeling (135, 136). VC is characterized by transdifferentiation of VSMCs to osteoblast-like cells where they express a number of bone matrix proteins (137). High plasma calcium phosphate product ($\text{Ca} \times \text{P}$) contributes to this active process and hyperphosphatemia is considered a key driver of VC in CKD. VC is observed due to imbalance between inhibitory and inducing mediators. The most relevant are OPN, osteoprotegerin (OPG), fibroblast growth factor-23 (FGF-23), bone morphogenetic proteins (BMP), matrix-GLA protein (MGP), CD73, aldosterone, fetuin, pyrophosphate, magnesium, and uremic toxins like Up₄A. Increased expression of OPN is an indicator of VC and transformation of VSMC to osteoblast-like cells (138). Unphosphorylated OPN enhances whereas phosphorylated OPN decreases VC (138). The amount of OPN can be pharmacologically modified by Mg^{2+} (139, 140). OPG is a decoy receptor for receptor activator of nuclear factor-kappaB ligand (RANKL) that inhibits RANKL-induced VC and bone loss (141). OPG^{-/-} mice display osteoporosis and VC. The phosphaturic hormone FGF-23 activates the FGFR1 receptor in the presence of Klotho (142). Klotho has FGF-23-independent phosphaturic actions. However, it is still unclear whether FGF-23 or Klotho exert direct effects on VSMC phenotype (143). Klotho-deficient mice display accelerated aging, hyperphosphatemia, osteoporosis, and VC. A human klotho mutation was associated with VC in a teenager (144). Both, systemic inflammation and CKD decrease Klotho expression. In CKD, FGF-23 levels increase more than a 100-fold in response to hyperphosphatemia, and elevated FGF-23 levels are predictive of cardiovascular events (145).

Bone morphogenetic proteins are crucial mediators of vascular remodeling and neovascularization (146). BMPs regulate calcification in mesenchymal progenitor cells for cartilage and bone (147) and modify expression of osteoblast markers like alkaline phosphatase in VSMC. The expression of BMPs is increased at VC sites. In addition, BMP and osteoblast homeoprotein Msx2 signaling pathway promotes the differentiation of myofibroblasts into the osteogenic pathway and enhances VC. BMP7 is a calcification inhibitor whereas BMP-2 promotes VC (148). Protein C and

S deficit and therapy with vitamin K-targeting anticoagulants are risk factors for calciphylaxis (calcific uremic arteriolopathy) (149). Vitamin K-dependent factors, including proteins C and S, require glutamic acid carboxylation to yield gamma carboxyglutamic residues (Gla) for activation. MGP is a vitamin K2-dependent calcification inhibitor. MGP^{-/-} mice die a few weeks after birth from aortic rupture due to massive calcification. Vitamin K deficiency has been linked to VC in *in vitro* and *in vivo* mouse studies (150). Warfarin, an anticoagulant decreases the active form of MGP and increases VC in CKD rats while vitamin K increased active MGP and prevented VC in warfarin-treated rats (151).

Aldosterone contributes to VC by activating PIT1-dependent osteo-inductive signaling (152). α 2-HS-glycoprotein/fetuin levels are decreased in CKD and associate with VC. Uremic toxins, such as IS, induce expression of osteoblast-specific proteins in human VSMC and associate with aortic calcification in CKD, whereas in hypertensive rats, it promotes aortic calcification (153). Leukocyte activation evoked by CKD directly contributes to VC and pyrophosphate acts as an inhibitor of VC (154). A low extracellular pyrophosphate level induces VC in murine model of progeria (155).

Recently, protective effects of VC by magnesium through multiple mechanisms by decreasing the intimal media thickness and aortic pulse wave velocity were described (139). In CKD rats, treatment with a magnesium-based phosphate binder significantly reduced aortic calcification compared to sevelamer-treated rats (156). However, randomized controlled studies should address the impact of magnesium administration in CKD patients.

Metabolic Derangements in CKD Modulating VC

In CKD, hyperphosphatemia, iatrogenic hypercalcemia, abnormal levels of PTH and FGF/23, diabetes, and inflammation may contribute to VC.

Hyperphosphatemia

High serum phosphate is associated to progression of VC and increased cardiovascular risks in patients with CKD and in dialysis. In the general population, serum phosphorus in the high normal range is associated with increased mortality (157). Exposure of VSMCs to high phosphorus concentrations results in loss of smooth muscle proteins and differentiation into osteogenic cells (158). Osteogenic transdifferentiation may end up in cell apoptosis with production of apoptotic bodies acting as nucleation sites for mineral deposition (159). Hyperphosphatemia-induced nanocrystals upregulate the expression of BMP-2 and OPN VSMC (160). Phosphorus binders are used to reduce phosphorus levels as a key factor in the management of CKD-MBD (mineral and bone disorder). The use of calcium-containing phosphate binders that induce positive calcium balance is associated with increased arterial calcification in the majority of studies (161). The amount of calcium binders required to control phosphate results in positive calcium balance (162) and most studies reveal that the progression of VC is slower with non-calcium-based binders as compared with calcium-containing binders. Excessive load of calcium may favor the development of VC directly or through an inhibition in PTH secretion with the consequent reduction of bone turnover.

Hypercalcemia

In CKD patients, hypercalcemia is usually a consequence of excess calcium administration or vitamin D overdosing and is associated to VC. High-calcium medium increases VSMC calcification (159) and potentiates the effect of phosphate. Calcium induces the release of matrix vesicles by VSMC that become calcified in the absence of MGP (163).

Hypermagnesemia

In dialysis patients, high serum magnesium concentration was associated with less VC. Low serum magnesium was associated with increased mortality of hemodialysis and non-dialysis CKD patients in observational studies (164). Magnesium-containing phosphate binder-induced positive calcium balance is associated with increased arterial calcification in the majority of studies (165). Many interventional studies showed that magnesium-based compounds are more effective in reducing the phosphate level that improve the survival and progression of VC (166).

Vitamin D

Vitamin D may have dose-dependent opposing effects on VC. Vitamin D3 increases calcium transport into the cells and upregulates calcification-enhancing genes, osteocalcin, osterix, and Runx2 (167). Protective effects of Vitamin D3 on VC may be expected by its re-differentiating and anti-inflammatory properties (inhibition of TGF β and IL-6) (168), as well as by increasing the expression of VC inhibitors (MGP and Osteopontin).

Experimental studies *in vitro* and *in vivo* have shown that high doses of calcitriol may induce VC (169, 170). Knockout mouse models of FGF-23 and Klotho, which exhibit increased arterial calcification, have elevated calcitriol levels (171). In mice, deficiency in both FGF23 and renal 1- α -hydroxylase appear to blunt arterial calcification (171). The effect of calcitriol on VC is mainly due to the increase in phosphate absorption that in renal failure results in hyperphosphatemia.

FGF-23-klotho

The hormone fibroblast growth factor-23 (FGF-23) induces phosphaturia and inhibits calcitriol synthesis and PTH production. Serum levels of FGF-23 increase early in the development of CKD. Several clinical studies demonstrated associations between higher levels of FGF23 and VC (172). However, neutralization of FGF-23 in uremic rats results in increased VC (173). A more recent human study including a large population showed that FGF-23 is not associated with VC (174). Some authors have not been able to identify klotho, co-receptor for FGF-23 in human arteries (174). Whether FGF23 has a direct effect on VC is still under debate.

Parathyroid hormone

Hyperparathyroidism is a major risk factor for medial calcification in renal disease (175). In uremic animals, PTH infusion produced osteogenic transdifferentiation of VSMCs with increased calcium deposition regardless of phosphate intake. The severity of medial calcium deposition in the aorta correlates with the levels of serum PTH in uremic rats with ovariectomy and with the loss of cortical bone. High and low PTH is associated with high turnover and adynamic bone disease, respectively. They both lead to decreased

bone formation, a situations in which the bone is not efficient in incorporating excess calcium and phosphorus (176). This may in turn increase the risk for soft-tissue calcifications.

Diabetes mellitus

In diabetic patients, VC is commonly seen in coronary and vascular arteries of lower limbs (177, 178). Monckeberg already described medial artery calcification in an autopsy series that was identified in older age, renal failure, and diabetes as the factors associated with calcifications (179). In patient with diabetes, increased serum creatinine concentration, older age, and poor glucose control are associated with VC and mortality. High cholesterol levels, smoking, and body mass index were not associated with increased VCs. In the *Ldlr*^{-/-} diabetic mice, a high fat diet produces aortic calcification with concomitant upregulation of osteogenic gene expression (180). Incubation of bovine VSMCs with high glucose led to osteogenic transdifferentiation and calcification (181). In diabetic arteriosclerosis, *Msx1* and *Msx2* promote vascular mineralization (182). Furthermore, elevation of glucose levels increased the BMP-2/*Msx2*-Wnt pathway, which promoted osteogenic differentiation (181). Thus, it is likely that high glucose may have direct effects on the osteogenic differentiation of VSMCs and *per se* VC formation.

Dyslipidemia

From clinical studies, lipids do not appear to play a major role on VC formation. *In vitro*, HDL inhibits the osteogenic differentiation; among the fatty acids, stearate, promoted mineralization, whereas inhibition of acetyl-CoA carboxylase reduced mineralization (183). As mentioned above, in diabetes, cells are loaded with FFAs. One of the abundantly found long-chain saturated fatty

acid is palmitic acid, induces the expression of BMP-2, *Msx2*, and OPN in human aortic smooth cells through the expression of long chain acyl-coA synthetase 3 and NF- κ B that resulted in osteoblastic differentiation (184). Oxidized lipids are involved in VC. Oxidized LDL promotes the calcification process in human coronary artery smooth muscle cells by upregulating the expression of mineral matrix proteins like osterix (*Osx*) and *Runx2* mediated through nuclear factor of activated T cells (185, 186).

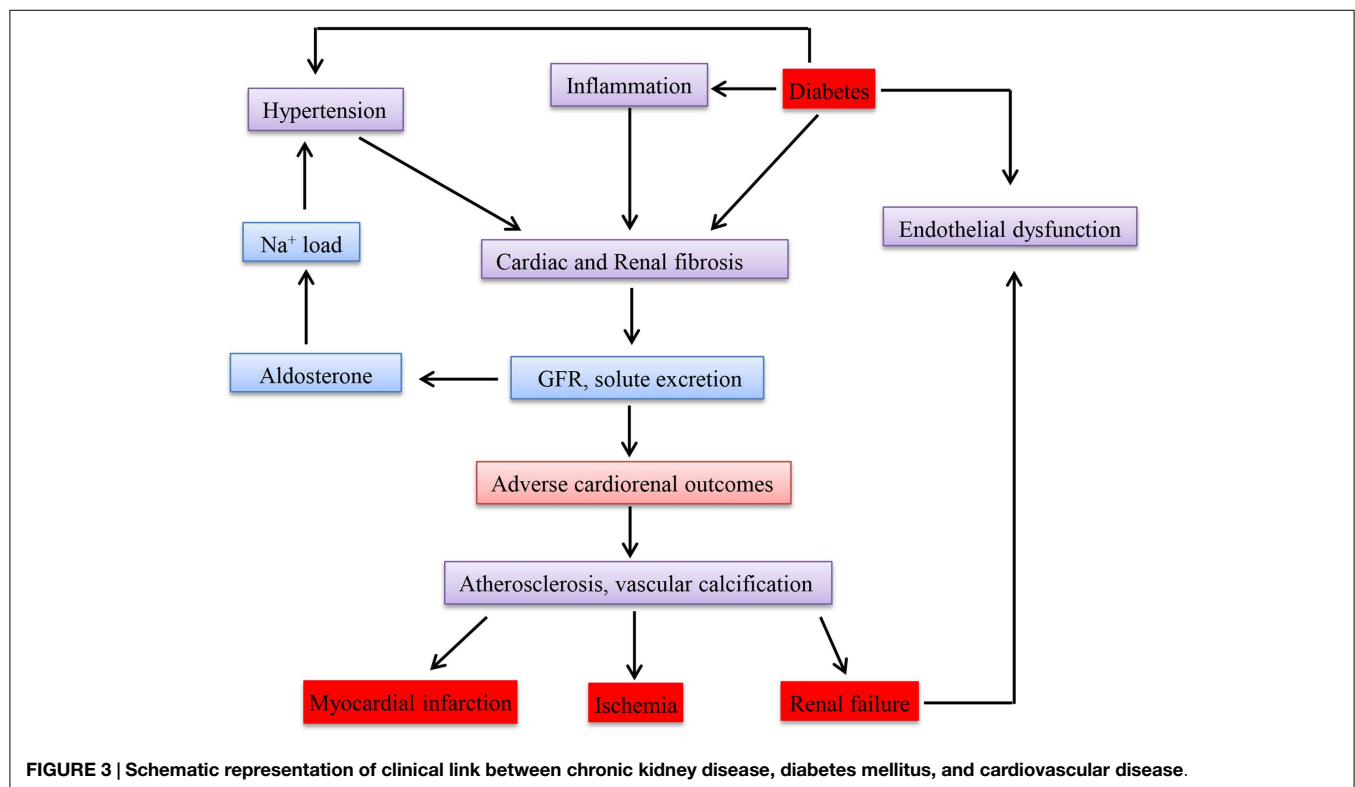
Inflammation-oxidative stress

Inflammatory cytokines and oxidative stress promote VSMC expression of osteogenic transcription factors. In a murine model of uremia, phenotypic changes in VSMCs occur prior to calcium deposition, accompanied by elastin degradation, possibly due to upregulation of proteases, including matrix metalloproteinases (187).

Oxidative stress and oxidized lipids induce osteochondrogenic differentiation of vascular cells *in vitro*. Oxidative stress induces *Cbfa1/Runx2* expression through modulation of PI3-kinase/Akt signaling (188). *In vitro* studies show that TNF- α induces calcification in VSMC. TNF- α promotes phosphate-induced calcification by reducing efflux of a calcification inhibitor, pyrophosphate, and its transport protein, ankylosis protein homolog (189). The important role of oxidant stress on VC is demonstrated in experiments showing that antioxidants block vascular cell calcification *in vitro*.

Clinical Link of the Diseases

Epidemiological studies have so far confirmed and reported that there is an association between CKD and CVD (190). Go



et al. (191) conducted a study on the relationship between GFR and cardiovascular events in a large group who had not undergone dialysis or transplantation and demonstrated an inverse relationship. The key mechanisms accounting for cardiovascular events apart from the traditional risk factors could be disturbances in mineral metabolism, anemia, ADMA (asymmetric omega NG, NG-dimethylarginine), inflammation, and oxidative stress, which are observed even in CKD. Whether CKD causes CVD or acts as a marker is still controversial (192). Due to disturbances in the metabolism in diabetes, the most effected cells are endothelial and β cells. Since the micro and macro vessels are lined with endothelial cells, the whole vascular system is damaged leading to CKD and CVD. Therefore, disturbance in one of these diseases will affect the other. **Figure 3** shows how these diseases are interlinked with each other in a clinical perspective.

Conclusion

The heart, kidney, and the vascular system are strongly related and help in maintaining hemostasis and cardiorenal equilibrium.

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Unfavorable outcomes are most common in CKD and for that reason it may deserve a specific approach to evaluate its progression. Due to the advancements in the basic research, many mechanistic pathways have been elucidated in the context of these diseases recently but none of the pathway could interpret the genesis of CKD. Therefore, a key factor in intervention for CKD patients is having an insight on the mechanisms of risk factors associated with it. Our knowledge about the underlying mechanism is increasing, but obviously still at its infancy. However, knowing the key components involved in the pathology of the diseases, their pathophysiological significance and their trend during acute phases, is essential as the basis for new prevention and treatment strategies to combat these diseases in the future.

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Animal models to study links between cardiovascular disease and renal failure and their relevance to human pathology

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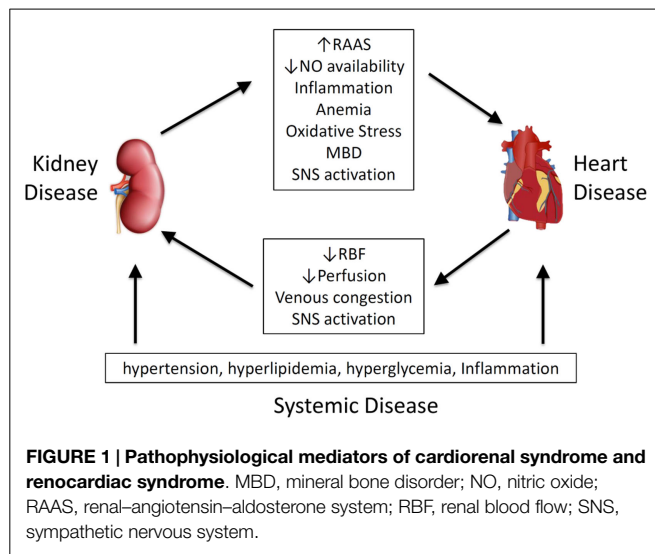
The close association between cardiovascular pathology and renal dysfunction is well documented and significant. Patients with conventional risk factors for cardiovascular disease like diabetes and hypertension also suffer renal dysfunction. This is unsurprising if the kidney is simply regarded as a “modified blood vessel” and thus, traditional risk factors will affect both systems. Consistent with this, it is relatively easy to comprehend how patients with either sudden or gradual cardiac and or vascular compromise have changes in both renal hemodynamic and regulatory systems. However, patients with pure or primary renal dysfunction also have metabolic changes (e.g., oxidant stress, inflammation, nitric oxide, or endocrine changes) that affect the cardiovascular system. Thus, cardiovascular and renal systems are intimately, bidirectionally and inextricably linked. Whilst we understand several of these links, some of the mechanisms for these connections remain incompletely explained. Animal models of cardiovascular and renal disease allow us to explore such mechanisms, and more importantly, potential therapeutic strategies. In this article, we review various experimental models used, and examine critically how representative they are of the human condition.

Keywords: animal models, cardiorenal syndrome, heart, kidney, review

Introduction

Patients with conventional risk factors for cardiovascular disease (CVD), like diabetes and hypertension, also suffer renal dysfunction. This is unsurprising if the kidney is simply regarded as a “modified blood vessel” with traditional risk factors likely to affect both systems and coexist (1). However, although a problem in one organ system affects the other, the prime mover in this loop may be occult. Animal models have helped us tease apart these associations but have consistently shown that impairment of one organ has detrimental effects on the other at functional, biochemical, and molecular level [reviewed in Ref. (2–5)]. First described by El-Atat et al. (6), these clinical interactions are now collectively known as the cardiorenal syndromes (CRS), which for many years were considered a single diagnostic group, and thus, may have limited or confounded many early mechanistic studies.

Given its importance, and the wide spectrum of primary disease and clinical presentation, a number of classification systems have been proposed. Ronco et al. have classified CRS on time frame (acute, chronic or secondary) and which organ is involved first (heart or kidney) recognizing five different types of the CRS or renocardiac syndromes (RCS) (7, 8). Other approaches are based



on pathophysiological links (9–11) (**Figure 1**), arguing that effects are bidirectional (11), and that temporal differences in organ involvement are artificial (9).

As the natural history is sometimes slow and consequently difficult to explore in clinical trials, adequate experimental modeling of the clinical scenario is crucial to examining mechanisms and potential therapeutic strategies. In this review, we use the Ronco classification (7) to discuss animal approximations of the CRS.

Models of Cardiac Injury Causing Renal Dysfunction (Types 1/2)

Neurohumoral mechanisms have evolved to maintain a relatively constant blood volume and organ perfusion under continuously changing conditions (11). In the context of a failing pump (heart), vasopressor systems, like the sympathetic nervous system (SNS) and renin-angiotensin-aldosterone system (RAAS), are activated to maintain the hemodynamic balance (12). Vasoconstriction of the efferent artery helps maintain glomerular filtration rate (GFR) in low-output states, but the increased vascular resistance may reduce overall renal perfusion and cause intra-renal hemodynamic changes. This can occur acutely in the setting of abrupt hypotension [e.g., due to myocardial infarction (MI)] or chronically, which over time causes tubular hypoxia and apoptosis leading to a loss of nephron mass and function.

In animal models, MI, produced by ligation of the left anterior descending coronary artery has detrimental effects on the renal function over time. GFR significantly decreases after MI within 4 weeks, and deteriorates further at 16 weeks. Histological analysis reveals greater renal interstitial fibrosis and downstream transforming growth factor- β 1 (TGF β 1) signaling (smad2 phosphorylation) at all time-points (13).

In rodents, beta-adrenergic stimulation through multiple isoproterenol injections results in left ventricular (LV) fibrosis (14). It remains unclear if this interference with the SNS has renal consequences, consistent with CRS.

Hemodynamic changes are a well-recognized driving force in the pathophysiology of CRS. A reduction in cardiac output (forward failure) reduces renal blood flow leading to changes in distal tubular chloride and other solute content, resulting in increased renin release from the macula densa and activation of the RAAS (15). Surgical restriction of the carotid artery leads to aortic regurgitation (16) with both cardiac hypertrophy and albuminuria from 5 months (17).

Higher central venous pressures due to congestion may increase both interstitial pressure and efferent pressure (through a decreased afferent-efferent gradient) (5). In these circumstances, elevated venous pressures (backward failure) may reduce renal blood flow and consequently urine output more than a reduction in arterial pressure, ultimately leading to hypoxia and activation of the RAAS. Elevated renal venous pressure alone reduces renal arterial flow and GFR in the pig, with increases in plasma renin activity (18). While maintaining cardiac homeostasis initially, long-term activation of the RAAS eventually leads to progression and myocardial remodeling through fibrosis (3).

Models of Acute/Chronic Renal Disease Causing Cardiac Dysfunction (Type 3/4)

As chronic kidney disease (CKD) progresses, the SNS is stimulated as a result of renal ischemia, activation of the RAAS and suppression of nitric oxide (NO) synthesis. This results in hypertension, left ventricular hypertrophy (LVH), and progressive LV dilatation (12). Early cardiac hypertrophy, and subsequent fibrosis due to fibroblast activation (19, 20), are final common contributors to organ dysfunction irrespective of the nature of the initial injury.

Cardiac Pathologies in Renal Models

Sub-total nephrectomy, often termed 5/6 nephrectomy, is probably the most established method of modeling progressive renal failure seen with loss of renal mass. Although commonly performed in the rat, similar rabbit and mouse models exist but appear less reliable. Rather than mimicking a renal disease *per se*, sub-total nephrectomy parallels the consequences of reducing functional nephron number. The predominant pathological abnormalities within the remaining kidney are glomerulosclerosis and tubulointerstitial fibrosis (21). It is important to appreciate that two quite different models are encompassed by the expression “5/6 nephrectomy”, namely an ablation model widely used for the study of CRS and a less common ligation model. In the former, one kidney is removed along with ~50% of the contralateral kidney by polar excision 1–2 weeks later, with the rate of renal decline very closely related to the amount of tissue excised (21). In contrast to human chronic kidney disease (CKD), although LVH is a consistent feature (22) and resembles that seen in early human CKD (23), severe hypertension is not usually a feature of this model. Nevertheless early LV diastolic dysfunction is seen with commensurate increases in heart weight (corrected for body weight), and myocyte cross sectional area (24, 25). Other documented pathologies include increased myocardial artery wall thickness, capillary density (26) and interstitial fibrosis (24). Similar changes are seen in mice, but tend to be more strain dependent (4).

Modeling CKD-Specific Risk Factors

While traditional cardiovascular risk factors are highly prevalent in patients with CKD, results of clinical trials focusing on controlling such factors have been largely disappointing, and thus, non-traditional risk factors associated with CKD are being increasingly explored.

Anemia

Anemia is a feature of most CKD and related to erythropoietin deficiency (27), yet it is unclear whether it is a mediator of CRS or simply a marker of disease progression (11). There is some experimental evidence that correction of anemia preserves both renal and cardiovascular function. Cardiomyopathy occurs after IV injection of anthracycline antibiotics, such as adriamycin (28, 29), with renal fibrosis and pronounced loss of renal function accompanying LV enlargement (29). In the doxorubicin model administration of darbepoetin can increase Hb to control levels and significantly attenuates the renal dysfunction, renal interstitial fibrosis and LV weight (29). Likewise, erythropoietin improves cardiac function post-MI, an effect seemingly related to the promotion of neovascularization (30). Effects in a combined cardiac-renal model have not been examined.

Inflammation

Progressive renal impairment is characterized by a chronic inflammatory state with elevated tissue and circulating concentrations of cytokines including interleukin-6 (IL-6) and tumor necrosis factor- α (TNF α). The origin of inflammation in renal disease is multifactorial, and involves reduced clearance of pro-inflammatory cytokines, reactive oxygen species and effects of comorbid conditions, such as diabetes (12). Inflammation is also a risk factor for MI and death in uremic patients (31) and a marker of severity and progression of heart failure (32). Experimentally, increased inflammatory cytokine mRNA expression is seen in the heart after renal ischemia-reperfusion injury (33). Dietary phosphate overload directly induces systemic increases in serum and tissue TNF α in a model of adenine-induced chronic renal failure (34). Conversely, renal macrophage infiltration and inflammatory cytokine expression is significantly increased from as early as 3 days post-MI (35). Activation of inflammasome pathways have been seen in both acute and CKD, with infiltrating macrophages specifically implicated (36). It is interesting to note that statins may reduce effects of inflammation in both the kidney and heart and may be protective (37).

Nitric oxide

The nitric oxide synthase (NOS) inhibitor asymmetrical dimethyl arginine (ADMA) accumulates in renal failure (38) and contributes to a reduction in NO systemically and intra-renally. NOS inhibition with N-nitro-L-arginine administration (L-NAME) depletes NO and both exacerbate renal dysfunction, and induce permanent cardiac dysfunction in rats with sub-total renal nephrectomy (39). Further evidence for the role of NO comes from those studies showing that chronic administration of L-NAME on its own also induces heart and kidney damage similar to that found in CRS (40). The model is characterized by a

progressive increase in BP over 10 weeks with severe proteinuria, glomerulosclerosis, and tubulointerstitial fibrosis, and elevation in serum creatinine (40). Further, SNS activation is a recognized feature of the L-NAME model. Proteinuria and cardiac hypertrophy induced by chronic L-NAME treatment is abrogated by bilateral renal sympathetic denervation, but not hydralazine, even when blood pressure and NO depletion are equivalent (40).

Oxidative stress

Reactive oxygen species, produced as a result of redox reactions in various cells, have been recognized as key chemical mediators causing cellular damage and organ dysfunction in both CVD and CKD. There is growing evidence that oxidative stress is one of the central mediators of CRS. Increased oxidative stress is seen in patients with cardiac (41) and renal failure (42) and the reduced availability of NO impairs vasodilatation, and reduces renal perfusion (43).

It is now widely hypothesized that interactions between the RAAS system, the SNS and inflammation, may all potentiate CRS through excessive oxidative stress pathways (44). Consistent with this, experimental studies have identified several dysregulated pathways in heart failure and in CKD that lead to increased oxidative stress. Dahl salt-sensitive rats show increased LV NADPH oxidase activity, which is normalized by Angiotensin II blockade (45). In rats with sub-total nephrectomy, mitochondrial respiration in the heart is dysregulated, with cardiomyocytes isolated from uremic animals more susceptible to oxidant induced cell death than their normal counterparts (46).

Protein-bound toxins

Uremic toxins, such as indoxyl sulfate (IS), appear to accelerate the progression of CKD via profibrotic and oxidative pathways. Oral administration of IS in sub-totally nephrectomized rats induced renal tubular injury, renal interstitial fibrosis and glomerular sclerosis, leading to functional impairment (increased serum creatinine and blood urea nitrogen). These changes are associated with increased renal expression of profibrotic genes, such as TGF β 1, tissue inhibitor of metalloproteinases-1, and procollagen α 1(I). Glomerulosclerosis and renal impairment has also been demonstrated in sub-totally nephrectomized rats receiving indole (47). The presence of IS, not indole, in the urine of indole-loaded animals confirms the protein metabolite hypothesis of IS production (47). IS has effects on cardiac myocytes and can cause cardiac hypertrophy and fibrosis (48, 49).

Neurohormonal disturbance

SNS over-activation is observed early in CKD, stemming from renal ischemia, raised angiotensin II levels, and suppression of NO amongst other causes. It is deleterious causing hypertension, LVH and eventually ventricular dysfunction and dilatation (12). Renal denervation in small animals can be achieved both surgically and chemically. These techniques have been widely used to study SNS activation and the relationship to hypertension in various experimental models of renal disease including sub-total nephrectomy (50) and deoxycorticosterone acetate (DOCA)-salt hypertension (51). Sympathectomy directly prevents onset and

progression of albuminuria after chronic cardiac volume overload caused by aortic regurgitation (17).

Mineral bone disorder

The burden of excess CVD in patients with CKD is partly attributed to systemic disturbances in mineral metabolism and changes in bone histomorphometry. This is frequently accompanied by soft tissue calcification (52) especially within the arterial wall, where it is associated with significant mortality and morbidity (53). Patients with CKD also have a preponderance of medial arterial calcification (MAC), as well as greater calcification of intimal lesions. Pathophysiologically, aortic MAC is linked to alterations in vessel compliance, which exposes the heart to changes in vascular compliance and resistance changes in systolic pressure leading to LVH and myocardial fibrosis (54). Impaired aortic recoil results in lower diastolic pressures and a widened pulse pressure and reduced perfusion of coronary arteries, leading to sub-endocardial ischemia (55). Loss of vessel compliance may also impact on renal autoregulation (56). Although not a consistent finding in man (57), rodent models of renal failure demonstrate a convincing relationship between vascular stiffness and calcification scores (58, 59).

Three rodent animal models are commonly employed to study mineral bone disorder (MBD) and its cardiovascular sequelae in the context of CKD: 1) phosphate/vitamin D loading post 5/6 nephrectomy (60); 2) adenine-induced renal failure (61); and 3) the mouse electrocautery model of CKD (62) in strains with a genetic predisposition to vascular calcification (e.g., LDLR^{-/-} or apoE^{-/-}). While these models consistently generate biochemical changes, calcification phenotypes are inconsistent due to differences in diet, study duration and the genetic background of the animals. Indeed, some inbred rodent strains (e.g., C57BL/6 mice and Sprague-Dawley rats) are surprisingly calcification resistant. Without concurrent calcitriol administration and/or high phosphate feeding (>1% diet), vascular calcification after 5/6 nephrectomy is only apparent after 24 weeks (63). The mouse electrocautery model shows variable CKD, and mild hyperphosphataemia, even with high phosphate feeding. Interestingly, induction of CKD in transgenic atherosclerotic-prone animals only imparts a modest increase in aortic calcium relative to non-uremic littermates. Induction of milder renal impairment (equivalent to CKD Stage 2) by less intensive cautery of one kidney followed by contralateral nephrectomy (64) provides compelling evidence of the development of MBD in early CKD. The generation of uremic mice with adenine-enriched diets has gained considerable interest; here, animals develop advanced CKD, hyperphosphataemia (even without dietary phosphate loading), severe hyperparathyroidism despite normocalcaemia, and MAC within 4 weeks when on a 0.75% adenine diet (65, 66).

Crucially however, the MBD animal models discussed, thus far are generated by acute injury and rely on the consequent development of CKD. To date, few models of spontaneous CKD with well-characterized MBD and vascular calcification have been described. One such model, heterozygous Han:SPRD (Cy^{-/-}) rats (a model of autosomal dominant polycystic kidney disease) develop slowly progressive CKD, hyperphosphataemia, hyperparathyroidism and bone abnormalities but the vascular

calcification generated is not progressive and only present in a subset of animals even after 38 weeks (67). Other models of spontaneous CKD (e.g., the Col4a3 null mouse model of human autosomal-recessive Alport syndrome), despite showing convincing biochemical evidence of CKD-MBD, do not exhibit a consistent vascular calcification phenotype (68).

A plethora of *in vitro* and *in vivo* studies have evoked the now widely accepted view that vascular calcification is a highly regulated and principally cell-mediated phenomenon that recapitulates many features of physiological ossification (69). There is strong evidence of osteochondrocytic differentiation of vascular cells in the calcified intimal plaques of high-fat fed LDLR^{-/-} and ApoE^{-/-} mice (70, 71), as well as in the arterial media of adenine and mineral stressed 5/6 nephrectomy models (72, 73). However, while de-differentiation of VSMC to a synthetic phenotype is found in some knockout models deficient in calcification inhibitors (e.g., matrix Gla protein (MGP)^{-/-} mice) (74), it is not a consistent finding (e.g., fetuin-A-deficient mice) (75). Moreover, mutations in the regulators of mineralization can manifest themselves quite differently in rodents and man. For instance, MGP^{-/-} mice have massive MAC. In humans, however, inactivating mutations in MGP (Keutel syndrome) exhibit infrequent arterial calcification (76). Conversely, inactivating mutations in Enpp1 encoding the pyrophosphate synthesizing ectoenzyme nucleotide pyrophosphatase/phosphodiesterase results in the devastating syndrome, Generalized Arterial Calcification of Infancy (77). Ablation of homologous gene, *Npps*, in mice however, results in ossification of the spinal ligaments and peri-articular calcification but with relatively minor arterial involvement and only a modestly shortened lifespan (78).

Finally it is worth noting that despite considerable enthusiasm for the role of VSMC phenotype switching as a major mechanism for vascular calcification, the evidence for this phenomenon in human CKD is currently limited to a subset of patients on hemodialysis (79, 80). Indeed, even in this setting, evidence of such changes appear conspicuously absent at some vascular sites exclusively affected by medial calcification (81). An explanation for this variable disease penetrance is not currently forthcoming, although it should also be stressed that analyses to date have been on small-to-medium sized muscular arteries that are generally free from intimal/atherosclerotic disease involvement and which may, therefore, not be representative of changes occurring in some larger elastic vessels (e.g., aorta). This may in part also explain the apparent disparity with findings in some animal models of uremia, where studies have mainly centered on changes in VSMC phenotype in the aorta and where phenotypic switching would appear to occur relatively early in disease progression (64).

Cardiac and Renal Involvement in Systemic Disease (Type 5 CRS)

In many cases cardiac and renal pathologies are common to a system-wide perturbation. Relevant models include metabolic syndromes, such as hypertension, diabetes, obesity, liver disease, myeloma, lupus, and other autoimmune disease. Self-evidently these models align closely with the traditional risk factors seen in CVD, and reflect both chronic and acute causes.

Chronic Conditions

Hypertension

A number of experimental models of hypertension exist, including amongst others, inbred models of inherited primary hypertension [spontaneously hypertensive rat (SHR), Milan hypertensive rat, dahl salt-sensitive rat, and transgenic models over expressing renin (mRen2)]. While these consistently display cardiac pathologies, only some have parallel changes in renal function (82), with high pre-glomerular resistance relatively protective in selectively bred SHRs (83). Whilst inherited hypertension is a rare but recognized cause of hypertension in humans (82), in animal models correction of hypertension *per se* is not sufficient to prevent progression of experimental CRS (84).

Diabetes

Several models of diabetes have shown simultaneous cardiac and renal dysfunction including accelerated models, developed in an attempt to more closely mimic the human condition. The transgenic (mRen-2) rat overexpresses the murine renin gene (85) with elevated angiotensin II activity. Streptozotocin (STZ) induced beta-cell destruction in the mRen-2 rat is an established model of diabetes and its complications. The major advantage being that these rodents develop functional and structural pathology closely mimicking that seen in advanced human diabetic nephropathy (85) and cardiomyopathy (86). In a similar manner, SHR made diabetic with STZ replicate the confounding hypertension seen in diabetic nephropathy (87). However, despite a similar rise in blood pressure, unlike the diabetic mRen2 rat, these animals do not usually progress to renal failure.

STZ administration in the atherosclerotic (ApoE^{-/-}) mouse model accelerates both diabetic renal pathology and atherosclerosis, a major risk factor for MI ischemia (88).

Obesity

Obese Zucker (OZ) rats (readily available commercially, and extensively studied) are characterized by mild glucose intolerance and peripheral insulin resistance similar to that found in humans with Type 2 diabetes. These abnormalities precede the development of albuminuria and glomerular injury and animals show a parallel deterioration in cardiac output and renal function (89). Ultrastructural studies have shown cardiomyopathy in both the OZ rat, and in mice with a similar mutation (db/db mouse) (90). A comprehensive analysis in the db/db mouse has shown that albuminuria/glomerulopathy and cardiac contractile dysfunction appear after 2–4 months of hyperglycemia (91).

Acute Conditions

Liver Disease

Both acute cholestatic and chronic fibrotic liver disease cause renal and cardiac dysfunction. Bile duct ligation causes acute and chronic renal (92) and cardiac dysfunction (93). Administration of carbon tetrachloride (CCl₄) is a model of acute (or if repeated, chronic) hepatic failure causing widespread disruption to cardiac and renal function, although CCl₄ generates oxidant injury directly in other organs (94). Other relevant experimental models include toxin induced murine models, as well as some murine models of autoimmune hepatitis and primary biliary cirrhosis (95).

Lupus

The classic murine model of lupus (as a paradigm for other autoimmune disease) includes genetically predisposed crosses (New Zealand Black crosses) and toxin (pristane) induced models (96). Nevertheless, many of the murine models fail to fully replicate the multisystem manifestations of human lupus, and whilst most replicate lupus nephritis, other organs like skin and arthritis are inconsistently affected, although the NZB murine model does seem to develop pericardial, epicardial and myocardial inflammation (97). Because the murine model is not altogether representative of the human condition, other models including canine and porcine models have been developed (98, 99).

Compound Effect of Combining Renal and Cardiac Pathologies

A less common experimental scenario looks at the effect of over-laying renal impairment on cardiac disease, and the reverse. For example sub-total nephrectomy accelerates pathological cardiac remodeling post-MI when performed 4 weeks after infarction with worse ejection fraction in those animals with renal impairment (100). Similarly when insults are reversed e.g., performing a sub-total nephrectomy a week before MI, LV damage is worse and associated with worse creatinine clearance (101) and renal blood flow, and more proteinuria and glomerulosclerosis. Sub-total nephrectomy followed by MI once renal injury is firmly established leads to more pronounced damage in both organs (102).

In sub-totally nephrectomized rats, temporary ligation of the descending branch of the left coronary artery after 3 weeks resulted in a larger area of cardiac necrosis (devoid of mitochondrial oxidation) than sham ligated paired controls. Infarcts after coronary artery ligation (CAL) were larger in animals with even modest renal impairment (84). A major disadvantage of these models is that experimental mortality is high. Although the effect is mild, reducing renal mass by uninephrectomy is also often used as a means of accelerating renal disease, but this is not in itself sufficient to produce a cardiac phenotype (103).

Conclusion

No animal model in isolation reproduces the complexity of different CRS (Table 1). Nevertheless, animal models have provided valuable insights into the pathogenesis of CRS in all its forms (5). Like their counterpart clinical trials, animal studies have highlighted the mechanistic importance of non-traditional risk factors. To this end the RAAS system, SNS, indirect and direct effects of the uremic toxins, anemia, inflammation, neurohormonal factors and disturbances in mineral handling and bone turnover are all implicated causatively. However, although the small animal models frequently employed in these studies are readily amenable to further genetic manipulation and intervention, induction of injury is generally acute and unphysiological, and these models often fail to faithfully recapitulate the pathological features of human CRS.

In this article, we have discussed a range of models that can be used to mimic the mechanisms of human renal and

TABLE 1 | Animal models of cardiorenal and renocardiac syndromes, and relative advantages and disadvantages of each.

Model	Technique	Mechanism	Advantages	Disadvantages	Reference
PRIMARY CARDIAC DISEASE					
Coronary artery ligation (CAL)	Surgical	Myocardial ischemia (MI)	Widely used, well characterized	Variable renal pathology	(13, 35)
Aortic regurgitation	Surgical	Cardiac volume overload		Mild renal pathology	(16, 17)
PRIMARY RENAL DISEASE					
Sub-total nephrectomy (SNx)	Surgical	Uremia, renal insufficiency	Relevant to CKD in general, well characterized	Highly variable if not performed uniformly	(21)
SIMULTANEOUS CARDIAC AND RENAL DISEASE					
SNx followed by CAL	Surgical			High mortality	(101)
SNx followed by CAL with established CKD	Surgical		Clinical relevance		(102)
CAL followed by SNx	Surgical			High mortality, poorly characterized	(100)
CAL and uninephrectomy	Surgical		Lower mortality than SNx, and more reproducible	Unrepresentative of chronic kidney disease	(103)
Anthracycline antitumor antibiotics (e.g., adriamycin)	IV injection	Toxicity	Simple. Simultaneous cardiac and renal pathologies	Off target toxicity, cardiac and renal dose responses differ	(29)
		Anemia	Simple	Mild anemia	(29)
L-NAME	IV injection	NOS inhibition, SNS activity	Simultaneous cardiac and renal pathologies		(40)
MODELS OF SYSTEMIC DISEASE WITH CARDIAC AND RENAL PATHOLOGIES					
Spontaneously hypertensive rat (various inbred strains)	Spontaneous	Hypertension, RAAS		Uncommon cause of human hypertension	(82)
Zucker rat (inbred rat strain with leptin receptor deficiency)	Spontaneous	Dyslipidemia	Approximates type 2 diabetes	Leptin receptor mutations are rare in humans	(89)
db/db mouse (leptin receptor mutation)	Spontaneous	Dyslipidemia	Approximates type 2 diabetes	Leptin receptor mutations are rare in humans	(91)
Diabetic mRen2 rat (STZ diabetes in transgenic renin overexpressing rat)	Spontaneous, IV injection	hyperglycemia, hypertension, RAAS	Accelerated type 1 diabetes, simultaneous cardiac and renal functional changes	Hypertension is primary rather than secondary to diabetes	(85)
Lupus	Spontaneous			Unrepresentative of human condition	(96)
Hepatic bile duct ligation	Surgical			Off target pathology	(92, 93)
BONE MINERAL DISORDERS					
Phosphate/vitamin D loading post-SNx	Surgical, oral intake	Mineral bone disorder	Widely used, well characterized	Slow, high mortality, poorly reproducible with complications, unphysiological	(60)
Adenine	Oral intake	Mineral bone disorder	Simple, rapidly progressive	Substantial weight loss (dehydration)	(61)
Electrocautery in LDLR ^{-/-} or apoE ^{-/-} mice	Surgical, spontaneous	Mineral bone disorder	Good models of atherosclerosis	Not widely available, poor models of arteriosclerosis (lack of intimal calcification).	(62)
Han:SPRD ^{+/-}	Spontaneous	Mineral bone disorder	Spontaneous, mimics chronicity of process	Not widely available, no bone phenotype, mild calcification phenotype	(67)

CAL, coronary artery ligation; L-NAME, N-nitro-L-arginine administration; NOS, nitric oxide synthase; MI, myocardial infarction; RAAS, renin angiotensin aldosterone syndrome; SNx, sub-total nephrectomy.

cardiac disease, and examined how representative they are of the human condition. While not perfect, careful and ethical use of animal models offers the opportunity to examine the complex interactions seen in CRS in an accelerated time frame.

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Diversity and inter-connections in the CXCR4 chemokine receptor/ligand family: molecular perspectives

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CXCR4 and its ligand CXCL12 mediate the homing of progenitor cells in the bone marrow and their recruitment to sites of injury, as well as affect processes such as cell arrest, survival, and angiogenesis. CXCL12 was long thought to be the sole CXCR4 ligand, but more recently the atypical chemokine macrophage migration inhibitory factor (MIF) was identified as an alternative, non-cognate ligand for CXCR4 and shown to mediate chemotaxis and arrest of CXCR4-expressing T-cells. This has complicated the understanding of CXCR4-mediated signaling and associated biological processes. Compared to CXCL12/CXCR4-induced signaling, only few details are known on MIF/CXCR4-mediated signaling and it remains unclear to which extent MIF and CXCL12 reciprocally influence CXCR4 binding and signaling. Furthermore, the atypical chemokine receptor 3 (ACKR3) (previously CXCR7) has added to the complexity of CXCR4 signaling due to its ability to bind CXCL12 and MIF, and to evoke CXCL12- and MIF-triggered signaling independently of CXCR4. Also, extracellular ubiquitin (eUb) and the viral protein gp120 (HIV) have been reported as CXCR4 ligands, whereas viral chemokine vMIP-II (Herpesvirus) and human β 3-defensin (HBD-3) have been identified as CXCR4 antagonists. This review will provide insight into the diversity and inter-connections in the CXCR4 receptor/ligand family. We will discuss signaling pathways initiated by binding of CXCL12 vs. MIF to CXCR4, elaborate on how ACKR3 affects CXCR4 signaling, and summarize biological functions of CXCR4 signaling mediated by CXCL12 or MIF. Also, we will discuss eUb and gp120 as alternative ligands for CXCR4, and describe vMIP-II and HBD-3 as antagonists for CXCR4. Detailed insight into biological effects of CXCR4 signaling and underlying mechanisms, including diversity of CXCR4 ligands and inter-connections with other (chemokine) receptors, is clinically important, as the CXCR4 antagonist AMD3100 has been approved as stem cell mobilizer in specific disease settings.

Keywords: chemokine, signaling, CXCR4, CXCL12, MIF, CXCR7, ACKR3, ubiquitin

Introduction

The CXC chemokine receptor CXCR4 is well known for its role in the homing of progenitor cells into the bone marrow. Upon injury or stress or CXCR4 blockade, progenitor cell mobilization to the periphery is enhanced (1). These functions are based on the role of CXCR4 in mediating the chemotaxis and/or arrest of CXCR4-expressing progenitor cells triggered by the CXCR4 ligand CXCL12, which is secreted by bone marrow stromal cells, but also increased in the periphery at sites of injury or cell stress. Similarly, CXCR4 influences trafficking of other cell types such as lymphocytes and neutrophils, but also CXCR4-positive cancer cells. Based on these functions, CXCR4 has been widely studied in different diseases. For example, blocking the CXCL12/CXCR4 axis with the CXCR4 antagonist AMD3100 or Plerixafor is clinically approved for the mobilization of hematopoietic progenitor cells in combination with granulocyte-colony stimulating factor (G-CSF) in patients with non-Hodgkin's lymphoma and multiple myeloma (2). Furthermore, CXCR4 has been intensively studied in conditions of injury and ischemia, such as vascular restenosis upon stent implantation or myocardial infarction, respectively, as reviewed in detail recently elsewhere (1). Also, CXCR4 has been under intensive investigation in the area of cancer and different auto-immune diseases such as rheumatoid arthritis and systemic lupus erythematosus (2, 3). Mostly, these roles of CXCR4 have been linked to its capacity to bind its cognate ligand CXCL12.

However, it has become clear that CXCR4-mediated signaling is more complex as initially thought, as also macrophage migration inhibitory factor (MIF) and extracellular ubiquitin (eUb) have been shown to bind CXCR4 and induce intracellular signaling (4, 5). Furthermore, the chemokine receptor ACKR3 (previously CXCR7) has added to the complexity of CXCR4 signaling due to its ability to bind not only CXCL11 but also CXCL12 and to interact with MIF, regulating ligand availability for CXCR4 and evoking CXCL12- and MIF-triggered signaling independently from CXCR4 (6, 7). Also, the endogenous, antimicrobial protein human β 3-defensin (HBD-3) was identified as a novel antagonist of CXCR4, blocking CXCL12-induced CXCR4 signaling (8). Moreover, CXCR4 binds the exogenous HIV protein gp120, and is therefore crucially involved in HIV infection (2, 9). On the other hand, CXCR4 is antagonized by the viral macrophage inflammatory protein-II (vMIP-II). vMIP-II is a CC chemokine expressed by Kaposi's sarcoma-associated herpesvirus, functions as an antagonist for multiple chemokine receptors, and is employed by the virus to escape the host immune system (10, 11).

Here, we will summarize the structural characteristics of the chemokine receptor CXCR4, its chemokine ligands CXCL12 and MIF, as well as non-canonical and non-host ligands eUb and HIV gp120. We will review and discuss current knowledge on intracellular signaling through CXCR4 and biological effects triggered by each of these CXCR4 ligands, and will elaborate on how ACKR3 modulates classical CXCR4 signaling triggered by CXCL12. Also, we will discuss the antimicrobial protein HBD-3 as well as the viral chemokine vMIP-II as an endogenous, respectively, viral antagonist for CXCR4 (8, 10), summarize the efforts that have been undertaken in developing CXCR4 antagonists for clinical use, and summarize clinical trials that are currently ongoing and

that target CXCR4 in different pathologies. Getting insight into the signaling mechanisms of CXCR4 by different ligands, modulation by CXCR4-containing receptor complexes and resulting biological effects is important to understand the potential merits of CXCR4 inhibition in the context of different pathologies, but also to be able to foresee potential side effects of long-term interference with CXCR4.

Structural Characteristics Affecting CXCR4 Functional Properties

The Chemokine Receptor CXCR4

Chemokines carry conserved cysteine motifs and are defined by those cysteine "signature motifs." This has led to a categorization into four classes of chemokines, i.e., the CXC, CC, C, or CX₂C chemokines where C is a cysteine residue and X any amino acid (aa) residue (12) (**Box 1**). Chemokine receptors represent a subclass of G protein-coupled receptors (GPCRs). Since chemokine receptors are classified according to the respective ligand classes, CXCR4 belongs to the family of CXC chemokine receptors (12, 13). Historically, CXCR4 was termed leukocyte-derived seven-transmembrane domain receptor (LESTR) following its cloning from a cDNA library of human monocytes in 1994 (14). Alternative names were cluster of differentiation 184 (CD184) and fusin, the latter defined as a co-factor for the fusion and entry

BOX 1 | Chemokines and chemokine receptors.

Chemokines are 8–10 kDa small proteins that belong to the superfamily of cytokines and induce chemotactic cell migration by binding to their corresponding receptors. In this way, chemokines mediate the formation of tissues, e.g., during embryogenesis or wound healing, as well as the recruitment of immune cells out of the bloodstream to sites of injury and infection (17). Chemokines are classified into four conventional subgroups according to the number and positioning of certain conserved N-terminal cysteine residues. The C-chemokines exhibit only one N-terminal cysteine, while the CC-family has two cysteines localized side by side. In the CXC chemokine family, the two cysteines are spaced from each other by one amino acid, whereas in CX₂C chemokines they are separated by three amino acids (12, 18). Additionally, a family of chemokine-like function (CLF) chemokines was defined to exhibit typical chemokine activities in the absence of a prototypical N-terminal cysteine motif (19).

Chemokines mediate their effects by binding to corresponding chemokine receptors, whose nomenclature aligns with their ligands, i.e., CR, CCR, CXCR, and CX₂CR. More generally, chemokine receptors are divided into two groups: the GPCRs, which signal via G proteins and induce various cellular functions including cell migration or leukocyte arrest, and the atypical chemokine receptors (ACKRs), which are structurally homologous to the GPCRs but are unable to couple to G proteins due to a lacking cytosolic DRYLAIV motif, and fail to induce classical chemokine signaling. Because ACKRs efficiently internalize their ligands, they can function, on the one hand, as chemokine decoy receptors and, on the other hand, they concentrate chemokines in hard-to-reach domains (20). For example, chemokine transcytosis across biological barriers mediated by the ACKR "DARC" on venular endothelial cells was shown to result in apical retention of chemokines and enhanced leukocyte migration across DARC-expressing monolayers (21).

Some chemokines exclusively bind to one receptor, while others bind a variety of receptors. Inversely, some chemokine receptors only bind one ligand, while others bind several chemokines (12, 18). This variability is referred to as "redundancy" or "promiscuity." In addition, chemokine receptor expression varies between different cell types. This capacious complexity of the chemokine/chemokine receptor network enables the fine-tuned recruitment of defined cell types to their place of destination (18).

of HIV-1 (15). The term CXCR4 was proposed by Oberlin et al. in 1996, who were the first to describe CXCL12 as a ligand for this receptor (16).

CXCR4 is an evolutionary conserved protein with 89% similarity between the human [352 aa] and mouse (359 aa) ortholog (22). Ubiquitous expression of CXCR4 has been detected in bone marrow, lymph nodes, liver, lung, brain, heart, kidney, thymus, stomach, pancreas, spleen, ovary, and small intestine (23). Similar to other GPCRs, CXCR4 has an extracellular N-terminus (34 aa for CXCR4), seven transmembrane alpha helices connected by three extracellular and three intracellular loops (ICL), and a C-terminus that is located in the cytoplasm (**Figure 1A**). However, analysis of crystal structures of CXCR4 in complex with the small-molecular antagonist IT1t and the cyclic peptide antagonist CVX15 in 2010 revealed specific differences in the orientation of the alpha helices compared with other available GPCR structures, mostly at the extracellular side (24). One important difference is the extension of the extracellular end of helix VII, which is two helical turns longer as in other GPCR structures and allows a disulfide bond of Cys274 in helix VII with Cys28 in the N-terminal region. This disulfide bond, together with and among chemokine receptors highly conserved disulfide bond between extracellular loop 2 (ECL2, Cys186) and the extracellular end of alpha helix III (Cys109), is essential for CXCL12 binding by shaping the entrance to the ligand-binding pocket (24). Compared to the extracellular half of CXCR4, which overall substantially differs from other GPCRs, the intracellular part of CXCR4 is structurally fairly similar to the intracellular half of other GPCRs. As exception, the intracellular part of helix VII of CXCR4 is one turn shorter compared to other available GPCR structures. Furthermore, the CXCR4 crystal structure lacks the short alpha helix VIII present

in many GPCRs and also lacks in its C-terminus a palmitoylation site, which in many GPCRs attaches to the lipid membrane (24). Crystallization studies revealed CXCR4 to exist as a homodimer, however, left open the possibility of CXCL12/CXCR4 binding in a 1:1 or 1:2 ligand:receptor stoichiometry (24). Recently, a combination of computational, functional, as well as biophysical methods supported a 1:1 over a 1:2 CXCL12:CXCR4 binding stoichiometry (25). This was recently further supported by the crystal structure of CXCR4 in complex with the high-affinity antagonist vMIP-II encoded by Kaposi's sarcoma-associated herpesvirus (as discussed in more detail later). CXCR4 interacted as a dimer with vMIP-II in a 1:1 stoichiometry (11). In addition to homodimerization, CXCR4 can also form dimers with ACKR3 and other receptors, as discussed in detail later.

Upon ligand binding, CXCR4 is internalized by endocytosis and degraded in lysosomes, mediated by a degradation motif (SSLKILSKGK) in its C-terminus and ubiquitination of vicinal lysine residues (28).

Endogenous Ligands of CXCR4: CXCL12, MIF, and eUb CXCL12

The chemokine CXCL12 or stromal cell-derived factor-1 (SDF-1), which is the best known ligand of CXCR4, i.e., the “cognate” ligand of CXCR4, is highly conserved between mouse and human (>92%), suggesting an evolutionarily important role (29). Six isoforms of human CXCL12 have been defined, with CXCL12- α and CXCL12- β representing the major (“classical”) isoforms, and CXCL12- γ , CXCL12- δ , CXCL12- ϵ , and CXCL12- ϕ being less well characterized. CXCL12 isoforms are generated by differential splicing events. All isoforms share the first three exons,

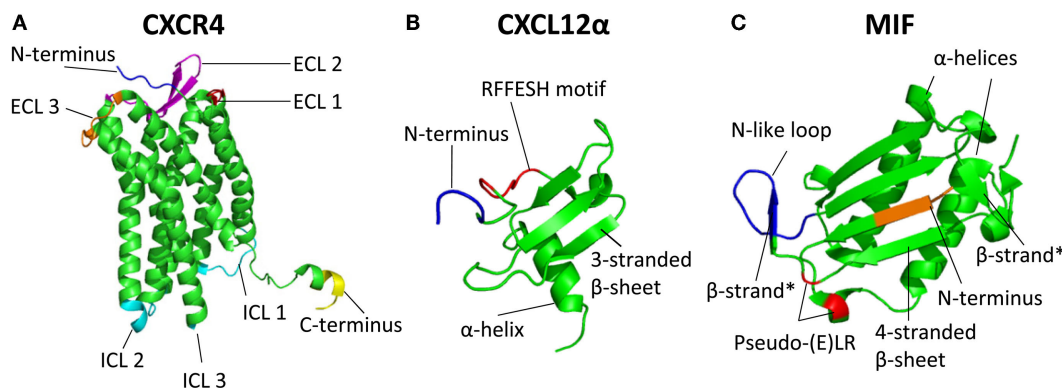


FIGURE 1 | Three-dimensional structure of the chemokine receptor CXCR4 and its ligands CXCL12 and MIF. (A) Crystal structure of CXCR4 [Protein data bank identifier 3ODU; (24)]. Included in the crystal structure are amino acids (aa) 27 to 319 of the 352 residues of CXCR4. CXCR4 can exist as a homodimer; here, only chain A is depicted. The N-terminal aa 27–31 are depicted in blue, extracellular loop 1 (aa 100–104) in red, extracellular loop 2 (aa 174–192) in purple, and extracellular loop 3 (aa 267–273) in orange. Intracellular loops (ICL) 1 (aa 65–71), ICL2 (aa 140–144), and ICL3 (aa 225–230) are depicted in cyan. The C-terminal aa 315–319 are shown in yellow. The degradation motif SSLKILSKGK (aa 324–333) cannot be depicted here due to the shortened sequence in the crystal structure. **(B)** Crystal structure of CXCL12 isoform α [Protein data bank identifier 3GV3; (26)].

Included in the crystal structure are aa 4–67 (with aa counting referring to the mature CXCL12). Here, a CXCL12 monomer is depicted with the N-terminal aa 4–7 in blue. The RFFESH motif (aa 12–17), which is the most important binding motif for CXCR4 in the CXCL12 core, is depicted in red. Each monomer includes a three-stranded β -sheet and one α -helix. **(C)** Crystal structure of MIF [Protein data bank identifier 3DJH; (27)]. Included in the crystal structure are aa 2–114 (with aa counting including Met-1). A MIF monomer is depicted. The N-terminal aa 2–5 are shown in orange. Depicted in red is the pseudo-(E)LR motif (Asp45–X–Arg12), the N-like loop (aa 48–57) is depicted in blue. Each monomer includes two antiparallel α -helices and a four-stranded β -sheet. Two additional, short β -strands (aa 48–50 and aa 107–109) can be found in each monomer stabilized by trimer formation, and are indicated with “ β -strand*.”

with observed differences in the fourth exon only. The CXCL12- α pro-protein (before N-terminal processing and secretion) is an 89 aa protein, while the other isoforms have extensions at the C-terminus with an additional 4 aa (CXCL12- β), 20 aa (CXCL12- γ), 51 aa (CXCL12- δ), 1 aa (CXCL12- ϵ), and 11 aa (CXCL12- ϕ) (1, 30). Not all isoforms show an identical expression pattern, with CXCL12- γ in adult mice mainly expressed in brain, heart, and the endothelium of vessels, compared to a broader expression pattern of CXCL12- α (31). On functional level, most research has focused on CXCL12- α , and the isoforms CXCL12- β and even to a higher extent - γ , - δ , - ϵ , and - ϕ have still been poorly characterized. However, functional differences have been revealed between the different isoforms, with, for example, CXCL12- γ binding the receptor CXCR4 with only low affinity and triggering reduced chemotaxis *in vitro* compared to CXCL12- α (31, 32). Also, the γ isoform has been shown to be present in the nucleus of mouse cardiac tissue by transcription of a distinct mRNA lacking the N-terminal signal peptide responsible for chemokine secretion (as explained in more detail below), suggesting specific intracellular functions different from the extracellular functions of the α and β isoforms (33). Furthermore, an isoform-specific role of CXCL12 has previously been suggested in the context of cerebral ischemia, where leukocyte infiltration was associated with endothelial CXCL12- β but not - α (34). In comparison to the human system, there are only three CXCL12 isoforms described in mouse. These are Cxcl12- α , - β , and - γ , which correspond to the respective human isoform counterparts, with only a single homologous aa substitution (Val to Ile substitution at aa 18 in the mature CXCL12 protein) from human to mouse (32, 33, 35).

The CXCL12 “pro-protein” contains a signal peptide of 21 aa at the CXCL12 N-terminus, which is cleaved off before secretion of the mature, biologically active CXCL12 protein. In the literature, residue numbers of important motifs of CXCL12 are numbered starting from Lys-22 in the pro-protein, now being counted as Lys-1 in the mature protein. The CXCL12 residue numbers mentioned in this manuscript are numbered accordingly.

The structure of the mature CXCL12 protein is characterized by a three-stranded β -sheet that is packed against an α -helix (**Figure 1B**) and extends to a six-stranded β -sheet in dimeric CXCL12 species (see below). The N-terminus of mature CXCL12, in particular the first two residues Lys-1 and Pro-2 (with aa indication referring to their position in mature CXCL12 throughout this manuscript), is essential for CXCR4 activation, as shown by the observation that loss of these first two residues completely abolished CXCR4 activation, while CXCR4 binding affinity was decreased 10-fold (36). A report by Crump et al. (36) and subsequent studies (18) support a so-called “two-site” model of chemokine binding to their receptors: “Site one” consists of the chemokine core domain and is responsible for docking of the chemokine to its receptor. In CXCL12, the most important core domain for CXCR4 binding is the so-called RFFESH motif (residues 12–17 in mature CXCL12). “Site two” consists of the N-terminus of CXCL12, more precisely especially Lys-1 and Pro-2, which activate CXCR4 signaling (36). The differential C-termini in the different CXCL12 isoforms are not involved in either of these site one or two interactions with CXCR4.

This “two-site” model has been proposed as a general functional mechanism of chemokines for a long time (18, 37). However, which residues of CXCR4 in particular are involved in site one and site two interaction with CXCL12 still remains to be elucidated in more detail. An important contribution to CXCL12 binding was revealed to occur through posttranslational sulfation of tyrosine residues in the CXCR4 N-terminus (Tyr-21, Tyr-12, Tyr-7). This increases the binding affinity of CXCR4 for CXCL12 through electrostatic interactions between acidic sulfated tyrosines within CXCR4 and basic residues within CXCL12 (38, 39) and is expected to contribute to “site one” interaction between CXCR4 and CXCL12. More specifically, sulfated Tyr-21 was recently predicted to interact with the N-loop- β 1 strand junction within CXCL12 based on the crystal structure of CXCR4 bound to the viral chemokine vMIP-II (11). Furthermore, this latter study revealed within the β 3 strand of vMIP-II a stretch of four residues (RQVC, aa 48–51) that interacted with CXCR4 residues D22 and E26 and that was previously shown to be important for interaction of CXCR4 with vMIP-II (11). These same residues RQVC are conserved in CXCL12 (aa 47–50), also previously shown to be important for CXCL12/CXCR4 binding, and structural modeling predicted their involvement in CXCL12/CXCR4 interaction comparably to vMIP-II/CXCR4 interaction (11). Furthermore, the crystal structure of CXCR4 in complex with IT1t as competitive antagonist of CXCL12 revealed as important ligand/receptor contact points the acidic residues Asp-187, Glu-288, and Asp-97 in CXCR4, which were previously also shown to be important for CXCL12 binding (24). With respect to the “site two” interaction important for receptor signaling, the crystal structure of CXCR4 in complex with the antagonistic peptide CVX15 was suggested to reveal a “site two” interaction of the CXCL12 N-terminus (aa 1–8 KPVSLSYR) with CXCR4, with preliminary modeling studies suggesting that CXCL12 Lys-1 could integrate into the CXCR4 binding pocket and interact with available acidic aa (24). Together, these findings underline the notion that important insights into the interaction of CXCR4 with its ligand CXCL12 can be deduced from available crystal structures of CXCR4 with other ligands or small-molecule antagonists.

Mature CXCL12 is intrinsically unstable and truncated variants of mature CXCL12 missing 2,3,5 or 7 N-terminal aa have been identified in human blood, with only non-truncated CXCL12 able to induce intracellular calcium flux and chemotaxis of stem cells *in vitro* (40). Proteases able to inactivate CXCL12 by cleaving off N-terminal residues from CXCL12 *in vitro* include dipeptidyl peptidase 4 (DPP4, also known as CD26), matrix metalloproteinase 2, neutrophil elastase, and cathepsin G (**Box 2**). For example, CXCL12 has a half-life of less than 1 min for truncation by DPP4 *in vitro* (41). This intrinsic instability of CXCL12 could at least partly contribute to the short life-time of CXCL12 *in vivo*, with wild-type CXCL12 effectively cleared from mouse blood within 1 h of intravenous application (42).

In addition, fast clearance of CXCL12 from blood seems to be partially mediated by sequestration of CXCL12 to heparan sulfate on the surface of endothelial cells through the BBXB heparin sulfate-binding motif (aa) KHLK in position 24–27; with B = basic aa and X = any other aa) in the first strand of the β -sheet of CXCL12 (50); a CXCL12 mutant that failed to bind heparan

BOX 2 | Instability of CXCL12.

Signaling of the CXCL12/CXCR4 axis is tightly regulated. One way how signaling can be terminated is by proteolytic cleavage of CXCL12. Particularly interesting is dipeptidyl peptidase IV (DPP4, also known as CD26), an enzyme that cleaves off the first two residues of CXCL12 (43), as also shown in human serum (44). Removal of these first two amino acids is sufficient to completely abolish CXCL12-induced CXCR4 activation despite an only 10-fold reduction in CXCR4 binding (36). DPP4, which can be membrane-expressed in addition to its soluble form, also co-localizes and co-immunoprecipitates with CXCR4 (45), which further establishes a specific role for this protease in the regulation of CXCR4 signaling.

Furthermore, matrix metalloproteinase 2 (MMP-2) is capable of cleaving the first four N-terminal amino acids of CXCL12, and was shown to impair CXCR4 signaling in the context of recruitment of neural progenitor cells (46). MMP-2 was first shown to inactivate CXCL12 in *in vitro* experiments by McQuibban et al., alongside the MMP family members MMP 1, 3, 9, 13, and 14 (47). Also, neutrophil elastase, released from mononucleated blood cells or polymorphonuclear neutrophils, can cleave the first three N-terminal residues of CXCL12 inducing its inactivation. In addition, it can cleave the N-terminus of CXCR4, which significantly reduces CXCR4 binding to CXCL12 (48). Finally, cathepsin G has been shown to inactivate CXCL12 by cleaving the first five residues at the N-terminus (49).

sulfate proteoglycans was retained up to 60% in the blood of mice 6 h after injection (42). Of note, the C-terminus of the CXCL12- γ isoform encompasses an additional four overlapping BBXB heparan sulfate-binding motifs, which mediate strong absorption of CXCL12- γ on the plasma membrane after secretion through interaction with cell membrane glycosaminoglycans. This enables for efficient formation of chemokine gradients (“haptotactic gradients”) directing cell migration and angiogenesis *in vivo* with higher efficiency compared to CXCL12- α , despite a lower binding affinity to CXCR4 (31). Although also the CXCL12- β isoform contains one additional BBXB motif in its C-terminus, it does not show higher affinity to heparin sulfate compared to the α -isoform. Interestingly, a knock-in mouse line carrying a mutated *Cxcl12* that cannot bind heparin sulfate without effect on *Cxcr4* signaling by the α , β , or γ isoform showed an increased number of circulating hematopoietic progenitor cells, but a reduction in the number of cells infiltrating ischemic tissue after acute hindlimb ischemia and associated revascularization (51). On the other hand, a recent report revealed truncation of five or seven N-terminal residues from CXCL12 to increase its binding affinity to the glycosaminoglycan heparin. As both truncated variants were able to induce stem cell mobilization in mice although they displayed *in vitro* no or a negative effect on CXCL12-induced chemotaxis, respectively, these findings may suggest that N-terminally truncated CXCL12 variants may influence stem cell mobilization by regulating binding of CXCL12 to glycosaminoglycans rather than to CXCR4 (40). Together, these findings reveal an important role for glycosaminoglycans in regulating CXCL12 functions.

Finally, *in vitro* experiments indicated that CXCL12 may exist in both monomeric and dimeric forms in the extracellular space. Although recent findings support a 1:1 over a 1:2 ligand:receptor stoichiometry for interaction of CXCR4 with both CXCL12 (25) as well as vMIP-II (11), it remains unclear whether dimeric CXCR4, as revealed in crystal structures, would preferentially bind dimeric CXCL12 or rather two monomeric CXCL12 proteins (24). Of note, distinct CXCL12 oligomers have been

associated with differential downstream signaling, although with contradictory findings, as discussed in more detail later.

Migration inhibitory factor

Migration inhibitory factor is a 12.3 kDa small cytokine with chemokine-like properties. It is quasi-ubiquitously expressed in various tissues in mammals and its structure is highly conserved with about 90% sequence homology between mouse and human species (52). MIF consists of 115 aa, but the N-terminal methionine residue is posttranslationally removed after ribosomal synthesis in essentially all cells and organisms. Crystallographic studies revealed MIF as a homotrimer consisting of three monomers that each has two antiparallel α -helices and a four-stranded β -sheet. Two additional, short β -strands can be detected in each monomer which interact with the β -sheet of the adjacent subunits (53) (Figure 1C). However, different studies revealed MIF to be able to exist as monomer, dimer, trimer, or even higher-order oligomers, potentially concentration-dependent, although the biologically relevant “active” oligomerization state of MIF is still elusive (53–55). As interference with MIF trimerization using the inhibitor ebselen increased MIF’s chemotactic capacity, it is however likely that the MIF homotrimer is not the only biologically active form (56). For example, MIF was recently identified as a chaperone molecule inhibiting the accumulation and mitochondrial association of misfolded superoxide dismutase SOD1, and gel filtration fractions of cellular lysates containing this inhibitory potential contained monomeric as well as oligomerized MIF (57). Of note, a chaperone function of MIF was recently suggested toward insulin to ensure full insulin function through effects on insulin conformation (58).

Migration inhibitory factor lacks a typical N-terminal leader sequence indicating that it is secreted by a non-classical secretion pathway (59). Because MIF is missing an N-terminal cysteine motif, it cannot be grouped into one of the four classical chemokine groups (C, CC, CXC, and CX₃C), which are classified by the presence and spacing of their N-terminal cysteine residues (Box 1). Nevertheless, MIF exhibits potent chemotactic properties through interaction with classical chemokine receptors and thus is a protagonistic member of the group of CLF chemokines (19, 60). MIF mediates its chemotactic effects by binding to the chemokine receptors CXCR2 and CXCR4 (4), as discussed in more detail below. MIF exhibits a structural motif (Asp45–X–Arg12, “DXR,” with aa numbering for MIF including Met-1 throughout this manuscript) similar to the conserved N-terminal ELR (Glu–Leu–Arg) motif of the cognate CXCR2 ligands, which is important for their efficient binding to CXCR2. In MIF’s so-called “pseudo-(E)LR motif,” the glutamate (Glu) is exchanged by an aspartic acid (Asp), representing a conservative substitution. Furthermore, the Asp and Arg residues in this motif are located in neighboring loops in 3D space, but show similar spacing as the authentic ELR motif (61). Mutations of Asp45 or Arg12 indeed abrogated MIF/CXCR2-mediated effects indicating the “pseudo-(E)LR motif” as structural determinant for MIF binding to CXCR2 (61). Additionally, an N-like loop including the aa from position 48 to 57 of the MIF protein was identified as being important for MIF-CXCR2 binding (62). These findings

suggest a two-site binding mechanism for MIF-CXCR2 interaction that is reminiscent of that of cognate CXCR2 ligands. Site one, consisting of the N-like loop and the CXCR2 N-domain, supposedly initializes the interaction, while the site two interaction between the pseudo-(E)LR motif and the extracellular loops of CXCR2 is assumed to lead to receptor activation (63). The interaction sites between MIF and CXCR4 still need to be identified. Additional structural characteristics of MIF related to its intrinsic catalytic oxidoreductase and tautomerase activities were recently discussed at length (19, 64) and will not be further reflected herein.

Extracellular Ubiquitin (eUb)

Ubiquitin is a small 8.6 kDa protein constitutively expressed in all eukaryotic cells which displays a very conserved structure with only little variance from insects to humans (65). Its tightly controlled expression is encoded by four genes in mammals (66). Because its amino acid structure includes seven lysines, ubiquitin can build poly-chains (67). Therefore, intracellular ubiquitin can appear as a free monomer, a free polyubiquitin chain, or as a monomer or a polyubiquitin chain conjugated to a substrate (66). Ubiquitin is well characterized as a post-translational protein modifier, with Lys-48-linked polyubiquitin chains binding covalently to lysine residues of target proteins to induce their degradation through the 26S ubiquitin-proteasome degradation pathway. However, ubiquitin also serves different degradation-independent functions affecting intracellular processes in a reversible way, as, for example, in regulating protein activity, subcellular localization, and interaction with other proteins. This is mediated by a structurally different ubiquitination of target proteins, involving the coupling of target proteins to ubiquitin monomers or polyubiquitin chains interlinked through ubiquitin lysines other than Lys-48 (e.g., Lys-63) [reviewed in Ref. (65)].

But ubiquitin is not only an intracellular protein modifier, but it is also a natural plasma protein and can be detected in urine (68). Nevertheless, not much attention has been paid to ubiquitin's "extracellular" actions. Although the existence of a cell surface receptor for ubiquitin was assumed since its discovery in 1975 (69), first evidence that CXCR4 may function as a cell surface receptor for ubiquitin was only revealed in 2010 upon studying the effect of CXCR4 antagonism or knockdown on binding of ubiquitin to CXCR4-positive cells (70). Studies concerning the structural determinants of the ubiquitin-CXCR4 interaction revealed by binding assays using fluorescently labeled ubiquitin indicated a two-site binding mechanism involving the flexible C-terminus of ubiquitin and its hydrophobic surface surrounding Phe-4 and Val-70. Within CXCR4, residues Phe-29, Phe-189, and Lys-271, which do not contribute to the CXCL12-CXCR4 interaction interface, seem to be important for ubiquitin-CXCR4 binding (71).

Gp120 as Exogenous Ligand of CXCR4

The HI-virus (HIV) is surrounded by an envelope consisting of a host cell-derived lipid bilayer and virus-encoded glycoproteins. To enter a new target cell, the virus membrane has to be fused

with the target cell membrane, a process mediated by the virus glycoprotein gp120 (**Box 3**) (72). Gp120 has a molecular weight of 120 kDa. Its secondary structure involves four surface-exposed loops which are formed by variable regions (V1-V4) with disulfide bonds at their bases. They are divided by five relatively conserved domains (C1-C5) (73). The envelope glycoproteins of HIV are encoded by the virus RNA *env*-gene (74). They are synthesized as precursors in the endoplasmic reticulum of the infected cell. After addition of asparagine-linked high-mannose sugar chains, the resulting glycoprotein gp160 is transported to the Golgi apparatus, where it is cleaved by cellular proteases into the functional envelope proteins gp120 and gp41 (75, 76). The mature glycoproteins gp41 and gp120 build up complexes which are translocated to the cell surface, where they are integrated into sprouting virions (77). Gp41 functions as a transmembrane protein and gp120 is the exterior envelope protein capping gp41 (78). The evolving so-called Env protein, a trimer of gp41-gp120 heterodimers, mediates target cell receptor binding (79). First, the CD4 binding site of gp120, consisting of the hydrophobic regions around Thr-257 and Trp-427 and the hydrophilic regions around Asp-368 and Glu-370, binds to CD4 (80). This binding induces rearrangement of V1 and V2 and subsequent of V3 (81), enabling binding of V3 to the co-receptor CXCR4 or CCR5 depending on the sequence of V3 (82). Gp120 binding to the chemokine receptors CXCR4 or CCR5 is thereby supposed to be the trigger for membrane fusion of the virus and the target cell, which is responsible for HIV infection (79). The mechanism of binding to chemokine receptors without exhibiting typical chemokine structures, like gp120 binding to CXCR4 or CCR5, is called chemokine mimicry. It is used by several viruses like pox or herpes viruses to strengthen their propagation by blocking chemokine action or triggering chemokine receptor signaling (83). Molecular mechanisms underlying gp120/CXCR4-induced cell death are discussed in more detail below.

vMIP-II and Human β 3-Defensin as Potent CXCR4 Antagonists

vMIP-II as Viral CXCR4 Antagonist

Chemokine mimicry, as described for gp120, is also involved in the interaction of CXCR4 with vMIP-II. vMIP-II is a chemokine-like protein encoded by the Kaposi's sarcoma-associated herpesvirus 8, with about 40% similarity to mammalian chemokines (10). It was described as a potent antagonist for several CC and CXC chemokines and their receptors, including

BOX 3 | Gp120.

Gp120 is the envelope protein of the HI-virus, which is responsible for the entry of the virus into cells (79). Hence, it is a main virulence factor of HIV. The position of gp120 outside the virus membrane, bound to another envelope protein gp41, enables binding to the CD4 receptor of the target cell (77). After subsequent structural rearrangement of gp120, it can bind to one of the HIV co-receptors, CXCR4 or CCR5, via chemokine mimicry (83). This is the crucial step leading to membrane fusion and consolidation of the virus with its target cell, thereby enabling infection (79). Furthermore, gp120 induces cellular apoptosis, partly through CXCR4. Molecular mechanisms underlying gp120/CXCR4-mediated apoptosis are discussed in more detail elsewhere in this review.

CXCR4, for which it competes with CXCL12 for receptor binding. However, in contrast to CXCL12, vMIP-II cannot induce Ca^{2+} mobilization from intracellular stores, nor induce chemotaxis of human monocytes (10). Zhou et al. revealed in 2000 that the N-terminus of vMIP-II, particularly the first five residues, is essential for binding to CXCR4 (84). Interestingly, native vMIP-II was able to inhibit HIV-1 viral entry into a CD4-expressing U87 cell line *in vitro* by antagonizing CCR3, CCR5 as well as CXCR4 (10). A peptide corresponding to residues 1–21 of vMIP-II showed inhibition of HIV-1 gp120-mediated cell–cell fusion only via CXCR4 (84). Together, these findings implicated vMIP-II as a promising lead molecule for anti-HIV drug development. vMIP-II-derived peptides are also being investigated as CXCR4 inhibitors in the context of cancer therapy (85).

HBD-3 as Endogenous CXCR4 Antagonist

Human β 3-defensin was proposed as a novel, endogenous antagonist of CXCR4 in 2006 by Feng et al. (8). The β -defensins are a group of antimicrobial peptides present on mucosal epithelium, which are also upregulated in the presence of HIV-1 and block HIV replication by direct binding of virions and blocking of the HIV co-receptor CXCR4 (8). HBD-3 competes with CXCL12 for CXCR4 binding, as shown by the inhibition of CXCL12-induced T-cell chemotaxis, ERK1/2 activation, and Ca^{2+} mobilization upon HBD-3 treatment (8). Confocal microscopy showed that HBD-3 treatment induced internalization of CXCR4 in a T-cell line, however HBD-3 did not trigger downstream signaling such as Ca^{2+} mobilization or ERK phosphorylation, nor chemotaxis (8). The structural features of HBD-3 important in CXCR4 antagonism were unraveled by the same group in 2013, partly by structural comparison with CXCL12. HBD-3 is a protein of 45 aa, with six conserved cysteine residues (Cys-11, 18, 23, 31, 40, and 41) forming three disulfide bonds, which stabilize the protein (86, 87). Substituting these cysteine residues with uncharged aa, generating a “linearized” HBD-3, completely abrogated the inhibitory effect of HBD-3 on CXCL12-triggered Ca^{2+} mobilization (87). Similar effects were observed upon substituting the cationic residues Lys8, Lys32, and Arg36, resembling the residues Lys-1, Arg8, and Lys-12 in CXCL12, with neutral ones; substituting the positively charged C-terminus with negatively charged residues; as well as removing the first 10 N-terminal residues (87). Of note, such structural insights into the CXCR4 antagonism by HBD-3 could stimulate the development of novel CXCR4 antagonists.

Biological Processes and Signaling Triggered by CXCL12 Through CXCR4

The CXCL12/CXCR4 axis is involved in a plethora of biological processes, including, for example, progenitor cell homing and mobilization, neutrophil homeostasis, embryonic development, and angiogenesis. These biological effects are mediated by complex signaling mechanisms, including classical GPCR signaling, β -arrestin recruitment,

or the activation of the JAK/signal transducer and activator of transcription (STAT) pathway. Also, oligomerization of CXCR4 or CXCL12, as well as interaction of CXCR4 with other signaling molecules affects the outcome of CXCL12/CXCR4 signaling. Each of these aspects will be discussed in the following section.

Biological Processes Regulated by the CXCL12/CXCR4 Axis

The CXCL12/CXCR4 axis plays an important role in the homing of hematopoietic stem and progenitor cells (HSPCs) in the bone marrow, and their mobilization to the periphery in conditions of stress or injury. Homing of HSPCs is mediated by CXCL12 secretion by endothelial cells in the bone marrow sinusoids as well as by bone marrow stromal cells. CXCR4-positive HSPCs flowing through the bloodstream are triggered to firmly adhere to the endothelium of bone marrow sinusoids through CXCL12/CXCR4-induced integrin activation (88), followed by their migration into specialized bone marrow niches. Constant trafficking of a small number of HSPCs from the bone marrow to the periphery occurs in normal physiological state, however in certain conditions such as injury or stress, an increased release of HSPCs into the bloodstream can be observed. Since in turn CXCL12/CXCR4 signaling is essential for bone marrow retention of HSPCs, interference with this pathway was shown to trigger HSPC mobilization (89). There are several proposed mechanisms of CXCR4 interference in the context of HSPC mobilization, as discussed in more detail recently (1). For example, the commonly used stem cell mobilizing reagent G-CSF was shown to induce the cleavage of the CXCR4 N-terminus on HSPCs, leading to diminished chemotaxis and arrest, and reduced retention of HSPCs in the bone marrow (90). *In vitro*, such CXCR4 cleavage could be induced by the neutrophil proteases neutrophil elastin and cathepsin G (90), although the *in vivo* relevance requires further validation.

In addition to HSPC homeostasis, the CXCL12/CXCR4 axis has been implicated to be involved in progenitor cell survival and proliferation (91, 92). Also, similar to its effect on HSPCs, CXCL12/CXCR4 signaling regulates the homeostasis of CXCR4-positive neutrophils and mediates their homing in the bone marrow through the constitutive expression of CXCL12 by bone marrow stromal cells (93). The retention of neutrophils in the bone marrow by CXCL12/CXCR4 can be blocked by the CXCR4 antagonist AMD3100 (Plerixafor), which has been used to correct neutropenia in patients (94).

Since CXCR4 is crucial in stem cell homeostasis, it is not surprising that it has essential functions in embryonic development. In fact, knockout of *Cxcr4* in mice causes several lethal birth defects, such as defective trafficking of HSPCs from the fetal liver to the embryonic bone marrow, impaired B-lymphopoiesis, impaired vascularization, and abnormal cerebellum development (95, 96). In addition to its role in vascularization during development, the CXCL12/CXCR4 also plays an important role in angiogenesis in the context of ischemia, as discussed in more detail recently (1). CXCL12 expression was shown to

be upregulated in conditions of hypoxia in a HIF-1-dependent way, resulting in increased chemotaxis and adhesion of CXCR4-positive progenitor cells to ischemic tissue (97). In a mouse model of myocardial ischemia, CXCL12 treatment leads to increased levels of vascular endothelial growth factor (VEGF) and enhanced neo-angiogenesis, associated with reduced infarction size after myocardial infarction (98). Also, the transplantation of CXCL12-overexpressing endothelial progenitor cells during myocardial infarction in rats could increase neo-angiogenesis (99). In ischemic preconditioning, which exerts cardioprotective effects, the levels of *Cxcr4* mRNA were increased in cardiac myocytes and fibroblasts, suggesting also a local protective effect of the CXCL12/CXCR4 axis. Indeed, the *in vivo* administration of CXCL12 leads to a decrease in myocardial infarct size associated with signaling through the anti-apoptotic kinases ERK 1/2 and AKT in cardiac cells (99).

Related to its role in angiogenesis, chemotaxis (100, 101), and cell proliferation (100, 102), CXCL12/CXCR4 signaling has also been linked to different pathologies including tumor progression and metastasis, as discussed in more detail later.

CXCL12/CXCR4 Binding Triggers G Protein-Coupled Signaling

CXCR4 is classified as a GPCR, indicating that one of the main pathways triggered by CXCR4 stimulation involves G protein-coupled signaling (Figure 2). The G protein complex is a heterotrimeric complex, composed of a $G\alpha$, $G\beta$, and $G\gamma$ subunit (Box 4), and is associated with CXCR4 and the inner leaflet of the plasma membrane. CXCR4 is mainly coupled to the $G\alpha_i$ subunit, which after dissociation of the $G\alpha\beta\gamma$ complex upon CXCR4 stimulation, inhibits adenylyl cyclase activity, and triggers mitogen-activated protein kinases (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathway activation (96). The $G\beta\gamma$ subunit leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to diacylglycerol (DAG) and inositol triphosphate (IP₃) by phospholipase C (PLC) and subsequent mobilization of Ca²⁺ ions from intracellular stores (103, 104). This could also be considered a downstream effect of $G\alpha_i$ activity, since the inhibition of $G\alpha_i$ activity by its potent inhibitor pertussis toxin has been reported to lead to decreased Ca²⁺ mobilization from intracellular stores (103, 105). Although CXCR4 is most likely primarily coupled to

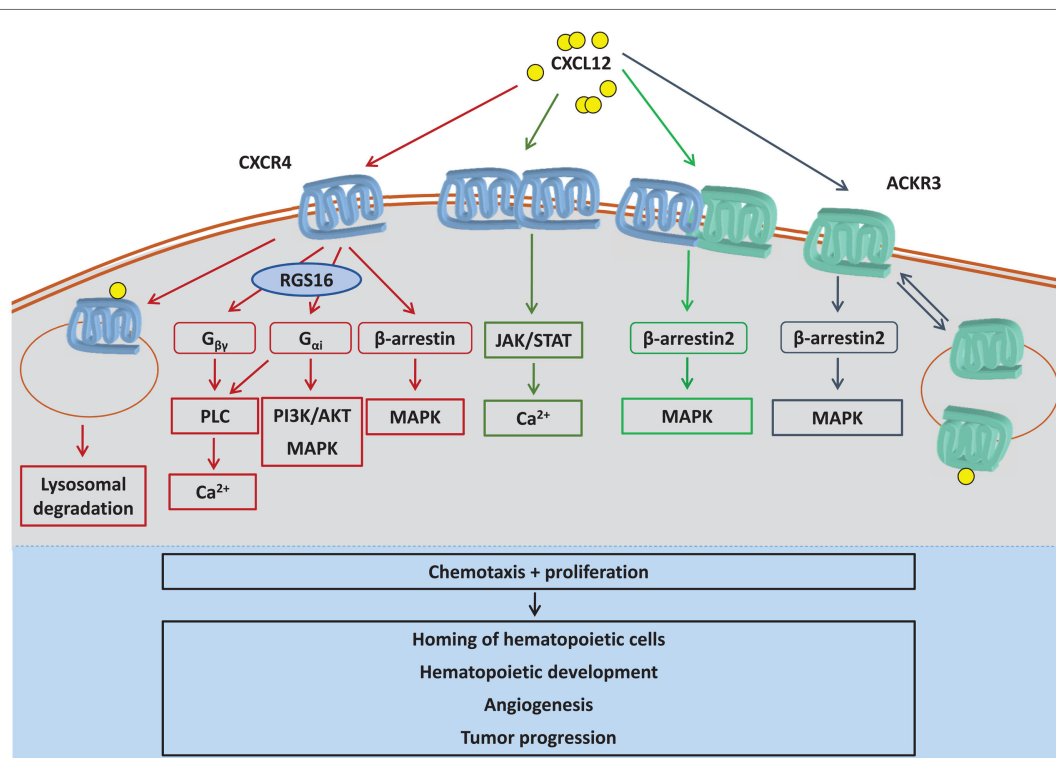


FIGURE 2 | CXCL12-induced signaling pathways. CXCL12 can trigger intracellular signaling by binding to CXCR4 monomers, CXCR4 homodimers, ACKR3, or CXCR4/ACKR3 heterodimers. CXCR4 preferentially activates G protein-mediated signaling, which is negatively regulated by RGS16. The atypical chemokine receptor ACKR3 (previously called CXCR7) functions as a CXCL12 scavenger and also signals via β -arrestin. Also, complex formation between CXCR4 and ACKR3 shifts CXCL12-induced signaling away from classical G protein signaling to β -arrestin signaling. By CXCL12-induced dimerization, CXCR4 has also been reported to induce JAK/STAT signaling. Whereas CXCR4 is mostly degraded after CXCL12-elicited

internalization, ACKR3 is continuously internalized and recycled to plasma membrane independent of ligand binding, a process that also promotes CXCL12 degradation. CXCL12 is known to induce chemotaxis and proliferation, supporting several downstream biological processes such as hematopoietic development, angiogenesis, or tumor progression. AKT, protein kinase B; Ca²⁺, calcium ions; $G\alpha$, G protein subunit α ; $G\beta\gamma$, G protein subunit $\beta\gamma$; JAK, janus kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; RGS16, regulator of G protein signaling 16; STAT, signal transducer and activator of transcription.

BOX 4 | G protein-coupled (GPCR) signaling.

G protein-coupled signaling is a major signaling pathway in most cell types. The heterotrimeric G protein complex can interact with so-called GPCRs and is composed of the three subunits $G\alpha$, $G\beta$, and $G\gamma$. In its non-active form, nucleotide guanosine diphosphate (GDP) is bound to the G protein complex. Upon ligand binding and subsequent activation and conformational change of the GPCR (107), GDP is replaced by guanosine triphosphate (GTP) and the three G protein subunits dissociate into a GTP-bound $G\alpha$ monomer and a $G\beta\gamma$ dimer (108), each triggering distinct intracellular signaling pathways. After G protein activation, GTP is again hydrolyzed to GDP through an intrinsic GTPase activity of the $G\alpha$ subunit, which in turn leads to re-association of the $G\alpha\beta\gamma$ -trimer/GPCR complex and hence termination of GPCR signaling (96, 109). The G protein subunits can be divided into several classes, with more than 16 different $G\alpha$ isoforms, 5 $G\beta$ isoforms, and 14 $G\gamma$ isoforms [reviewed in Ref. (110)]. The $G\beta\gamma$ subunit leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to diacylglycerol (DAG) and inositol triphosphate (IP₃) by phospholipase C (PLC) and subsequent mobilization of Ca²⁺ ions from intracellular stores (103, 104). The $G\alpha$ proteins are the main signal transducers, and association of GPCRs with different $G\alpha$ isoforms leads to activation of different downstream signaling mediators. For example, $G\alpha_s$ induces cyclic AMP (cAMP) production, activating protein kinase A (PKA) and thus the transcription factor cAMP-responsive element-binding protein (CREB). Both $G\alpha_s$ and $G\alpha_i$ are involved in activating PLC and subsequent mobilization of Ca²⁺ ions from intracellular stores, as well as inhibiting adenylyl cyclase activity and triggering activation of MAP kinases, the phosphatidylinositol-3-kinase (PI3K) pathway, and the NF- κ B pathway (106).

$G\alpha_i$, recent reports suggest that CXCR4 associates with other $G\alpha$ subunits, i.e., $G\alpha_q$ or $G\alpha_{12}$, each of which has been associated with different intracellular signaling cascades (Box 4) (106).

RGS16 as a Negative Regulator of CXCL12/CXCR4 Signaling

G protein-coupled signaling triggers diverse downstream signaling pathways and hence needs to be tightly regulated. Signaling termination is initiated by hydrolysis of $G\alpha$ -bound GTP to GDP by an intrinsic GTPase activity of the $G\alpha$ subunit, with subsequent re-association of the inactive G protein trimer/receptor complex (96, 109). This GTPase activity can be further enhanced by so-called regulators of G protein signaling (RGS), which bind to the activated $G\alpha$ subunit following GPCR stimulation. The family of RGS proteins consists of at least 37 members that all share a conserved 120 aa GTPase-accelerating domain (111), termed the RGS box (109). The family member RGS16 was identified as a negative regulator of CXCL12–CXCR4 signaling in 2005 (112). Overexpression of RGS16 leads to reduced CXCL12-induced migration of megakaryocytes and reduced activation of MAPK and the kinase AKT, whereas knockdown of RGS16 increased CXCR4 signaling (112). Furthermore, down-regulation of RGS16 expression by microRNA126 in endothelial cells was shown to increase CXCR4 signaling, leading to an auto-regulatory positive feedback loop through increased production of CXCL12 (113).

Involvement of the JAK/STAT Pathway in CXCL12/CXCR4 Signaling

Upon binding of CXCL12, CXCR4 can dimerize and becomes phosphorylated at intracellular tyrosine residues by rapid recruitment and activation of the Janus kinases JAK2 and JAK3. These phosphorylated tyrosines mediate the binding of STAT proteins,

which are phosphorylated by the CXCR4-bound JAK kinases, leading to STAT dimerization and initiation of the STAT signaling pathway (114) (Figure 2). The phosphorylation of CXCR4 by JAK2/3, and hence activation of the JAK/STAT pathway, is unaffected by treatment with the $G\alpha_i$ -specific inhibitor pertussis toxin. However, it is interesting to note that the complex of CXCR4 with JAK2/3 does not dissociate in pertussis toxin-treated cells, as it would normally do. This implies that the $G\alpha_i$ protein could be involved in the recycling of the JAK/STAT receptor complex by uncoupling of JAK2/3 from CXCR4 (114). The precise mechanism for this effect however remains unidentified.

The activation of the JAK/STAT pathway leads to diverse cellular effects, including Ca²⁺ mobilization from intracellular stores, which shows again the complex interplay with G protein-coupled signaling. In fact, employing a JAK-specific inhibitor, it could be shown that the association of $G\alpha_i$ to CXCR4 is dependent on JAK, further supporting a co-dependent mechanism of action between members of the JAK/STAT pathway and G protein-coupled signaling (115). Although activation of the JAK/STAT pathway by CXCR4 has interesting implications for future research, only limited data on this interaction are available to date.

Beta-Arrestin Modulates CXCL12/CXCR4 Signaling

Apart from classical signaling through G protein activation, CXCR4 has also been shown to induce β -arrestin-mediated signaling (Figure 2). β -Arrestin exists in two isoforms, β -arrestin-1 and β -arrestin-2, which have historically been described as terminators of G protein-coupled signaling. Signaling is terminated by recruitment of β -arrestin to the receptor site, whereby G protein coupling to the receptor is sterically hindered (116). In addition, β -arrestins facilitate the internalization of the receptor by acting as an adaptor for $\beta(2)$ -adaplin and clathrin, transporting the receptor to clathrin-coated pits for endocytosis (117). Interestingly, high levels of intracellular CXCR4, located in early and recycling endosomes, can be found constitutively in HSPCs, independent of ligand binding. This suggests that constitutive endocytosis of CXCR4 and possibly CXCR4 recycling to the cell membrane, mediated by clathrin-coated vesicles, are important mechanisms in HSPC regulation and trafficking (118). Similar findings were reported in fetal mesenchymal stem/stromal cells (119).

However, β -arrestin is not only involved in G protein-coupled signaling termination and GPCR internalization, but also initiates signaling itself. One of the first findings pointing to signaling mediated by β -arrestin was that β -arrestin acts as an adapter protein between SRC family tyrosine kinases and a GPCR, which in turn leads to activation of the MAP kinases ERK 1/2 (120). Since then, a variety of β -arrestin-mediated signaling effects have been described, including scaffolding for AKT, PI3K, and phosphodiesterase 4 (PDE4) in the context of specific receptors [reviewed in Ref. (116)]. G protein-independent activation of ERK 1/2 through the β adrenergic receptor (β 2AR) was reported in 2006 by Shenoy et al. (121). Treatment of HEK293 cells with isoproterenol, which activates β 2AR signaling, led to distinct ERK1/2 activation outcomes: rapid ERK1/2 activation sensitive to pertussis toxin treatment and therefore $G\alpha_i$ protein-dependent,

as well as a slower, more sustained ERK1/2 activation which was insensitive to pertussis toxin and thus G_{α_i} protein-independent, and which could be abrogated by siRNAs against β -arrestins (121). These findings show that the involvement of β -arrestin-mediated signaling in the context of GPCRs should not be underestimated. When it comes to the function of β -arrestin in regard to CXCL12, it clearly emerges that this aspect cannot be investigated without taking the interaction of CXCR4 and ACKR3 into account (122–124), as discussed in more detail below.

Homo-Oligomerization of CXCR4 and CXCL12

An important event in the activation of GPCRs is receptor dimerization, a process that is of great interest for drug targeting (125). Crystallization of CXCR4 in complex with the antagonists IT1t and CVX15 or the viral protein ligand vMIP-II revealed the existence of a CXCR4 dimer (11, 24). Also, stable, constitutive dimerization and even higher-order oligomerization of CXCR4 in the absence of a ligand could be shown in fluorescence resonance energy transfer (FRET) experiments (126, 127), and binding of the ligand CXCL12 was reported to even further increase CXCR4 oligomerization in FRET experiments (128).

Not only CXCR4, but also CXCL12 can form homodimers, and crystallization studies of CXCR4 left open the possibilities of CXCL12 interacting as monomer as well as dimer with a CXCR4 dimer (24). Of note, the oligomerization state of CXCL12 has been reported to influence signaling, however with conflicting data presented. Ray et al. report on a bias for monomeric CXCL12 to initiate G protein-coupled signaling and for dimeric CXCL12 to rather promote recruitment of β -arrestin-2 to CXCR4, as shown *in vitro* in breast cancer cells (129). By contrast, Drury et al. showed in colon carcinoma cells that dimeric CXCL12 has merely any effect on β -arrestin, whereas monomeric CXCL12 recruited β -arrestin. In this study, both CXCL12 forms however mobilized intracellular calcium and inhibited adenylyl cyclase signaling, showing activation of G_{α_i} signaling (130). These findings show that a clear-cut distinction of the dimerization state of CXCL12 and its relation to specific downstream signaling pathways cannot (yet) be made. Furthermore, to which extent dimerization of CXCL12 is favored over the monomeric form *in vivo* still remains open and is believed to be highly tissue-dependent (129).

Interaction of CXCR4 and CXCL12 with Other Signaling Mediators

Apart from homodimerization (24, 126, 127), CXCR4 forms a functional MIF receptor complex with CD74 (131) and can form heterodimers with the chemokine receptor ACKR3 (previously CXCR7), modulating CXCL12/CXCR4 signaling as will be discussed in detail below. Furthermore, oligomerization of CXCR4 with CCR5 and CCR2, chemokine receptors important for HIV infection, has been shown by FRET and co-immunoprecipitation experiments (132–137).

Also, it could be shown that CXCR4 forms a heterodimer with the T-cell receptor (TCR) upon stimulation of T-cells with CXCL12, leading to several cellular outcomes such as ERK activation, increased Ca^{2+} levels, gene transcription, and cytokine production (138). For example, a particular factor regulated by

CXCL12 signaling in a TCR-dependent way is RasGRP1, a Ras guanine-nucleotide exchange factor, which activates ERK and has important functions in autoimmunity and immunodeficiency (139). Interestingly, the heterodimerization of TCR and CXCR4 was found to be crucial for CXCR4 endocytosis in T-cells (140).

Furthermore, in an intriguing study conducted by Wagner et al., it was shown that in a mouse model of hindlimb ischemia, the administration of a TLR2 blocking antibody had similar pro-angiogenic effects compared with administration of CXCL12 and induced the activation of both ERK1/2 and AKT, the canonical signal transduction pathways of CXCR4. Immunoprecipitation experiments revealed an interaction of TLR2 and CXCR4 in endothelial cells and it was proposed that the effects of the TLR2 blocking antibody were mediated through CXCR4. Indeed, upon CXCR4 knockdown or G protein inhibition, the observed pro-angiogenic effects of the TLR2 blocking antibody were abolished, supporting a role for a functional interaction of CXCR4 with TLR2 in mediating pro-angiogenic effects of TLR2 blocking (141).

Furthermore, recent research implicates a functional interaction of a CXCR4/ACKR3 dimer with the androgen receptor, regulating CXCL12-dependent cellular motility in a prostate tumor cell line (142). Also, heteromerization of CXCR4 with $\alpha 1A/B$ -adrenergic GPCR has been reported recently in HeLa cells and human vascular smooth muscle cells (143). In addition, interactions of CXCR4 with CD4, lipopolysaccharide receptor, Epstein–Barr virus-encoded GPCR BILF1, kappa-type opioid receptor, and delta-type opioid receptor have been described [reviewed in Ref. (144)].

Finally, not only CXCR4 but also CXCL12 has been shown to interact with other signaling mediators affecting CXCL12/CXCR4 signaling: high mobility group box 1 (HMGB1), a damage-associated molecular pattern released from damaged cells, was revealed to form a heterocomplex with CXCL12, shifting the efficiency of CXCR4 activation to lower concentrations and mediating inflammatory cell recruitment *in vivo* (145).

Together, these findings show that the CXCL12/CXCR4 axis is not monogamous, but is connected to a variety of other signaling receptors.

Biological Processes and Signaling Triggered by the MIF/CXCR4 Axis

Migration inhibitory factor is involved in the progression of diverse acute and chronic inflammatory diseases including septic shock, atherosclerosis, septic lupus erythematosus, bladder pain, and allergic diseases such as eosinophilic esophagitis (19, 146–149). On the other hand, MIF also exhibits protective functions, e.g., in liver fibrosis and myocardial ischemia-reperfusion injury, associated with the activation of the protective kinases AMPK and PKC ϵ (150–153). Recently, a novel protective role of MIF was revealed in the developing cerebral cortex upon tissue damage, with MIF upregulated upon cell death and stimulating the proliferation of microglia in the developing cerebral cortex (154).

CD74, the surface-expressed form of MHC class-II-associated invariant chain, has been identified as a MIF receptor. In a signaling complex with CD44, MIF/CD74 interaction leads to ERK1/2

and AKT phosphorylation promoting cell survival and proliferation (155–157) (**Figure 3A**). The chemotactic properties of MIF are mediated via binding to the chemokine receptors CXCR2 and CXCR4 (4), in part by interaction with CD74. Interaction of MIF with CXCR2 has been shown to mediate the recruitment and arrest of monocytes and neutrophils (4, 158), with MIF–CXCR2-induced signaling discussed in detail previously (19). Via CXCR4, MIF has been shown to recruit many cell types, including T-cells, B-cells, eosinophils, endothelial progenitor cells, mesenchymal stromal cells, as well as cancer cells (4, 148, 159–162). Furthermore, MIF–CXCR4 interaction increases in experimental bladder inflammation (163), with PAR4-induced abdominal hypersensitivity shown to occur through MIF and at least partially also through CXCR4 on the urothelium (149). However, the underlying signaling events of MIF–CXCR4 association are only partially enlightened.

In T- and B-cells, MIF-induced cell migration is G_{12} -dependent and MIF induces cytosolic Ca^{2+} -influx (4, 161). Furthermore, MIF triggers the rapid activation of integrins, as shown in T-cells (4), and which induces cell arrest by binding to integrin ligands presented by, e.g., activated endothelium. In B-cells, both MIF- and CXCL12-induced migration are dependent on the participation

of the tyrosine kinase ZAP-70 (161). ZAP-70 was originally noted for its involvement in the initiation of the antigen-dependent T-cell response by phosphorylating the scaffold proteins LAT and SLP-76 (164). During T-cell migration triggered by CXCL12, ZAP-70 regulates directionality by interacting with talin, which acts as an integrin scaffold for F-actin (165). Whether ZAP-70 exerts these same functions in MIF-induced signaling remains to be unraveled.

In colon cancer cells, the MIF/CXCR4 axis induces an aggressive phenotype by inducing proliferation, adhesion, migration, and invasion of these cells, which is related to metastasis (160, 166). Similarly, the MIF/CXCR4 axis was recently shown to be the main axis mediating the recruitment of mesenchymal stromal cells to tumors and underlying their invasion capacity, both *in vitro* as well as in a pulmonary metastasis model, and this was linked with signaling through the MAP kinases MEK1/MEK2, upstream of ERK1/2 (162). On the other hand, CXCR4-expressing human rhabdomyosarcoma cells do not only show phosphorylation of ERK1/2 and AKT, but also enhanced cell adhesion after stimulation with MIF, the latter preventing their release into the circulation and thereby inhibits metastasis (7). Therefore, the role of MIF in metastasis seems to be ambivalent maybe dependent on tumor

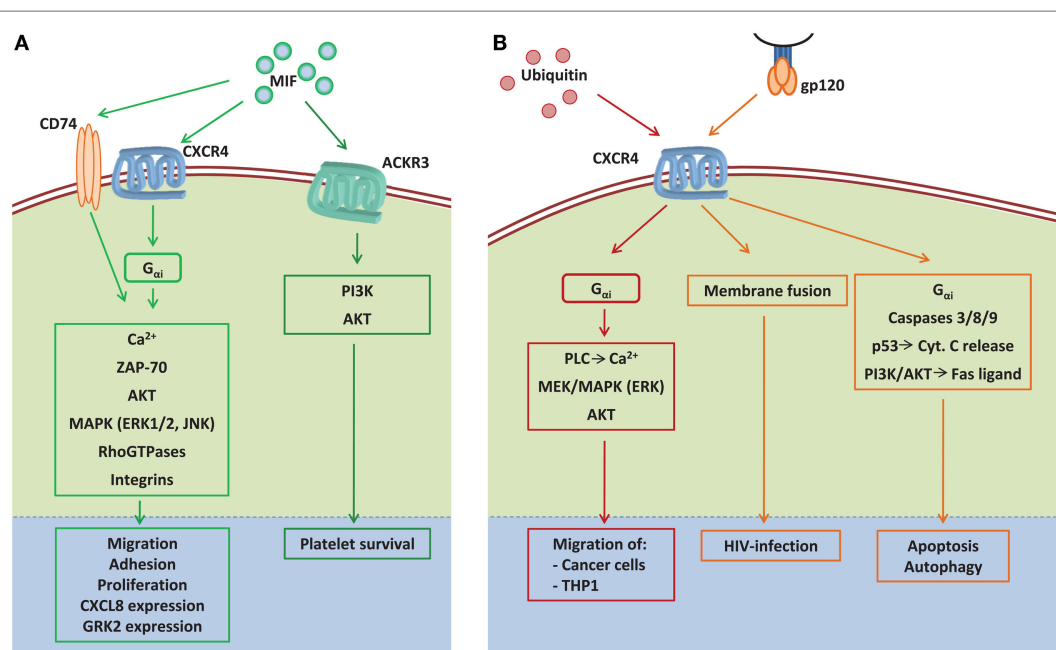


FIGURE 3 | The signaling network induced by the non-canonical CXCR4 ligands MIF, extracellular ubiquitin, and gp120. (A) MIF induces signal transduction by binding to the CXCR4, which can form a receptor complex with CD74 under certain conditions, or by binding to the atypical chemokine receptor 3 (ACKR3) (previously called CXCR7). MIF binding to CXCR4 triggers cytosolic Ca^{2+} influx, integrin activation, and ZAP-70 activation via $G_{\alpha i}$, resulting in cell adhesion, migration, proliferation, and GRK2 expression. By binding to the CXCR4/CD74 signaling complex, MIF activates ERK1/2 and AKT signaling promoting cell survival and proliferation. Additionally, the MIF–CXCR4/CD74 interaction promotes JNK phosphorylation, which results in enhanced CXCL8 expression. By binding to ACKR3 MIF induces platelet survival via the activation of PI3K and AKT. **(B)** By binding to CXCR4, ubiquitin induces the migration of cancer as well as THP1

cells through the activation of $G_{\alpha i}$ -dependent PLC-induced Ca^{2+} influx, MAPK, and AKT phosphorylation. The HIV glycoprotein gp120 uses CXCR4 as co-receptor for fusion of viral and host cell membrane, which results in HIV infection. In addition, gp120 can induce cell death via CXCR4 by activating caspases, p53-dependent cytochrome C release, and PI3K/AKT-dependent Fas ligand expression. AKT, protein kinase B; Ca^{2+} , calcium ions; Cyt. C, cytochrome C; ERK1/2, extracellular signal-regulated kinases 1/2; $G_{\alpha i}$, G α -protein subunit α ; gp120, glycoprotein 120; GRK2, G protein-coupled receptor kinase 2; JAK, janus kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MIF, macrophage migration inhibitory factor; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; THP1, monocytic cell line; ZAP-70, zeta-chain-associated protein kinase 70.

type. Interestingly, MIF, which is secreted in response to hypoxia by different tumor cells, was reported to induce CD11b⁺GR1⁺ myeloid cell migration via CD74/CXCR4 and CD74/CXCR2 complexes, as well as through p38 and PI3K activation (167). Because CD11b⁺GR1⁺ myeloid cells have gained much attention for their role in tumor immunity suppression as well as for their ability to promote angiogenesis (168, 169), MIF could support tumor growth by recruiting CD11b⁺GR1⁺ myeloid cells.

In addition, tumor-originated MIF leads to enhanced accumulation of interleukin-17-producing subsets of tumor-infiltrating lymphocytes by binding to CXCR4, which can affect patient prognosis (170). The clinical relevance of these interleukin-17-producing lymphocyte subsets for cancer seems to be cancer type-dependent. While these subsets are beneficial in ovarian cancer, the number of interleukin-17-producing subsets negatively predicts the outcome of patients with hepatocellular carcinoma (171, 172).

In 2009, Schwartz et al. could show that CXCR4 and CD74 form a functional MIF receptor complex, which induces MIF-dependent AKT phosphorylation that can be blocked by anti-CXCR4 as well as by anti-CD74 antibodies (131) (**Figure 3A**). After binding to this complex, MIF is internalized by clathrin/dynamin-dependent endocytosis leading to endosomal AKT signaling (173). This CXCR4/CD74-complex also mediates MIF-dependent JNK activation via the PI3 and SRC kinases leading to c-Jun and AP-1 activation to upregulate gene expression of CXCL8 in T-cells (174).

Despite its chemotactic activity, MIF was originally described as a substance which inhibits the random movement as well as directed migration of macrophages, hence its name “macrophage migration inhibiting factor” (175, 176). Later on, this was explained by a desensitization effect of MIF toward migration induced by other chemokines, as also shown, e.g., MCP1/CCL2- and CXCL8-induced migration (4, 177). Recently, it was suggested that MIF inhibits migration of human monocytic U937 cells through CXCR4 in the absence of CD74 via a perturbation of RHO GTPase signaling. MIF/CXCR4 interaction was shown to induce G protein-coupled activation of RHO-A followed by subsequent inactivation. In addition, RAC1 was transiently inactivated while Cdc42 showed cyclic activation and inactivation. Together, these results were suggested to contribute to MIF-induced migration inhibition in U937 cells. Furthermore, they indicate that CXCR4 can mediate MIF signaling in the absence of CD74, in addition to its function as MIF co-receptor in complex with CD74 (178).

Finally, increased levels of MIF, as observed in plasma of diabetic patients, also enhance the expression of GRK2 in cardiomyoblasts, an effect mediated through CXCR4 (179). As an upregulation of GRK2 in cardiomyocytes precedes the development of heart failure (180), this finding might suggest that MIF could promote heart failure by amplifying GRK2 expression.

CXCR4 Signaling Triggered by the Non-Canonical Ligands eUb and gp120

Extracellular Ubiquitin (eUb)/CXCR4 Signaling

Opposite to the well-characterized functions of ubiquitin as intracellular signaling molecule, as discussed earlier, only little attention has been paid to its extracellular actions. eUb can be

found in human serum in concentrations varying in response to different diseases. The cellular source and the underlying release mechanism remain unclear (181, 182), but active secretion of ubiquitin was assumed after increased ubiquitin concentrations were measured in the supernatant of Ba/F3 B-cells and 293T human embryonic kidney cells after transfection with an ubiquitin expression vector (183). Furthermore, it has been shown that eUb can be taken up by cells (184), and induces Ca²⁺ flux after binding to THP1 monocytes. Ubiquitin-induced Ca²⁺ flux could be inhibited by the use of the G protein inhibitor pertussis toxin, indicating that ubiquitin signals via a GPCR. By gene silencing and the use of the CXCR4 antagonist AMD3100 Saini et al. showed that eUb signals via CXCR4 and leads to Ca²⁺ flux and reduced cAMP levels (70). Ubiquitin-induced Ca²⁺ flux can also be attenuated by the PLC inhibitor U73122, indicating that PLC plays a role in ubiquitin-induced signaling events (185) (**Figure 3B**).

Extracellular ubiquitin has chemotactic properties inducing the migration of different cancer cell types as well as THP1 cells via CXCR4, PLC, AKT, and the MEK/ERK pathway (186–188). The effects of ubiquitin and the canonical CXCR4 ligand CXCL12 are partially synergistic: co-stimulation with suboptimal ligand concentrations leads to enhanced Ca²⁺ flux, without synergistic effects on cAMP levels, AKT and ERK1/2 phosphorylation or chemotactic responses (188). In 2011, ubiquitin has been characterized as a substrate for the ubiquitously expressed cell surface protein “insulin degrading enzyme” (IDE), cleaving the C-terminal di-Gly aa from ubiquitin (189). This modification modulates CXCR4-mediated ubiquitin signaling. Reduced IDE expression enhances the ubiquitin-induced reduction in cAMP levels and reinforces the chemotactic activity of ubiquitin (186). Modulation of ubiquitin by IDE could thereby contribute to the fine-tuning of CXCR4-mediated cell functions.

Gp120/CXCR4 Signaling

Binding of gp120 on the surface of the HI-virus to CD4 and the HIV co-receptors CCR5 or CXCR4 leads to the fusion of the viral and the host cell membrane (79). Also, binding of gp120 to CXCR4 is involved in HIV-mediated apoptosis of infected and uninfected lymphocytes (190), neurons (191), cardiomyocytes (192), hepatocytes (193), and different cancer cells (194, 195). The underlying signaling events are controversially discussed (**Figure 3B**). CXCR4 signaling is typically mediated via the Gα_i protein. But in case of gp120/CXCR4-mediated apoptosis, the signaling pathway seems to be Gα_i protein-independent, at least in T-cells (196, 197), whereas gp120/CXCR4-induced apoptosis of breast cancer cells (194) and hepatocytes (193) depends on Gα_i protein involvement. Also, the participation of caspases seems to be cell type-dependent: caspases 8 and 9 play an essential role in gp120/CXCR4-induced apoptosis of cardiomyocytes (192); gp120/CXCR4-induced apoptosis of hepatocytes is caspase-independent (193); but T-cells seem to display a caspase-independent pathway as well as a pathway involving caspases 3 and 9 (197, 198). By the use of CD4 inhibitors or CD4 mutants, different studies reported that gp120/CXCR4-induced apoptosis is CD4-independent. By contrast, CD45 was observed to be important for gp120/CXCR4-mediated T-cell apoptosis, and through interaction with CXCR4, CD45 is involved in Fas ligand activation via

the PI3K/AKT pathway (199). Gp120 expression on the surface of infected cells can also cause apoptosis by binding to a CD4/CXCR4-expressing cell. The resulting “fused cell,” also called syncytium, undergoes apoptosis mediated by mitochondrial membrane permeabilization and cytochrome c release involving p53 activation (200, 201).

To gain more insight into the molecular mechanisms leading to gp120/CXCR4-mediated apoptosis of lymphocytes, Molina et al. performed a proteomic analysis of immune cells after coculture with cells expressing gp120. This approach showed that most proteins involved in gp120/CXCR4-mediated apoptosis can be linked to degradation processes, redox homeostasis, metabolism, or cytoskeleton dynamics (202). Not only apoptosis but also autophagy of CD4 T-cells can be induced by gp120 binding to CXCR4, but the underlying signaling events remain unclear (203). Because gp120/CXCR4-induced lymphocyte death leads to pathological immunodeficiency, a lot of research is still ongoing to identify a CXCR4 antagonist that would selectively prevent the gp120/CXCR4 interaction and thereby HIV-triggered disease progression (204).

ACKR3 Influences Signaling through CXCR4

Atypical chemokine receptor 3 (ACKR3) (originally named RDC-1 or CXCR7) was first described in 1989 as a putative GPCR cloned from thyroid complementary DNA (205). Further evidence for RDC-1 belonging to the seven-transmembrane-spanning receptor family was obtained by sequence analysis (206). The notion that RDC-1 might be part of the CXC family of chemokine receptors was brought up by Heesen et al., who reported in 1998 a 55% nucleic acid similarity between mouse RDC-1 and rabbit CXCR2 (207). In 2005, Balabanian et al. identified the orphan receptor RDC-1 as another receptor for CXCL12 and hence suggested to rename it to CXCR7 according to the chemokine nomenclature (6). In 2014, the name was changed into ACKR3 (12). Of note, ACKR3 was reported to have a ~10-fold higher affinity for CXCL12 compared with CXCR4 (6, 36, 208). This finding has considerably changed the view of CXCL12/CXCR4 signaling, as CXCR4 was until then believed to be the only receptor for CXCL12, and since, ACKR3 has been under intensive investigation.

ACKR3 is expressed in a variety of tissues, including, for example, embryonic neuronal and heart tissue, hematopoietic cells, and activated endothelium (209). Its expression has also been linked to a variety of cancers, including, for example, breast and lung tumors (210), brain metastases (211), and renal cell carcinoma (212). Apart from cancer research, ACKR3 has been implicated in other diseases, for example in acute coronary syndrome, in which platelets from patients showed enhanced surface expression of ACKR3 but not CXCR4 (213).

CXCL12 as a Ligand for ACKR3 and Interrelation with CXCL12/CXCR4 Signaling

With ACKR3 as a high-affinity receptor for CXCL12 (6, 36, 208), ACKR3 functions as a scavenger receptor for CXCL12, thereby downtuning classical CXCL12/CXCR4 signaling. On the other

hand, ACKR3 is also able to induce CXCL12 signaling independently from CXCR4 (6, 214–216), as will be discussed in more detail below. It is currently thought that ACKR3 does not *per se* induce G protein signaling upon binding of CXCL12, based on which the previous name CXCR7 was recently renamed into ACKR3 based on its classification in the group of ACKRs that do not signal through G proteins (12), although this has been challenged by others (217).

ACKR3 continuously cycles between the plasma membrane and intracellular compartments without or with ligand binding, and in contrast to CXCR4, ACKR3 is not degraded after internalization (209) (**Figure 2**). ACKR3 internalization is dependent on β -arrestin and also involves constitutive ubiquitination for correct trafficking of ACKR3 (218).

Co-expression of CXCR4 with ACKR3 resulted in heterodimerization independent of ligand binding (219). This was associated with a constitutive recruitment of β -arrestin-2 to the CXCR4/ACKR3 complex, with simultaneous down-regulation of $G\alpha_i$ -mediated signaling as shown by a cAMP reporter gene assay as read-out of $G\alpha_i$ signaling (123) (**Box 4; Figure 2**). Based on these results, Decaillot et al. postulated that the CXCR4/ACKR3 heterodimer down-tunes classical CXCL12/CXCR4-triggered G protein-coupled signaling by preferentially triggering the recruitment of β -arrestin and hence inducing β -arrestin-mediated signaling pathways, including activation of the MAP kinases ERK1/2, p38 and SAPK. The activation of these pathways is enhanced by binding of CXCL12 to the receptor complex, which leads to an increased recruitment of β -arrestin compared to the interaction of CXCL12 with CXCR4 alone (123). Of note, CXCR4/ACKR3 heterodimers form as efficiently as CXCR4 homodimers, indicating that these mechanisms are of equal importance (219).

Furthermore, recent reports showed additional negative regulatory functions of ACKR3 toward classical CXCL12/CXCR4 signaling: an agonist of ACKR3 promoted the dimerization of ACKR3 with CXCR4, which in turn led to internalization and degradation of CXCR4 and inhibition of CXCL12-induced tube formation (124). Also, ACKR3 binding to CXCL12 but also to its other ligand CXCL11 (as discussed below) enhances internalization of the chemokine ligand/receptor complex and results in lysosomal degradation of the chemokine ligand (209, 220), which again negatively affects CXCL12/CXCR4 signaling. Together, these findings have led to the current idea that the CXCR4/ACKR3 complex results in decreased classical signaling of CXCL12/CXCR4 through G protein-coupled pathways and initiation of β -arrestin signaling through ACKR3.

Of note, ACKR3 preferentially sequesters the monomeric form of CXCL12, whereas dimeric CXCL12 showed significantly lower binding to ACKR3 *in vitro* and in a breast cancer xenograft model (129). However, how the oligomerization state of CXCL12 influences downstream signaling remains controversial, as discussed earlier. Taking into account the findings by Drury et al. who showed in colon carcinoma cells that monomeric but not dimeric CXCL12 preferentially recruited β -arrestin to CXCR4 (130), one could hypothesize that enhanced β -arrestin signaling through ACKR3 is due to preferential binding of monomeric CXCL12. However Ray et al. reported that monomeric CXCL12 actually promotes $G\alpha_i$ signaling through CXCR4, whereas dimeric

CXCL12 rather recruited β -arrestin (129). This shows that these mechanisms still need to be further investigated and could possibly be cell type-dependent and influenced by the build-up of different receptor complexes, as, for example, homomeric or heteromeric interactions involving CXCR4, ACKR3, and even CD74.

Furthermore, different reports come to the conclusion that CXCL12 can signal through ACKR3 independently of CXCR4, further diversifying the potential mechanisms of the CXCL12 signaling network (Figure 2). The migration of neural progenitor cells was shown to be regulated by CXCL12, both through the well-established mechanism of CXCR4 but also independently of CXCR4 via ACKR3 (216). Similarly, CXCL12 can induce T-cell migration through ACKR3 independent of CXCR4 (6, 215), and in cortical interneurons MAPK activation can be induced by CXCL12/ACKR3 independent of CXCR4 (214). However, the idea that ACKR3 is merely a scavenger receptor modulating CXCR4 function and an ACKR not coupled to classical G protein signaling was in turn challenged by Ödemis et al., who showed that ACKR3-mediated effects on ERK and AKT activation in rodent astrocytes and human glioma cells were pertussis toxin-sensitive, and hence mediated by G protein activation (217).

In conclusion, the previous view of classical CXCL12/CXCR4 signaling needs to take into account active signaling moderation by ACKR3. Whether CXCL12 signaling is mediated through CXCR4, ACKR3, or both receptors in conjunction seems to be cell type-dependent (221). Interestingly, a recent report described CXCL12 to stimulate CXCR4 internalization on platelets, which in turn led to increased ACKR3 surface expression. This CXCR4-dependent increased ACKR3 surface exposure had anti-apoptotic effects on platelets (222). These findings reveal a highly interesting, novel pro-survival mechanism of CXCL12, and also demonstrate that CXCR4 and ACKR3 can work in close conjunction to trigger specific biological effects. However, signaling through CXCR4 vs. ACKR3 can also have opposite biological effects. For example, it was recently shown that ACKR3 is upregulated in liver sinusoidal endothelial cells after acute liver injury and in cooperation with CXCR4 induces liver regeneration through the production of pro-regenerative angiocrine factors. By contrast, chronic liver injury increased CXCR4 expression, overwhelming ACKR3 signaling and promoting a pro-fibrotic response instead (223).

CXCL11 as a Ligand for ACKR3

In 2006, ACKR3 (at that time still called CXCR7) was also identified as a receptor for the chemokine CXCL11, which was previously believed to exclusively bind to CXCR3 (208, 224). This chemokine was discovered in 1998 by Cole et al. by sequence analysis of cDNAs derived from cytokine-activated primary human astrocytes (224), and based on experiments named as interferon-inducible T-cell alpha chemoattractant (I-TAC). Later on, this name was replaced by CXCL11, according to the common chemokine nomenclature (225, 226). Interactions of CXCL11 with CXCR3 have been intensively investigated, and have, for example, been associated with several pro- as well as anti-tumorigenic effects (227). By contrast, the CXCL11/ACKR3 relationship is less well studied. Yet, it clearly emerges that CXCL12, CXCL11, and their common receptor ACKR3 form an interactive network.

For example, local administration of CXCL11 in a mouse model of colorectal cancer enhanced tumor growth. Without exogenous CXCL11 stimulation, blocking endogenous CXCL11 or CXCL12 alone did not influence tumor growth and angiogenesis, whereas the combined inhibition almost completely abrogated tumor angiogenesis, providing evidence for the proposed close relationship of these chemokines (228).

Potential Alternative Ligands for ACKR3: MIF and gp120

Apart from the binding of CXCL12 and CXCL11, ACKR3 has also been implied as a receptor for MIF (Figure 3A). For example, an anti-ACKR3 antibody inhibited the adhesion of rhabdomyosarcoma cells in response to MIF in an *in vitro* assay (7). Furthermore, a recent report indicated MIF to interact with both CXCR4 and ACKR3 on the platelet surface, although biochemical receptor binding evidence is currently still elusive. Although MIF could also induce CXCR4 internalization similar to CXCL12, MIF was not able to induce downstream ERK phosphorylation upon CXCR4 binding and also failed to induce subsequent upregulation of ACKR3 externalization, likely due to lack of CD74, which is not expressed in platelets. However, MIF-induced platelet survival through ACKR3 but not CXCR4, as well as activation of the PI3K-AKT pathway (229). The injection of MIF in a mouse model led to decreased thrombus formation after arterial injury, which could be abrogated by a ACKR3-blocking antibody. *In vitro*, reduced thrombus formation by MIF was mediated through both CXCR4 and ACKR3 (229).

Finally, the similarity of ACKR3 to CXCR4 in ligand binding is also apparent by the ability of both receptors to act as co-receptors for several strains of HIV in combination with CD4 (230). While for CXCR4, binding of the HIV envelope glycoprotein gp120 is a well-established mechanism for viral entry (231), interaction of HIV envelope proteins to ACKR3 has not yet been demonstrated by biochemical receptor binding assays.

Involvement of (Defective) CXCR4 Signaling in Pathological Settings

Since CXCR4 signaling is induced by different ligands and affects important biological processes, it is not surprising that CXCR4 is also involved in a plethora of pathological events, such as HIV infection (15, 179), WHIM syndrome (232), as well as diverse cancer types (96).

As already introduced before, CXCR4 is important for HIV entry into T-cells of infected patients, and this conclusion is underlined by the fact that administration of the CXCR4 antagonist AMD3100/Plerixafor can stop virus replication (9). Therefore, CXCR4 antagonists are still under thorough investigation for their potential therapeutic value in HIV infection (233).

The warts, hypogammaglobulinemia, infection, and myelokathexis (WHIM) syndrome was first described in 1990 by Wetzler et al. (234). WHIM syndrome is characterized by severe neutropenia despite having abundant mature myeloid cells in the bone marrow, which is termed myelokathexis (232). Gain-of-function mutations in the *CXCR4* gene were identified as the underlying cause of this autosomal-dominant syndrome in

2003 (232, 235), corresponding to the fact that CXCR4 mediates neutrophil retention in the bone marrow (93). A highly interesting finding on WHIM was published this year; McDermott et al. report a case in which a patient with WHIM was spontaneously cured by chromothripsis, an intensive deletion and rearrangement process in chromosomes. Here, the defective *CXCR4* gene was randomly deleted in a hematopoietic stem cell that repopulated the myeloid but not the lymphoid lineage, leading to complete remission of the patient (235).

Furthermore, CXCR4 is the chemokine receptor most widely expressed in malignant tumors (236). It plays a role in a variety of cancer types, and has been linked with cancer cell proliferation and metastasis to bones and lymph nodes through both CXCL12 and MIF, as described above (96, 160, 166). For example, activation of CXCR4 induces leukemia cell trafficking and homing to the bone marrow (237) and is critical for the growth of both malignant neuronal and glial tumors (238).

In contrast to pathological effects of CXCR4 signaling, CXCR4 also exerts important protective functions in the context of disease. For example, the CXCL12/CXCR4 axis exerts cardioprotective effects after myocardial ischemia by enhancing the incorporation of progenitor cells in the infarcted region and promoting survival of cardiomyocytes (98, 99, 239). On the other hand, CXCR4 also promotes the recruitment of inflammatory cells to the infarcted heart (240, 241) and has in this context been linked to an increase in infarct area (240). This indicates a double-edged role of CXCR4 in myocardial ischemia (241), which has also been described for MIF (242), as discussed in detail in recent reviews (1, 243), and warns for a careful evaluation of effects of CXCR4 antagonists in clinical trials.

Development and Clinical Use of CXCR4 Antagonists

Since CXCR4 signaling is involved in a plethora of pathological processes, small-molecule antagonists directed against CXCR4 are of great interest for medical treatment. A large variety of drug candidates and lead compounds targeting CXCR4 have been discovered over the last years, with several classes of chemical compounds being investigated. While peptide-derived compounds were the earliest anti-CXCR4 agents under investigation, they had poor pharmacokinetic properties. Nevertheless, these compounds were essential for the development of a basic pharmacophore model to further develop more intricate smaller molecules antagonizing CXCR4. These include cyclic pentapeptide-based antagonists, indole-based antagonists, tetrahydroquinolines-based antagonists, para-xylyl-enediamine-based compounds, guanidine-based antagonists, quinoline derivatives, and various other compounds, as reviewed in detail recently (2). The first and so far only CXCR4 antagonist that was approved by the Food and Drug Administration (FDA) is AMD3100, a bicyclam compound marketed under the brand name Plerixafor (Genzyme Corporation). It is being used in combination with G-CSF for the mobilization of HSPCs for autologous transplantation in patients with non-Hodgkin's lymphoma (244). The use of HSPCs, derived by treatment with G-CSF and AMD3100, has essentially replaced bone marrow as a source of stem cells for both autologous and

allogeneic transplantation, providing greater safety for the patient (245). In addition to the FDA-approved Plerixafor, other CXCR4 antagonists are being evaluated in ongoing clinical trials. To date, nine other clinical trials with CXCR4 antagonists are listed in the database at clinicaltrials.gov (search term "CXCR4") (Table 1).

In addition to ongoing clinical trials, several different CXCR4 antagonists are under investigation in *in vitro* and *in vivo* experimental research. For example, TG-0054 has recently been investigated for its therapeutic potential in an animal model of myocardial infarction. It could be shown that the administration of TG-0054 after induction of myocardial infarction leads to a mobilization of mesenchymal stem cells, preventing left ventricular dysfunction and causing a decrease in inflammatory cytokine levels (246). A phase II clinical trial to assess the pharmacokinetics and safety of TG-0054 for the mobilization of stem cells in patients with multiple myeloma, non-Hodgkin lymphoma, or Hodgkin disease has been completed by the end of 2014; however, the results have not been published to date (clinical trials NCT01458288).

Since AMD3100 is currently the only FDA-approved CXCR4 antagonist, it is being used in multiple clinical trials (search term "AMD3100" at clinicaltrials.gov reveals 112 hits). Additionally, researchers are constantly aiming to improve AMD3100, for example, efforts to increase its anti-HIV properties by functionalizing the phenyl moiety are still ongoing (233). Mechanistically, AMD3100 prevents the binding of CXCL12 to CXCR4 and thus CXCR4 downstream signaling. AMD3465, an analog of AMD3100, similarly prevents CXCL12 binding to CXCR4 with

TABLE 1 | Ongoing clinical trials with CXCR4 antagonists as listed in the database at clinicaltrials.gov (search term "CXCR4").

Drug	Clinical trial phase	Being tested in	Sponsor
BMS-936564	Phase 1	Multiple myeloma	Bristol-Myers Squibb
BKT140	Phase 1/ phase 2	Multiple myeloma	Biokine Therapeutics Ltd
BL-8040	Phase 1/ phase 2	Chronic myeloid leukemia	Sheba Medical Center
POL6326	Phase 2	Large reperfused ST-elevation myocardial infarction	Polyphor Ltd.
BMS-936564	Phase 1	Acute myelogenous leukemia; diffuse large B-cell leukemia; chronic lymphocytic leukemia; follicular lymphoma	Bristol-Myers Squibb
AMD11070/ AMD070	Phase 1/ phase 2	HIV infections	National Institute of Allergy and Infectious Diseases (NIAID); AIDS Clinical Trials Group
MSX-122	Phase 1	Solid tumors	Metastatix, Inc.
POL6326	Phase 1	Mobilization of hematopoietic stem cells in healthy volunteers	Polyphor Ltd.
AMD070	Phase 1	HIV infections	National Institute of Allergy and Infectious Diseases (NIAID); AIDS Clinical Trials Group

an even 10-fold higher effectiveness (247), however did not yet receive FDA approval. Of note, AMD3100 was previously reported to be able to enhance CXCL12 binding to ACKR3 and to induce basal as well as CXCL12-induced β -arrestin recruitment to ACKR3 above concentrations of 10 μ M, warranting AMD3100 not only to interfere with CXCR4 signaling but also to induce ACKR3 signaling at high concentrations or modulate CXCL12 scavenging through ACKR3 (248).

Concluding Remarks

In the last decade, many new insights in the CXCR4 signaling cascade have been revealed, both in terms of the ligands to which CXCR4 binds, the different signaling pathways that are initiated downstream, the formation of heteromeric receptor complexes involving CXCR4, as well as the interaction of other receptor complexes with the CXCR4 cascade. This will considerably help to understand the functions of CXCR4 and its ligands both in physiological settings as well as in disease. However, there is still a lot of haziness that remains to be clarified. For example, the specific residues of CXCR4 involved in the site 1 and site 2 interaction with CXCL12 vs. MIF are still unknown, as is its affinity to CXCL12 compared to MIF binding in physiological settings, and thus the potential competition between CXCL12 and MIF for CXCR4 binding. Furthermore, both CXCL12 and MIF can interact with ACKR3, and, despite the initial view that ACKR3 merely serves as a chemokine decoy receptor, first reports have now indicated that both MIF- and CXCL12-induced ACKR3 signaling regulate important biological processes, and it can be expected that additional functions of these signaling pathways in both physiological as well as pathological conditions will be revealed. Also, the roles of CXCR4, CXCL12, and MIF in certain physiological or pathophysiological settings may be

very complex depending on the cell type, microenvironment, and perhaps potentially inter-individual settings. For example, CXCR4, CXCL12, as well as MIF have been associated with a pro- as well as anti-inflammatory role after myocardial infarction (98, 99, 239–242), and also in the context of injury-induced restenosis, CXCR4 has been associated with detrimental as well as protective effects (249, 250), as discussed in more detail recently by Döring et al. (1). Based on the fact that CXCR4 can interact with ACKR3 and CD74, but also other receptors as TLR2, it could be speculated that these double-edged functions may result from the involvement of differential receptor complexes that initiate different intracellular signaling pathways. Revealing differential interaction sites for CXCR4 with CXCL12 vs. MIF, and differential signaling pathways that mediate pro- vs. inflammatory properties could reveal new strategies to target only specific aspects of the CXCR4 signaling cascade, while leaving others unaffected to prevent unwanted side effects.

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MIF and CXCL12 in cardiovascular diseases: functional differences and similarities

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Coronary artery disease (CAD) as part of the cardiovascular diseases is a pathology caused by atherosclerosis, a chronic inflammatory disease of the vessel wall characterized by a massive invasion of lipids and inflammatory cells into the inner vessel layer (intima) leading to the formation of atherosclerotic lesions; their constant growth may cause complications such as flow-limiting stenosis and plaque rupture, the latter triggering vessel occlusion through thrombus formation. Pathophysiology of CAD is complex and over the last years many players have entered the picture. One of the latter being chemokines (small 8–12 kDa cytokines) and their receptors, known to orchestrate cell chemotaxis and arrest. Here, we will focus on the chemokine CXCL12, also known as stromal cell-derived factor 1 (SDF-1) and the chemokine-like function chemokine, macrophage migration-inhibitory factor (MIF). Both are ubiquitously expressed and highly conserved proteins and play an important role in cell homeostasis, recruitment, and arrest through binding to their corresponding chemokine receptors CXCR4 (CXCL12 and MIF), ACKR3 (CXCL12), and CXCR2 (MIF). In addition, MIF also binds to the receptor CD44 and the co-receptor CD74. CXCL12 has mostly been studied for its crucial role in the homing of (hematopoietic) progenitor cells in the bone marrow and their mobilization into the periphery. In contrast to CXCL12, MIF is secreted in response to diverse inflammatory stimuli, and has been associated with a clear pro-inflammatory and pro-atherogenic role in multiple studies of patients and animal models. Ongoing research on CXCL12 points at a protective function of this chemokine in atherosclerotic lesion development. This review will focus on the role of CXCL12 and MIF and their differences and similarities in CAD of high risk patients.

Keywords: cardiovascular disease, atherosclerosis, chemokines, macrophage migration-inhibitory factor, CXCL12

Introduction

Worldwide, cardiovascular disease (CVD) is the leading cause of death, accounting for more than 15 million deaths annually (1, 2). CVD is a collection of various diseases, but the most common and most severe are coronary artery disease (CAD) and cerebrovascular disease. In a high percentage of patients, these diseases eventually result in a myocardial infarction (MI) or stroke, respectively. CVD has not only a major impact on personal health, but also the economic burden is quite high. Besides the high mortality rates, patients who do survive are often hospitalized or should receive

lifelong treatment, leading to high healthcare costs. In the United States alone, these costs are even more than \$312 billion per year, indicating the magnitude of economic burden caused by CVD (3). Finally, the social burden on the direct environment of CVD patients should not be underestimated.

In recent years, or even decades a lot of research has been performed to better understand the exact pathology behind CVDs. Currently, atherosclerosis, a chronic inflammatory disease mainly affecting medium and large-sized arteries, has been identified as the main underlying cause of CVD (4). Upon activation of the vascular endothelial layer, lipids, immune cells, and cell debris start to accumulate in the vessel wall, forming initial lesions. These lesions will over time progress and grow in size, thereby partially or even fully occluding the artery. More often, however, the full occlusion of the vessel is caused by a rupture of the atherosclerotic lesion resulting in thrombus formation (4). Excessive growth or rupture of the lesion both result in ischemic areas in downstream tissues. Most commonly this occurs in arteries from the heart or the brain, leading to MI or stroke, respectively. To date, there is still no absolute suitable therapy available to cure or reverse atherosclerosis. Better understanding of this pathology will increase the ability to prevent it, and create opportunities to develop therapeutic strategies to cure it, or at least slow down the disease progression.

This review will give a short overview of the current knowledge about atherosclerosis, mainly focusing on the inflammatory aspects and the role of chemokines. Subsequently, the role of two important inflammatory mediators that recently have been connected with CVD and atherosclerosis will be discussed and put into clinical perspective, namely the chemokines CXCL12 and macrophage migration-inhibitory factor (MIF, **Table 1** and **Figure 1**).

Atherosclerosis

Risk Factors

Already a multitude of risk factors have been described for CVDs, mainly derived from epidemiological studies. Main risk factors include psychosocial factors, hypertension, changes in low-density lipoproteins (LDL) and high-density lipoproteins (HDL) cholesterol levels, physical inactivity, smoking, obesity, lack of fruits and vegetables consumption, and alcohol and diabetes mellitus (20–22). All these risk factors vary greatly in prevalence and potency to influence CVD and are often combined in patients with severe atherosclerosis, supporting the concept that atherosclerosis is a multifactorial, complex disease (22). A worldwide case-control study showed that these nine factors combined account for more than 90% of the cardiovascular risk (21), where smoking and abnormal lipids already account for two-thirds of the risk. Additionally, many patients, especially high risk patients, have genetic predispositions for atherosclerosis (23). Some of these risk factors should be relatively easy to prevent with life-style adjustments. However, due to the multifactorial characteristics of the disease, identification of the precise mechanisms of action, whereby these risk factors influence atherosclerosis, is a key and crucial step in the road toward therapeutic strategies. This way for example statins, lipid modulating drugs, have been developed

that lower LDL cholesterol levels and thereby inhibit the progression of atherosclerosis. Recently, Nahrendorf and Swirski very nicely reviewed the possible influence of various risk factors on the crosstalk between hematopoiesis, immune cells, and the cardiovascular system (22).

Atherosclerosis Initiation

Formation of atherosclerotic lesions occurs predominantly at predisposed sites, i.e., sites of disturbed laminar flow, like bifurcations and curvatures (24), thereby disturbing the normal, quiescent state of the endothelium. The resulting increased permeability of the endothelial layer leads to the accumulation of lipids, more specifically LDL, in the subendothelial layer of the arterial wall (25). LDL is very susceptible to oxidation, mainly caused by myeloperoxidase, lipoxygenases or by reactive oxygen species such as HOCl, resulting in oxidized-LDL particles (oxLDL) (26). The modification of LDL can activate the endothelial cells (ECs) and tissue-resident macrophages (27). Subendothelial/extravasated LDL will be oxidized and taken up by resident macrophages via scavenger receptors (SRs), mainly SR-A, SR-B1, and cluster of differentiation (CD) 36 (28, 29). Intracellularly, oxLDL will be hydrolyzed into free cholesterol and fatty acids in late endosomes (30). Free cholesterol is subsequently transported to the endoplasmic reticulum where it undergoes re-esterification to cholesteryl esters by the acyl-CoA:cholesterol ester transferase (ACAT) enzyme (31). This accumulation of cholesteryl esters will transform the macrophages into foam cells, a characteristic hallmark of early atherosclerosis. Not only the uptake and storage of cholesterol are disturbed, but also the excretion mechanisms are disturbed. ATP binding cassette transporters A1 and G1 (ABCA1 and ABCG1) are the major contributors to this cholesterol efflux through reverse cholesterol transport (RCT) (32, 33). Cholesterol will efflux toward HDL, making HDL beneficial for atherosclerosis development (32). Normally, this efflux mechanism is upregulated upon lipid loading, however, during hypercholesterolemia this route is compromised via TLR4-induced downregulation of the cholesterol transporters, further favoring the conversion of macrophages to foam cells (34).

This macrophage activation, due to the lipid loading, together with the activation of ECs will lead to more vascular inflammation, by the secretion of various cytokines, chemokines, and adhesion molecules (35). The inflammation will result in the attraction of mainly monocytes but also other immune cells, like T- and B-lymphocytes and neutrophils to the site of injury and lesion formation (26). In humans, two main subsets of circulating monocytes have been described based on their expression of CD14 and CD16 (36). Over 90% of the circulating monocytes are CD14^{high}/CD16[−] and are called the classical monocytes (37). The other population, the non-classical monocytes are CD14^{low}/CD16^{high} (36). These distinct subsets also show differential expression of chemokine receptors (38). The classical monocytes express, for example, high levels of CCR2, a receptor for CCL2 (39). CX₃CR1 is expressed on both subtypes, but the expression on non-classical monocytes is twofold higher compared to that on the classical monocytes (40, 41). In mice, also two main subtypes of circulating monocytes can be identified, discriminated mainly by the expression of Ly6C, i.e., the Ly6C^{high} (classical) and

TABLE 1 | Overview of human studies on CXCL12 and MIF in CVD.

Study design	Outcome	Reference
CXCL12		
Genome-wide association studies in over 100,000 patients	CXCL12 locus on chromosome 10q11 is clearly associated with CAD, indicating that CXCL12 may be involved in CVD development	(5)
Western blot analysis and immunohistochemical analysis of human plaques	Atherosclerotic lesions express high levels of CXCL12, in contrast to vascular cells of healthy vessels, associating CXCL12 with CVD	(6)
Cohort study of 904 patients with CAD	Platelet CXCL12 expression is increased in angina patients, though clinical significance remains to be elucidated	(7)
Cohort study of 215 patients with symptomatic CAD undergoing percutaneous coronary intervention	CXCR4 and ACKR3 are more highly expressed on platelets from CAD patients, associating receptors of CXCL12 to CVD	(8)
Plasma CXCL12 evaluation of 60 CAD patients	Plasma CXCL12 levels and surface expression of CXCR4 in peripheral blood mononuclear cells are decreased in angina patients, indicating that CXCL12 could be beneficial for CVD	(9)
Cohort study of 785 patients undergoing angiography	Plasma CXCL12 levels are superior to traditional risk factors in predicting CAD outcomes	(10)
Evaluation of 1,000 patients hospitalized due to chest pain	Platelet-derived CXCL12 expression occurs fast after injury in CAD patients, as early as 30 min, indicating that CXCL12 might be very useful as biomarker	(11)
MIF		
Single nucleotide polymorphism (SNP) evaluation of 459 MI patients and healthy controls	MIF single nucleotide polymorphism (rs755622) is associated with MI risk	(12)
MIF analysis in healthy and diseased internal mammary arteries	MIF is abundantly expressed in human atherosclerotic lesions, throughout lesion development, associating MIF with CVD	(13)
Immunohistochemical analysis of human atherosclerotic plaques	MIF is associated with fibrous cap weakening, by inducing protease expression and activity, associating MIF with plaque instability	(14)
Evaluation of 286 patients with symptomatic CAD undergoing percutaneous coronary intervention	Plasma MIF levels are increased in CVD patients, associated with inflammatory marker expression	(15)
Prospective study of 617 patients with CAD	High plasma MIF levels are an independent risk factor for future coronary events in CVD patients with impaired glucose tolerance or type 2 diabetes mellitus, associating MIF with CVD development	(16)
Plasma MIF and Grem 1 evaluation in 286 patients with CVD	High plasma Grem1/MIF ratio is associated with CVD and the grade of plaque stability, indicating MIF as a possible novel risk marker in CVD patients	(17)
Evaluation of MIF levels in patients with chronic stable angina	MI patients have higher plasma MIF levels which are predictive of final infarct size and remodeling, suggesting a role for MIF as biomarker	(18)
Prospective case-control study nested in the EPIC-Norfolk cohort in people without prior history of MI or stroke	Association of MIF with MI risk or death due to CVD is not very strong in humans without prior history of CVD, indicating that more research is necessary before choosing MIF as therapeutic target	(19)

Ly6C^{low} (non-classical) monocytes (42). Similar to the human subpopulations mouse classical monocytes express high levels of CCR2, while non-classical monocytes express CX₃CR1 (43). Hypercholesterolemia, a common characteristic of atherosclerosis increases Ly6C^{high} monocyte levels (44, 45). Additionally, Ly6C^{high} classical monocytes form the main subtype that will migrate to the site of injury (46).

The mechanism of monocyte recruitment and adhesion consists of complex interactions between various adhesion molecules and chemokines. The exact role of various chemokines in atherosclerosis will be discussed later. In short, the monocyte adhesion cascade consists of several steps. The first step in this cascade is the capture and rolling phase, where various chemokines and selectins on the luminal side of the activated endothelium play a crucial role (35, 47). The second step is the firm adhesion of these monocytes to the endothelium. In this phase, vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1) present on the endothelium are essential, since they attach firmly to the integrins very late antigen 4 (VLA4) and lymphocyte function-associated antigen 1 (LFA1), respectively, present on the monocytes (47). VCAM1, ICAM1, and E-selectin form clusters to initiate cytoskeletal reorganization, necessary for subsequent transmigration (48). These clusters, also called docking structures, have been shown to be located on upright endothelial membrane processes and

are organized as the so-called trans migratory cups by tetraspansins (49). Monocyte transmigration across the endothelial layer is mainly directed by various chemokines and their receptors (50). Next to these chemokines, also the endothelial junction molecules, platelet endothelial cell adhesion molecule 1 (PECAM1), VE-Cadherin, and junctional adhesion molecules (JAMs) play a crucial role as regulators of EC permeability and leukocyte transmigration (51). Monocytes have two ways to transmigrate, a paracellular route and a transcellular route. The paracellular route leads monocytes through the endothelial junctions, whereas the transcellular route uses fusing vesicles in the endothelium cell cytoplasm for transmigration (52). Once inside the vessel wall, monocytes can differentiate into macrophages, driven by macrophage colony-stimulating factor (M-CSF) (53). Macrophages are a heterogeneous cell population, consisting of two main groups, the classically activated, inflammatory M1 macrophages and the alternatively activated, inflammation resolving M2 macrophages (54). Various cytokines play a role in this polarization of macrophages, such as IL-10 and interferon- γ (55). Both types of macrophages are present in atherosclerotic lesions, where the balance between them is of great importance for either plaque development or resolving inflammation (56). These attracted leukocytes will again be exposed to the oxidized-lipid-rich environment of the developing lesion, forming foam cells. Thereby a vicious circle of

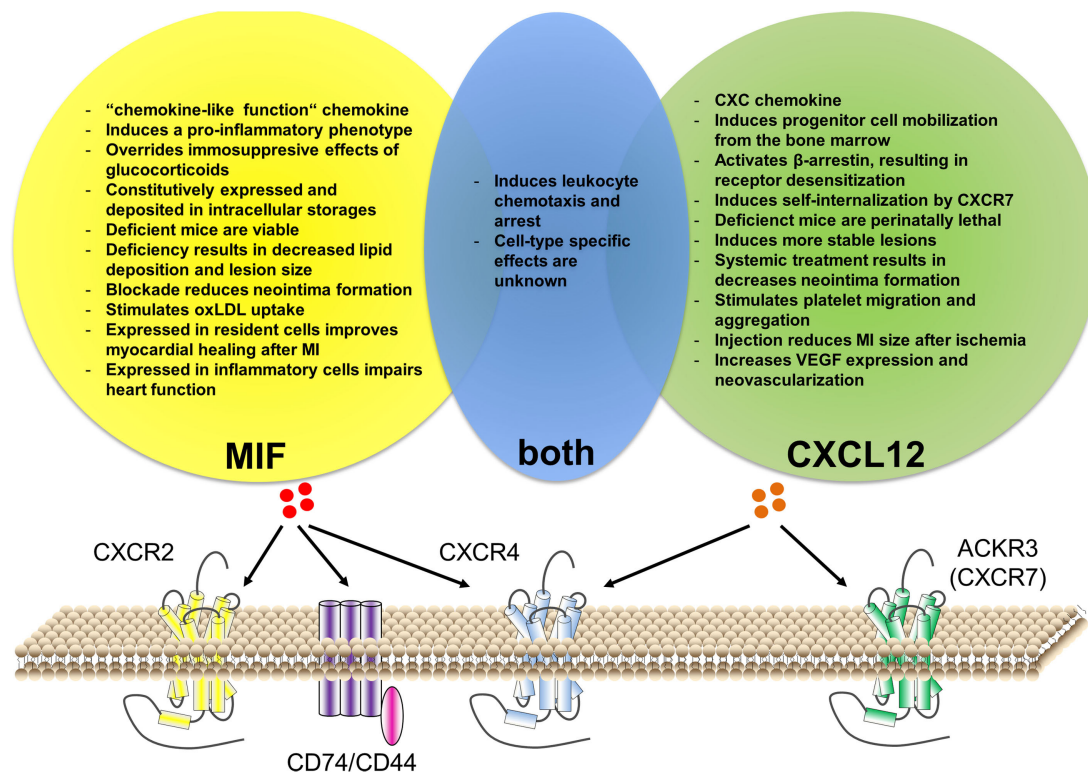


FIGURE 1 | Similarities and differences of CXCL12 versus MIF function in cardiovascular disease. Both CXCL12 and MIF can bind to CXCR4. Additionally, CXCL12 can bind to ACKR3 (CXCR7), while MIF binds to CXCR2 and CD74/CD44. Although MIF interaction with ACKR3 has been described for

platelets, it is still unclear whether this is via direct binding or via receptor heterodimerization. Both chemokines have an important role in leukocyte recruitment, although cell-type-specific effects remain unknown. Besides this, CXCL12 and MIF have individual functions associated with cardiovascular disease.

leukocyte attraction and lipid accumulation is formed, stimulating atherosclerosis development.

Plaque Progression

When lesional macrophages take up so many lipids and debris, many will eventually go into apoptosis. In early plaque development apoptosis is not considered detrimental, since neighboring macrophages will take up and eliminate the apoptotic debris, a process called efferocytosis (56). However, when plaque development progresses, the excessive uptake of lipids and debris continues and eventually leads to cellular stress and impaired efferocytosis (57). This will result in the accumulation of apoptotic debris and apoptotic macrophages will go into secondary necrosis, leading to the formation of the necrotic core which is characteristic of more advanced lesions (56, 58). The necrotic core will significantly contribute to the lesional inflammation, and thus progression, and also contains pro-thrombotic factors that will lead to a thrombus when it comes into contact with platelets (59). To prevent this from happening, a fibrous cap is formed between the necrotic core and the lumen, by deposition of mainly collagen and elastin by intimal smooth muscle cells (SMCs) (60). Various cytokines and growth factors, produced by leukocytes, are important for the migration of intimal SMCs to the intima and for the extracellular matrix production (60). Plaques with a big fibrous cap are considered to be more stable atherosclerotic

lesions, i.e., less prone to rupture. However, macrophages can also produce matrix metalloproteinases (MMPs), which are capable of degrading extracellular matrix proteins (59). Fibrous cap degradation and thus thinning makes the lesion more vulnerable and can eventually lead to a plaque rupture, releasing pro-thrombotic material into the bloodstream resulting in thrombus formation and obstruction of blood flow. This can cause ischemia to distal regions and result in a MI or stroke (58).

Chemokines in Atherosclerosis

Chemokines are the largest family of cytokines, consisting of small molecules (8–12 kDa) that exert chemotactic effects on cells (61). This large family is divided into four subclasses, being C, CC, CXC, and CX₃C. This classification is based on the position of the N-terminal cysteine residues (62). In addition to these four canonical chemokine classes, a fifth subclass consisting of molecules that share functional similarities with chemokines has emerged, referred to as “chemokine-like function” (CLF) chemokines (63). These non-canonical chemokines do exert some CLFs, but do not contain the specific N-terminal cysteine residue characteristic of canonical chemokines (63). Chemokines can bind to chemokine receptors, which are classified according to the chemokine they bind. Most chemokine receptors will activate G proteins and intracellular signaling upon binding and are therefore part of the G protein-coupled receptor family. However,

several receptors, the so-called atypical chemokine receptors, are G protein signaling independent and rather play a role in the scavenging of chemokines (64). Chemokines and chemokine receptors are expressed on various cell types, like ECs, SMCs, and leukocytes. Originally, chemokines were discovered for their capacity of directing leukocytes toward sites of inflammation (65). Thereby, chemokines also play a crucial role in atherosclerosis.

Chemokines already play a role in a very early stage of atherosclerosis development. Recently, it was shown that lysophosphatidic acid, a component of LDL mediates the release of CXCL1 from ECs (66). Studies in atherosclerotic prone ApoE^{-/-} mice fed a cholesterol-rich diet show that CXCL1 is not only important for the mobilization of the classical, or inflammatory, monocytes to the site of inflammation, but also important for neutrophil recruitment via the receptor CXCR2 (66–68).

The involvement of various chemokines and their receptors in monocyte recruitment has already been well described (69). However, during the recent decade these described involvements have been revised and are still being greatly debated. Using a highly sophisticated technique of transferring atherosclerotic aortic arches from ApoE^{-/-} mice into specific chemokine receptor knockout mice, it was shown that inflammatory monocytes require CCR2, CCR5, and CX₃CR1 to migrate into the atherosclerotic lesion, while the patrolling monocytes used CCR5 for recruitment (70). However, this view again changed recently using adoptive transfer experiments with pharmacological inhibition of the specific chemokine receptors. Here it was shown that the inflammatory Ly6C^{high} monocytes use CCR1 and CCR5 for recruitment, rather than the previously shown CCR2 and CX₃CR1 (68). CCR2-deficient mice on an atherosclerosis-prone background did, however, result in a significantly reduced lesion size in mice. Based on the adoptive transfer study and the fact that CCR2-deficient mice show reduced circulating monocyte counts, it can be suggested that the beneficial effect on atherosclerosis is due to its effects on monocyte release from the bone marrow, rather than direct effects on monocyte recruitment (71, 72). Also for CX₃CR1-deficient mice, it was observed that these animals have reduced atherosclerosis development, implicating CX₃CR1 in atherogenesis (73). However, instead of effects on monocyte recruitment, CX₃CR1 seemed to play an important role in cell survival. Thus, deficiency of this chemokine resulted in increased apoptosis of plaque macrophages, thereby reducing lesion development (73). CCR1 and CCR5, which do seem to be involved in leukocyte recruitment, both have several specific ligands, but also share ligands like CCL3 and CCL5. However, looking at the exact effects of both receptors on atherogenesis, there are distinct differences. CCR5 deficiency results in a clear reduction of diet-induced atherosclerosis in mice, while CCR1 deficiency increased lesion development (74, 75).

In the later stages of atherosclerotic lesion development, chemokines also still play an important role. The best-studied chemokine receptor in this stage, especially with respect to plaque regression, is CCR7. It has been shown in various studies that CCR7 is necessary for the egress of macrophages during lesion regression (76–78). However, CCR7 in T cells seems to play a pro-atherogenic role. CCR7-deficient T cells show an impaired entry and exit capacity from atherosclerotic lesions (79). Combined,

CCR7 is thus involved in macrophage egress from lesions and T cell migration. In these later stages of lesion development also CXCL10 is crucially involved, especially in plaque stability. Inhibition of CXCL10 resulted in relatively more SMCs and a more stable plaque phenotype (80).

These results clearly show that the chemokine system plays an important role in all stages of atherosclerotic lesion development, but underlines that these interactions are very complex and elaborate. Additional research is still needed to even further elucidate the role of this system in atherosclerosis and CVD in general. Besides the already described chemokines involved in atherosclerosis, in the recent years, more and more research has been focusing on two yet undiscussed chemokines, being CXCL12 and MIF. The remainder of this review will specifically focus on the role of these two chemokines in atherosclerosis and CVD.

CXCL12

Ligand/Receptor Characteristics

CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is one of the 17 members of the CXC chemokine family (5). Structurally, this group of chemokines can be further subdivided into two groups, depending on the presence of a specific amino acid motif [glutamic acid-leucine-arginine (ELR)] before the first cysteine group (81). Interestingly, this subdivision showed also to be a functional separation since ELR-positive chemokines attract neutrophils, while ELR-negative chemokines, such as CXCL12, attract T lymphocytes and natural killer cells (82, 83). CXCL12 consists on its turn again of six isoforms, derived from alternative splicing (84). The classical isoforms are CXCL12- α and CXCL12- β , which are expressed throughout the body and so far functionally indistinguishable (84). Other isoforms are called CXCL12- γ , - δ , - ϵ , and - ϕ , which show a more restricted expression pattern and are until now much less studied. All isoforms share the same N-terminal sequence, but differ in the C-terminal region (84).

The most important receptor for CXCL12 is CXCR4, which is also expressed on a wide variety of cells (85, 86). CXCR4 is a G protein-coupled receptor and binding of CXCL12 will induce intracellular signaling via a classical heterotrimeric G protein (86). Receptor activation has been shown to trigger MAPK and PI3K signaling, but also calcium mobilization (87). Additionally, activation of CXCR4 results in β -arrestin recruitment, resulting in the endocytosis of CXCR4 and thus receptor desensitization (88).

More recently, a second receptor for CXCL12 was identified, being ACKR3 (CXCR7) which is highly expressed on monocytes and mature B cells (89). This receptor has even a 10-fold higher affinity for CXCL12 than CXCR4. Binding of CXCL12 to the receptor does, however, not result in the classical leukocyte chemotaxis response or coupling with G proteins to induce intracellular signaling. ACKR3 is implicated in cell growth and survival (90). However, the main function of ACKR3 seems to be as a decoy receptor, since receptor stimulation by CXCL12 enhances internalization of ACKR3 and thereby delivery of its ligands to the lysosomes for degradation (91, 92). This way, ACKR3 activation would reduce CXCL12/CXCR4 signaling. However, since CXCL12 scavenging also prevents the downregulation of CXCR4,

ACKR3 could also well be beneficially influencing CXCR4-mediated effects (93). However, ACKR3 stimulation has also been shown to result in downregulation of CXCR4 (94). On the other hand, CXCR4 seems also to be influencing ACKR3, since the widely used antagonist for CXCR4 AMD3100 has recently been shown to have agonistic effects on ACKR3 (95). ACKR3 can have intracellular signaling effects on MAPK, but these are purely β -arrestin mediated (96). Altogether, these results clearly show that there is a complex interaction between CXCL12/CXCR4 and ACKR3.

CXCL12 and Stem-/Progenitor-Cell Mobilization

The importance of CXCL12 in general has been clearly shown in mice that have a total CXCL12 deficiency. These animals die already perinatally due to major defects in hematopoiesis, vasculo-, cardio-, and neurogenesis (97). These embryonic defects are indicative for the important role of the CXCL12/CXCR4 axis in progenitor cell migration, but also for survival and chemotaxis of murine embryonic stem cells during embryogenesis (98). The role of CXCL12/CXCR4 in this mobilization from the bone marrow has already been well studied, not only for hematopoietic stem cells, but also for EC and SMC progenitor cells (99). In physiological conditions, hematopoietic stem cells are retained in the bone marrow by high expression of CXCL12 by stromal cells (97). In the clinic, modulation of the CXCL12/CXCR4 axis by granulocyte colony-stimulating factor (G-CSF) is already used to mobilize stem cells from the bone marrow to the circulation. G-CSF has various ways of modulating this axis, reviewed in (97). Additionally, the role of CXCL12/CXCR4 is confirmed by using the CXCR4 antagonist AMD3100, which results in decreased bone marrow CXCL12 levels, thereby favoring mobilization of stem cells (100).

CXCL12 in Atherosclerosis

Mobilization of hematopoietic stem cells, but also progenitor cells like endothelial progenitor cells (EPCs) and smooth muscle progenitor cells (SPCs), has also been implicated in various pathologies like atherosclerosis (101, 102). A recent study showed that injections of CXCL12 in mice developing atherosclerosis resulted in more stable lesions, characterized by more SMCs and a thicker fibrous cap (103). These plaque-stabilizing effects were mediated by an increased recruitment of SPCs to these lesions. Supporting this finding, direct injection of SPCs in mice reduces atherosclerotic lesion development and improves the stability (104). Besides SPCs, also EPCs were shown to be involved in atherogenesis, since infusion of EPCs, or AMD3100 treatment triggering EPC mobilization resulted in a beneficial effect on lesion regression (105). Together, these studies show atheroprotective effects of the CXCL12/CXCR4 axis, mediated by progenitor mobilization.

Besides these effects on progenitor cells, mediating beneficial effects on atherosclerosis, CXCL12/CXCR4 may also influence disease development by influencing various atherosclerosis-related cells. This is especially interesting, since CXCR4 is expressed on basically every cell-type related to atherogenesis, like monocytes, macrophages, neutrophils, ECs, SMCs, and T- and B-cells (106–110). However, until now there have been no

studies directly investigating the causal cell-type-specific effects of CXCL12/CXCR4 in relation to atherosclerosis. Though there are already various studies at least associating this axis with atherogenesis.

For macrophages, it has been shown that oxLDL, also in large amounts present in atherosclerotic lesions, upregulated CXCR4 expression which could contribute to macrophage migration (111). The CXCL12/CXCR4 signaling in macrophages was also implicated in macropinocytosis, indicating a possible influence on lipid accumulation in these cells (112). Furthermore, it has also been associated with neutrophils as it regulates the release of neutrophils from the bone marrow (113). Not only the release is mediated by this axis, but also the clearance of circulating neutrophils as senescent neutrophils shows increased expression levels of CXCR4-mediating effective clearance (114). By contrast, activated neutrophils downregulate CXCR4 levels, leading to postponed clearance (97). Treatment of ApoE^{-/-} mice with AMD3100 resulted in an increased neutrophil mobilization, thereby increasing atherosclerotic lesion areas (44, 45). ECs also release more CXCL12 after oxLDL stimulation (115). Additionally, laminar shear stress appeared to influence CXCR4 expression, where high shear suppresses CXCR4 expression (116). CXCL12 can also increase vascular endothelial growth factor (VEGF) expression in ECs, which promotes angiogenesis (117). As angiogenesis is inducing a more vulnerable plaque phenotype, CXCL12 could have lesion destabilization effects. Chemotaxis of both T- and B-cell is also positively influenced by CXCL12/CXCR4 (118–121). Besides CXCL12/CXCR4 effect on all these different cell-types, expression of CXCL12/CXCR4 has also been shown on platelets (6, 122). Platelets are the first to arrive at a site of vascular injury, where its glycoproteins Ib and IIb/IIIa engage surface molecules on the ECs, contributing to endothelial activation (4). Platelets also produce and store CXCL12 in their α -granules. Upon release, platelet-derived CXCL12 has been implicated in cell adhesion and chemotaxis (123). Furthermore, CXCL12 is able to induce platelet aggregation, a crucial step in thrombus formation after atherosclerotic plaque rupture (6). Platelets also express the receptor CXCR4, and blocking studies indicated that this receptor is crucially involved in the aggregation effects of CXCL12 (6). Additionally, CXCL12 is able to stimulate platelet migration and transmigration (124). Together, these results show that CXCL12/CXCR4 have interactions with many cells that are relevant in atherosclerosis and is thereby modulating atherosclerosis development. Recently, also a role for ACKR3 in atherosclerosis development has been described, showing that activation of ACKR3 by a synthetic ligand reduced lesion formation and ameliorated hyperlipidemia. ACKR3 seemed to play an important role in the regulation of blood cholesterol levels, by promoting VLDL uptake in adipose tissue (125).

CXCL12 in Atherosclerosis-Related Pathologies

As previously described, atherosclerotic plaques eventually, either block an artery by growth or by rupture and subsequent thrombus formation. The result is ischemia in downstream tissues. One of the most common places for this to occur is in the heart, resulting in a MI. It is known that hypoxia results in an upregulation of CXCL12 and CXCR4 (126). Various studies have revealed a

protective role for CXCL12/CXCR4 signaling after MI through survival effects on resident cardiomyocytes and recruitment of protective circulating cells (97). Direct injection of CXCL12, for example, reduced myocardial infarct size after ischemia, which was associated with increased neo-angiogenesis (127). Recruitment of progenitor cells was crucial for this improved vessel growth (128). Supporting a role for CXCL12/CXCR4 in MI are studies using AMD3100, an antagonist for CXCR4. However, results from these studies are quite contradictory. A study using a single injection of AMD3100 showed improved cardiac function and enhanced progenitor cell accumulation and neovascularization (129, 130). However, more chronic administration of AMD3100 showed reduced incorporation of progenitor cells and cardiac outcome after MI (131).

CXCL12/CXCR4 has also been shown to play a role in vascular restenosis. The main treatment of choice after arterial blockage is percutaneous coronary intervention, where a stent is placed at the site of lesion development. However, neointimal hyperplasia often causes restenosis of these stents mainly driven by SMCs, thereby again occluding the artery. Systemic treatment of mice that underwent wire-induced arterial injury with CXCL12 or CXCR4 antagonists showed clear reductions of neointimal size and SMC content (132, 133). This reduced SMC content was caused by a reduction in progenitor mobilization toward the site of injury (132, 133). Additionally, CXCR4 blockage reduced cellular proliferation at sites of neointimal lesions (134). A significant decrease in EPC mobilization was recently also observed using genetic EC-specific knockout of CXCR4, although mice showed larger neointimal lesions consisting of more inflammatory macrophages, but less SMCs (135).

Human and Clinical Implications for CXCL12

Genome-wide association studies showed clear associations of CXCL12 with CVD (5) (**Table 1**). Previously, it was already shown that vascular cells express high levels of CXCL12 in human atherosclerotic lesions, but not in normal vessels (6). Various human studies supported the idea that CXCL12 is a potential regulatory agent in atherosclerosis. A study, comparing plasma CXCL12 levels of angina patients with healthy controls, showed decreased levels of CXCL12 in the patient group (9). Patients with unstable angina had even lower CXCL12 levels than those with stable angina. Additionally, these patients showed decreased surface protein expression of CXCR4 in peripheral blood mononuclear cells, while the RNA expression was increased probably as compensatory mechanism (9). Combined, this study suggests anti-atherogenic properties of CXCL12. In contrast, another study observed significantly increased CXCL12 expression on platelets of stable angina patients, compared to healthy controls. Plasma CXCL12 levels also correlated with platelet activation (7), suggesting a more atherogenic and pro-thrombotic role for CXCL12. In addition, both CXCR4 and ACKR3 are more highly expressed on platelets from CVD patients, compared to healthy controls (8). It is suggested that platelet-derived CXCL12 expression occurs relatively fast after vessel injury, being as early as 30 min (136). Currently, used biomarkers for acute coronary syndrome, like troponin-I are much slower. Therefore determination of CXCL12 might be very useful as early additional biomarker (11).

Recently, it was shown that CXCL12 is also associated with heart failure (137), and that plasma CXCL12 levels are even superior to traditional risk factors in predicting adverse cardiovascular outcomes (10). Although there are still some contradictory clinical results, it is clear that CXCL12 does play a role in atherosclerosis and CVD.

Macrophage Migration-Inhibitory Factor

Ligand/Receptor Characteristics

Discovered almost 50 years ago, macrophage MIF was one of the first cytokines to be identified (138). MIF is part of the CLF chemokine family, as it is missing the typical N-terminal cysteines (139). The name is derived from its discovery as MIF-containing supernatant showed to be inhibitory for macrophage migration (140). At first, T cells were thought to be the main cellular source of MIF. However, since its discovery, expression of MIF has also been shown in other immunity cells like monocytes, macrophages, neutrophils, dendritic cells, and B cells (13, 141–146). In contrast to many other chemokines, MIF is constitutively expressed and deposited in intracellular storages. Thus, upon stimulation, MIF release does not require *de novo* synthesis (138). It has already been well described that MIF can directly or indirectly stimulate a large variety of pro-inflammatory molecules, including various cytokines and nitric oxide. Additionally, MIF was shown to override the immunosuppressive effects of glucocorticoids (147). MIF has been implicated in various acute and chronic inflammatory diseases, like sepsis, rheumatoid arthritis, and cancer (148–150).

The first receptor identified for MIF was CD74, the membrane-expressed form of invariant chain and an MHC class II chaperone (151). However, besides its role in antigenic peptide loading, CD74 can also be expressed in the absence of the MHC class II protein, thus exerting functions as membrane receptor (152). MIF binds with high affinity to CD74, although CD74 by itself is not able to induce intracellular signaling. Therefore, it requires the recruitment of signaling-competent co-receptors. CD44 was the first described co-receptor of CD74, able to mediate signal transduction (153). CXCR2 and CXCR4 have also been described as co-receptors for CD74 (154, 155). The combination of CD74 with CD44 has been linked with MIF's pro-inflammatory and anti-apoptotic functions by the activation of MAPKs (153, 156). CD74/CXCR2 complexes have been shown to be involved in MIF-mediated monocyte chemotaxis and arrest. In line, a role for CD74 in atherogenesis has been identified (157).

CXCR4 has already been discussed previously as receptor for CXCL12. It has been found, in monocytes, T cells and fibroblasts, that CXCR4 can also form heterodimers with CD74 and induce Akt signaling (155). CXCR4 has mainly been shown as the receptor responsible for MIF-induced T cell recruitment (154). Finally, CXCR2 has been described as important receptor for MIF. MIF/CXCR2 interaction mainly triggered the recruitment and arrest of monocytes. Furthermore, MIF/CXCR2 has been implicated in integrin activation, an important step in leukocyte recruitment. Recently, ACKR3 on platelets has also been described as receptor for MIF, although it is still not clear whether this is a direct ligand–receptor interaction or indirect interaction via receptor heterodimerization such as CXCR2/ACKR3 (158).

MIF in Atherosclerosis

Hyperlipidemia is one of the hallmarks of atherogenesis. It was shown that upon hyperlipidemia, MIF expression is greatly enhanced in cells crucial for atherosclerosis development, like ECs, SMCs, monocytes, and T cells (13, 159, 160). As atherosclerotic lesions progressed, MIF expression was even further increased. Combined, these data clearly implicate MIF not only in atherosclerotic lesion development, but also in plaque destabilization.

Leukocyte recruitment into atherosclerotic plaques is one of the most important processes during lesion development. *In vitro* adhesion assays under flow clearly showed an increased monocyte arrest of monocytes to aortic ECs upon MIF incubation (161). This was confirmed by using MIF neutralizing antibodies, which blocked the observed effects. Additionally, using small interfering RNA to inhibit endothelial MIF production, it was observed that MIF deficiency resulted in a decreased expression of E-selectin, ICAM-1, VCAM1, IL-8, and MCP-1, all important mediators of leukocyte recruitment (162). Bernhagen et al. clearly showed that MIF can also more directly trigger monocyte, neutrophil, and T cell arrest and chemotaxis in an integrin-dependent manner (154). They further implicated the receptors for MIF in this process, since the integrin activation resulted in the triggering of G α_i activities of CXCR2 in monocytes and neutrophils and of CXCR4 in T cells. Additionally, CD74 also contributes to monocyte recruitment by interacting with CXCR2 (154).

Various functional animal studies confirmed the role of MIF in atherosclerosis development. MIF-deficient mice on an atherogenic background showed significantly reduced lipid deposition and lesion size compared to control animals (163). This was accompanied by a decreased lesion cell proliferation, especially of SMCs. Additionally, neutralizing MIF with specific monoclonal antibodies showed a reduced lesion size and especially reduced intimal inflammation (160). MIF blockage was even showed to induce regression of already established atherosclerotic lesions (154). Additionally, MIF stimulates the uptake of oxLDL by macrophages and is associated with the expression of proteases (163), which can contribute to the lesion destabilization properties of MIF. Recently, also an important role for platelet-derived MIF was described (164). MIF was even shown to have a stronger chemotactic activity than CXCL12 and substantially contributed to monocyte adhesion to an endothelial layer. Although in contrast to CXCL12 secretion, MIF secretion from platelets was much slower and did not enhance platelet activation (164).

Studies with CXCR2-deficient mice also identified important roles for CXCR2 in monocyte recruitment into atherosclerotic lesions, showing reduced lesion size and lesional macrophage content (165). In atherosclerosis studies with other CXCR2 ligands, like CXCL1 and CXCL8, deficiencies did not exceed half the effect of the receptor deficiency on atherogenesis, suggesting the presence of other ligands that play a crucial role (166). In 2007, MIF was identified as ligand for CXCR2 with pro-atherogenic capacities (154). Combined, all these studies clearly identify MIF and its receptors as an important mediator of leukocyte recruitment and atherosclerosis development.

MIF in Atherosclerosis-Related Pathologies

As described earlier, restenosis occurs frequently after stent implantation, leading to early stent failure. Carotid artery wire injury methods are often used to model this disease, characterized by neointimal hyperplasia driven by SMC proliferation. Carotid artery injury resulted in a fast induction of MIF expression in SMCs and later on also in foam cell formation (161, 167). To determine the causal role of MIF in neointimal hyperplasia, antibody-mediated MIF blockage has been used in various studies. MIF blockage indeed resulted in a decreased medial cell proliferation, enhanced apoptosis, and smaller inflammatory cell content (167). Another study also showed a decreased macrophage content and foam cell formation upon MIF blockage (161). This was accompanied by an increase in SMC and collagen content in the neointimal areas, suggesting a more stable phenotype after MIF blockage (168).

Various studies also describe MIF as a protective factor in MI-ischemia-reperfusion injury (63). However, recently it has been shown that this effect is dependent on the cellular source of MIF. Global MIF deficiency protects the heart from post-infarct cardiac rupture and remodeling, by suppressing the leukocyte infiltration and thus inflammation (169). However, leukocyte-derived MIF exerts opposing effects by promoting the inflammatory response after MI (169). These compartmentalized and opposing effects are shown to be mediated by CXCR2 (170).

Human and Clinical Implications for MIF

In humans, MIF has been shown to be abundantly produced by various cells in different stages of plaque development, indicating an important role for MIF in early plaque development but also in more advanced complicated lesions (13) (**Table 1**). Later, it was observed in human lesions that MIF plays a more important role in vulnerable lesions, compared to fibrous lesions. MIF was associated with the weakening of the fibrous cap, by inducing MMP-1 expression and activity in SMCs (14). CVD patients, more particularly patients with acute coronary syndromes also showed enhanced plasma MIF levels. Plasma MIF levels from these patients were associated with inflammatory markers like CRP and IL-6, but also with the cardiac necrosis marker troponin-I (15). High plasma MIF levels have also been identified as an independent risk factor for future coronary events in patients with CVD and impaired glucose tolerance or type 2 diabetes mellitus (16). Lately, the Grem1/MIF ratio has been identified as novel marker associated with CVD and the grade of plaque stability. Grem1 was discovered as an endogenous inhibitor of MIF (17). Furthermore, human epidemiological studies supported a pro-atherogenic role of MIF, by showing that a MIF single nucleotide polymorphism was associated with an enhanced risk for MI (12). A large group of MI patients also had elevated MIF plasma levels and these levels were predictive of final infarct size and the extent of cardiac remodeling (18). Elevated MIF levels in these patients were already observed after 4 to 6 h after acute MI, which could be very beneficial for the early detection of MI since current used markers or only elevated after 6 to 12 h post-MI (18).

There are already several MIF inhibitors developed, which show protective effects in various inflammatory models (168).

Another attractive therapeutic strategy would be to directly target the receptors for MIF, CXCR2, or CXCR4, or to manipulate the ligand–receptor interaction. However, more research is first needed to fully elucidate these precise interactions. Although MIF seems like a suitable target for therapy and biomarker in patients, the use of MIF as biomarker in healthy persons should be approached with caution. Prospective data suggest that the relation between MIF and the risk of MI or death due to CVD in humans without prior history of CVD is not very strong (19).

CXCL12 and MIF Side by Side

Both CXCL12 and MIF play an important role in the development of CVDs. However, besides some common effects both chemokines vary functionally from each other, partly mediated by differential receptor usage (Figure 1). An important common function that both chemokines have is the induction of leukocyte chemotaxis and arrest. However, as MIF seems to have more pro-atherosclerotic effects, CXCL12 may have a protective function, although results are still contradictory at some level and future research should further elucidate the exact role of these chemokines in atherosclerosis. Regarding vascular restenosis the effects of CXCL12 and MIF are more equal, since blockade studies showed that inhibition of either CXCL12 or MIF has beneficial outcomes on neointimal hyperplasia. Additionally, blockade of CXCR4, the receptor for both CXCL12 and MIF, has been shown to reduce neointimal formation. Furthermore, both CXCL12 and MIF play a protective role in MI-ischemia-reperfusion injury. For CXCL12, this beneficial effect upon systemic CXCL12 injection was associated with increased recruitment of progenitor cells and neo-angiogenesis. However, the beneficial effects of MIF seem to be cellular source dependent as global MIF deficiency reduced inflammation, while leukocyte-derived MIF promoted the inflammatory response after MI.

Concluding Remarks

It has already been well described that chemokines play an important role in inflammation, atherosclerosis, and CVD. However, the exact involvement of all these chemokines remains very complicated and as research in this area advances, current ideas and dogmas may still change. In the recent years, more data are accumulating pointing toward crucial roles of these chemokines in atherosclerosis and CVD. There have also already been some studies describing the ligand–receptor interactions and the involvement of the receptors, CXCR2, CXCR4, and ACKR3 in different pathologies, although also in this respect further research is needed to identify cell-type-specific effects of CXCR4 for example, but also to clarify the triggered intracellular signaling. Due to the complexity of this chemokine system, one should be very cautious with designing chemokine-based therapeutics, since unwanted side effects may occur very easily. Therefore very specific targeting approaches, like antibodies or inhibitors, are needed to isolate a specific ligand–receptor interaction, perhaps even at a specific cell type.

Author Contributions

EV: drafting the manuscript; YD: concept and design, critical revision; CW: concept and design, critical revision.

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The importance of the CXCL12/CXCR4 axis in therapeutic approaches to diabetes mellitus attenuation

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The pleiotropic chemokine (C–X–C motif) ligand 12 (CXCL12) has emerged as a crucial player in several diseases. The role of CXCL12 in diabetes promotion and progression remains elusive due to its multiple functions and the overwhelming complexity of diabetes. Diabetes is a metabolic disorder resulting from a failure in glucose regulation due to β -cell loss and/or dysfunction. In view of its ability to stimulate the regeneration, proliferation, and survival of β -cells, as well as its capacity to sustain local immune-isolation, CXCL12 has been considered in approaches aimed at attenuating type 1 diabetes. However, a note of caution emerges from examinations of the involvement of CXCL12 in the development of diabetes and its complications, as research data indicate that CXCL12 displays effects that range from protective to detrimental. Therefore, as a beneficial effect of CXCL12 in one process could have deleterious consequences in another, a more complete understanding of CXCL12 effects, in particular its functioning in the cellular microenvironment, is essential before CXCL12 can be considered in therapies for diabetes treatment.

Keywords: CXCL12, CXCR4, CXCR7, diabetes mellitus, diabetic complications, pancreatic β -cells

Introduction

The worldwide prevalence of diabetes has increased six-fold over the past 20 years, with diabetes assuming the proportions of an epidemic. Therefore, research devoted toward improving the steps that could be undertaken in the prevention and treatment of diabetes and its complications is a scientifically and socially significant task. Diabetes mellitus is a complex metabolic disorder that is presented in two major forms, type 1 diabetes (T1D) and the more common type 2 diabetes (T2D). While these conditions have different etiologies, both types of diabetes are characterized by hyperglycemia resulting either from insufficient insulin levels as in T1D, or by an insensitivity of target cells to insulin as in T2D. In T1D, hyperglycemia occurs as a result of destruction of insulin-producing pancreatic β -cells in an autoimmune process. T2D is a metabolic disease characterized by β -cell dysfunction and peripheral insulin resistance. Genetic predisposition and environmental factors, such as diet, physical inactivity, and viral infections contribute to the etiology of diabetes. Early exposure to hyperglycemia predisposes individuals to the development of diabetic complications, a phenomenon which is referred to as “metabolic memory” (1). At present, diabetes management is focused on lowering hyperglycemia and treating its pathological consequences, rather than its initial triggers.

CXCL12 and its Receptors

Current research has positioned CXCL12 as an important molecule in potential treatment of diabetes and its complications. CXCL12 belongs to the CXC group of chemokines. It is a potent chemoattractant involved in angiogenesis, leukocyte trafficking, cancer, inflammatory disorders, atherosclerosis, and HIV pathology (2, 3). CXCL12 is a ligand for two transmembrane receptors: CXCR4 and CXCR7 (4, 5). Interaction between CXCL12 and its receptor CXCR4 induces downstream signaling involved in chemotaxis, cell survival, proliferation, increase in intracellular calcium, and gene transcription (3). CXCR7 is an atypical chemokine receptor that does not signal through the canonical G-protein pathway. CXCL12 binds CXCR7 with even higher affinity than CXCR4. CXCR7/CXCL12 interaction triggers CXCR7 association with β -arrestin 2 and CXCL12/CXCR7 internalization, implying capability of CXCR7 to decrease the level of CXCL12 from the surroundings (6). The observed promotion of cell survival, adhesion, and tumor growth by CXCR7 points to signaling pathways that lie downstream of this receptor (7). This may be in correlation with its ability to heterodimerize with CXCR4 and regulate CXCR4/CXCL12-mediated processes. The role of CXCR7 in mediating the anti-apoptotic effect of CXCL12 has been documented (8). CXCR7 has also emerged as a determinant of autoimmunity and β -cell destruction which underlies diabetic progression (9).

The Role of CXCL12/CXCR4 Axis in Diabetes Pathophysiology

Type 1 Diabetes

Type 1 diabetes is an autoimmune disease triggered by environmental factors in genetically susceptible persons. In T1D, pancreatic β -cells are targeted by the individual's own immune system resulting in reduced or complete elimination of insulin production. Discouraging, long-term studies of islet transplantation stress the need for new strategies to counteract autoimmunity, and CXCL12 has emerged as a key molecule in this process. CXCL12 plays a particularly important role in directing T cell migration and therefore in immune processes. Recruitment of autoreactive T cells into pancreatic islets leads to inflammation (referred to as insulinitis) that initiates T1D development. Several reports have revealed that neutralization of CXCL12 inhibits insulinitis and diabetes development (10, 11). It was proposed that retention of regulatory T cells (Tregs) in the bone marrow by CXCL12 disturbs the balance of T cells in favor of autoreactive T cells, which intensifies disease progress. However, a reverse effect of CXCL12 inhibition was reported by Aboumarad et al. who showed that a population of CXCR4+ T cells attracted by CXCL12 protects recipient mice from the adoptive cell transfer of diabetes (12). The beneficial effects of CXCL12 could be explained by several properties specific to this chemokine. CXCL12 induces bi-directional movement of T cells, toward lower concentration and away from higher CXCL12 concentration (13). CXCL12 also exerts a chemorepulsive effect on diabetogenic T cells, while mediating firm adhesion of normal T cells (14). Moreover, CXCL12 expression in islets was shown to cause the selective repulsion of autoreactive T cells and retention of Tregs at the site (15). Tregs play a crucial role

in suppressing autoimmunity and data support their relevance in T1D pathogenesis (16). It was reported that pancreatic lymph nodes (PLNs) of non-obese diabetic (NOD) mice lack Tregs, while the recovery of euglycemia in these mice was associated with the restoration of the Treg population in PLNs (17). The absence of Tregs correlates with the locally decreased expression of CXCL12, suggesting that improved function of the CXCL12/CXCR4 axis and subsequent retention of Tregs in the PLNs could serve as the basis for an alternative therapeutic approach for treating T1D. Selective repulsion of autoreactive T cells and attraction of Tregs have been proposed as a mechanism for a recently reported novel strategy in islet transplantation. It has been shown that immune-mediated rejection of transplanted islets could be delayed by their local immune-isolation achieved through coating or encapsulation of islets with CXCL12, thus excluding the need for systemic immunosuppression (18). Despite the evidence importance of this finding for T1D treatment, it should be noted that local immunosuppression achieved through CXCL12 has also been observed in cancer models where this mechanism protects cancer cells from immune attack (19).

Type 2 Diabetes

Type 2 diabetes is a metabolic disorder characterized by insulin resistance in adipose tissue, liver and skeletal muscle, and defective pancreatic insulin secretion. Experimental and clinical data describe diabetes as a chronic inflammatory disease (20). More than 20 years ago, it was shown that the pro-inflammatory cytokine TNF- α was capable of inducing insulin resistance (21). It is now known that the plasma levels of pro-inflammatory cytokines, such as TNF- α , IL-6 or IL-1 β , and chemokines are elevated in T2D patients, while *in vivo* studies have revealed that inhibition of key inflammatory cytokines protects rodents from insulin resistance (22). The inflammatory cytokines promote insulin resistance by interfering with insulin signaling through activation of JNK kinase and NF κ B pathways (23). Pancreatic β -cells respond to insulin resistance by increasing insulin secretion. However, when β -cells fail to compensate for increased insulin demands, T2D develops.

Chronic low levels of inflammation in the pancreas and insulin-responsive tissues of diabetics are accompanied by infiltration of lymphocytes and macrophages. The latter process is associated with a switch from an anti- to a pro-inflammatory profile. Namely, diabetes is linked to a disturbed balance between pro-inflammatory (Th1 and Th17) and anti-inflammatory (Th2 and Tregs) subsets of T cells in favor of the pro-inflammatory phenotype. As a result, Th1 and Th17 promote the polarization of M1 macrophages, which are the main producers of pro-inflammatory cytokines (24). CXCL12 has a controversial role in inflammation, as a result of its ability to orchestrate the trafficking of a variety of immune cells. Based on the reports describing CXCL12-promoted recruitment of immune cells to inflamed tissues in autoimmune diseases such as rheumatoid arthritis (RA) and lupus erythematosus in lung inflammation and inflammatory bowel disease (25), CXCL12 has been proposed to have a pro-inflammatory role. Also, CXCL12 recruits monocytes into adipose tissue, which after differentiation secrete pro-inflammatory cytokines in obesity. It was suggested that the

CXCL12/CXCR4 axis induces M1 macrophage accumulation, subsequent inflammatory cytokine production, and finally insulin resistance (26). However, CXCL12 was also found to possess an anti-inflammatory role by mediating T cell polarization toward Tregs, and by stimulating IL-10 production in anti-inflammatory M2 macrophages (27). Moreover, CXCL12 promotes migration of monocytes and their polarization toward the M2 phenotype (28), which points to the potential role of CXCL12 in reducing inflammation in diabetes. Once again, we should be aware that promotion of the anti-inflammatory Treg/M2 phenotype by CXCL12 is part of the mechanism involved in suppression of anti-tumor immunity mediated by this chemokine (29).

Given the strong correlation between inflammation and T2D, anti-inflammatory strategies for T2D treatment have been proposed and some have been clinically tested. Encouraging results were observed with salsalate, an inhibitor of the NF- κ B pathway, and IL-1 β antagonists. Potential targeting of CXCL12 for T2D treatment requires additional studies and a better understanding of the role of CXCL12 in T2D and inflammation.

Potential Utilization of the CXCL12/CXCR4 Axis in Diabetes Management Through Promotion of Pancreatic β -Cell Differentiation, Regeneration, and Survival

Current limitations in diabetes treatment have stimulated efforts toward β -cell replacement therapy. Preservation of β -cell mass, stimulation of β -cell differentiation from embryonic stem cells (ESCs), regeneration of the impaired endocrine pancreas from remaining β -cells, and cellular reprogramming of other endocrine or exocrine cell types in pancreas could provide a long-term solution in diabetes treatment (30–32). This strategy requires understanding of the molecular mechanisms that control β -cell maturation, growth, and survival.

The CXCL12/CXCR4 Axis in β -Cell Differentiation and Regeneration

CXCL12/CXCR4 signaling is crucial for β -cell differentiation and pancreatic islet genesis (31). CXCL12 is expressed in the gut endoderm and attracts CXCR4 expressing angioblasts which induce pancreatic and duodenal homeobox 1 (Pdx1) expression in the pre-pancreas region (33). Pdx1 plays a key role in the expression of neurogenin 3 (Ngn3) which is essential for the formation of all islet cell types (34). During human fetal β -cell development, CXCR4 is necessary for the *in vivo* differentiation of islet-like clusters into β -cells while CXCL12 directs the proliferation of epithelial endocrine precursors through activation of phosphatidylinositol (PI)-3 and Akt kinases (31). Expression of interferon (IFN) γ , which is under the control of the insulin promoter, promotes ductal hyperplasia and regeneration of new islets in the pancreas of transgenic mice (35), providing an excellent model for studying pancreas regeneration. When NOD mice were used as an IFN γ transgenic model, their pancreas displayed three- and four-fold elevated expression of CXCL12 and CXCR4, respectively, in comparison to non-transgenic NOD mice (36).

CXCL12 expression in IFN γ -NOD mice stimulated pancreatic ductal cell migration and activation of the Akt, Src, and extracellular signal regulated protein kinase (ERK1/2) in duct cells, revealing the essential role of CXCL12 in their survival, proliferation, and migration during pancreatic regeneration. These insights could help in developing new therapeutic protocols for stimulating the differentiation of pancreatic stem cells into β -cells, proliferation of existing β -cells and transdifferentiation of particular cell types in diabetic patients (31, 32, 37). Furthermore, *in vitro* treatment of human ESCs with appropriate signals could direct their differentiation into β -cells that could be transplanted in diabetic patients.

The CXCL12/CXCR4 Axis in β -Cell Survival

Diabetes-related studies have provided evidence for an important role of CXCL12 in anti-apoptotic and anti-necrotic protection of β -cells from diabetogenic agents. Transgenic mice overexpressing CXCL12 in β -cells are protected from streptozotocin (STZ)-induced diabetes via activation of Akt kinase (38). CXCL12-stimulated Akt signaling activates anti-apoptotic signals in β -cells through increased expression of the anti-apoptotic B cell lymphoma 2 (Bcl-2) protein and anti-apoptotic phosphorylation of the proapoptotic Bcl-2-associated death (BAD) protein. CXCL12-mediated induction of Akt activity also promotes activation and stabilization of beta-catenin/transcription factor 7-like 2 (TCF7L2) that contributes to the survival of isolated islets and INS-1 cells (39). Activation of the CXCL12/CXCR4 axis by STZ, cytokines and in thapsigargin injury of human and mouse islets promotes intra-islet GLP-1 production and enhances β -cell survival (40). It has been proposed that the paracrine action of CXCL12 from β -cells activates Akt in adjacent α -cells, promoting their proliferation and production of GLP-1 instead of glucagon. CXCL12 and GLP-1 signal from α -cells together control the growth and viability of β -cells. These findings raise the possibility that the anti-apoptotic/prosurvival CXCL12 and pro-growth GLP-1 signaling act either additively or synergistically to conserve or possibly enhance β -cell mass in response to injury.

CXCL12 overexpression considerably improves insulin expression and viability of isolated rat islet cells and Rin-5F pancreatic β -cells after hydrogen peroxide treatment (41). CXCL12 overexpression in pancreatic cells switches hydrogen peroxide-induced cell death from the necrotic to the apoptotic pathway through Akt kinase-mediated reduction of poly(ADP-ribose) polymerase-1 (PARP-1) activity (Figure 1). These findings are in correlation with the documented role of PARP-1 in necrotic cell death (42) and with the observation that pharmacological inhibition or genetic deletion of PARP-1 protects animals against chemically induced and spontaneous diabetes development (43). The anti-necrotic effect of CXCL12 could prevent an additional pro-inflammatory burden of β -cells provoked by necrosis and could therefore be used for diabetes treatment.

Transcriptional Regulation of the CXCL12 Gene

Considering the involvement of CXCL12 in β -cell differentiation, growth, and survival, an understanding of CXCL12 transcriptional regulation offers possibility to improve β -cell mass in diabetes. PARP-1 is involved in rat CXCL12 gene (*Cxcl12*)

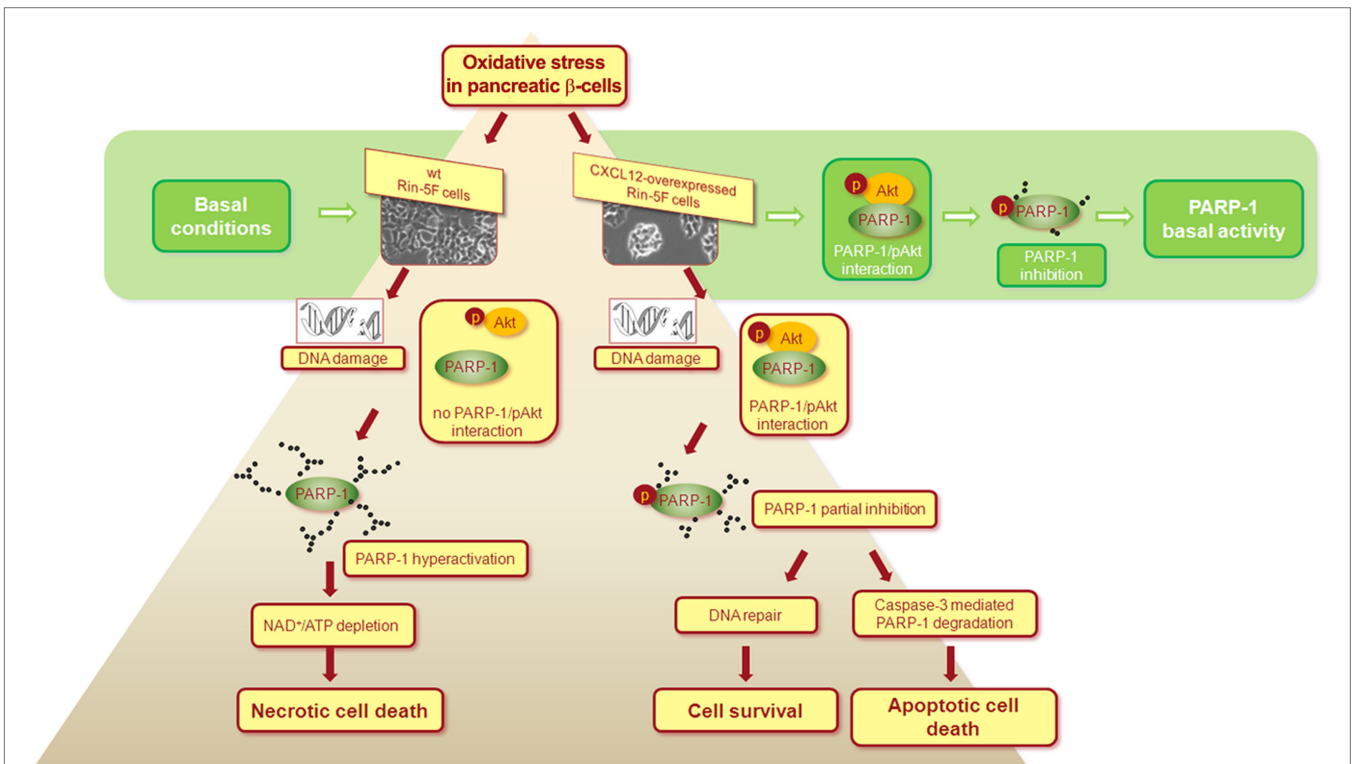


FIGURE 1 | Proposed mechanism of the CXCL12/Akt-mediated anti-necrotic effect that leads to pancreatic β-cells survival. Under basal conditions, interaction between activated Akt (pAkt) and PARP-1 leads to PARP-1 phosphorylation that results in partial inhibition of PARP-1 in wild type (wt) and CXCL12 overexpressing Rin-5F cells. After hydrogen peroxide-induced oxidative stress and in response to severe DNA damage in wt cells, the loss of pAkt-PARP-1 interaction allows PARP-1 hyperactivation, followed by extensive PARP-1 auto-poly(ADP-ribosylation), NAD⁺ and ATP

depletion, and final necrotic cell death. In CXCL12 overexpressing cells, pAkt-PARP-1 interaction persists after hydrogen peroxide treatment, maintaining partial inhibition of PARP-1. Consequently, cellular energy depletion is prevented and a switch from the necrotic to the apoptotic cell death is ensured. With CXCL12-mediated suppression of PARP-1 overactivation in stress conditions, cell still operates with the active PARP-1 that is essential player in many cellular processes. The mechanism is based on the findings presented in Ref. (41).

downregulation during the early stage of STZ-induced oxidative stress (44). During later stages of oxidative stress and intensive pancreatic β-cell injury, *Cxcl12* expression is upregulated by Yin Yang 1 (YY1). Multiple protein-protein interactions between C/EBPα, C/EBPβ, STAT3, p53, FOXO3a, and HMG I/Y transcription factors that bind to the *Cxcl12* promoter in the rat pancreatic Rin-5F cell line suggest that multi-subunit protein complexes are responsible for the regulation of *Cxcl12* transcription (44). Targeted stimulation or suppression of specific transcription factors involved in the regulation of genes engaged in the proper functioning of β-cells could improve therapeutic approaches in diabetes.

The Controversial Role of CXCL12 in Diabetic Complications

Along with the promotion of cell survival, the biological effects of CXCL12, such as angiogenesis induction and recruitment of bone marrow-derived progenitor cells suggest that this chemokine assumes a central position in tissue repair and regeneration. Since diabetes is accompanied by dysfunction and life-threatening damage of several organs, the potential of CXCL12 to create a microenvironment that supports repair processes is particularly

important. Indeed, CXCL12 accelerates wound healing in diabetes by recruiting endothelial progenitor cells (EPCs) and through improved angiogenesis (45, 46). However, the same injury response mechanisms could also promote disease progression, as in diabetic retinopathy, which starts with damage to small blood vessels in the eye and leads to reduced blood flow and ischemia. The ischemia promotes aberrant neovascularization that destroys the normal retinal architecture, causing impaired vision. In agreement with the fact that the expression of CXCL12 is controlled by hypoxia-inducible factor-1 (47), the level of CXCL12 increases as diabetic retinopathy progresses and contributes to angiogenesis by recruiting EPCs to the site of vascular injury (48). Blocking the function of CXCL12 prevents neovascularization and progression of proliferative retinopathy.

Diabetic nephropathy is characterized by the development of albuminuria with a subsequent decline in glomerular filtration rate, usually followed by failure of renal function. CXCL12 expression in the kidney increases during acute renal failure, resulting in homing of progenitor cells to the injured kidney (49). Although CXCL12 is considered as one of the major mediators involved in kidney repair after ischemic acute renal failure, data regarding the role of this chemokine in diabetic nephropathy

are limited. A study on the mouse model of T2D revealed that CXCL12 contributes to glomerulosclerosis, podocyte loss, and albuminuria, implicating the pathogenic role of CXCL12 in diabetic nephropathy (50). The same group proposed a novel strategy for the prevention of glomerulosclerosis in T2D. This is based on the protective effect of dual chemokine CCL2-CXCL12 blockage: inhibition of CCL2-mediated glomerular leukocyte recruitment and CXCL12-mediated loss of podocytes (51).








Diabetes dramatically increases the risk of various cardiovascular problems, including coronary artery disease with myocardial infarction and atherosclerosis (52). Although the importance of CXCL12 in cardiovascular disease has been intensively studied, current findings once again suggest a double-edged role of this chemokine in ischemic heart and atherosclerosis (53). Since this issue has been extensively reviewed elsewhere in Ref. (53), it will not be considered here in more detail. It is worth mentioning that genome-wide association studies revealed a significant association of two SNPs downstream of the *CXCL12* gene with cardiovascular disease (54).

The general conclusion regarding the role of CXCL12 in diabetic complications is that CXCL12 walks a thin line between protective and detrimental effects. Therapies that rely on either promotion of CXCL12 or blocking of its activity have been suggested for a variety of conditions. The above-mentioned findings indicate that CXCL12-based therapy should be used with extreme caution and by precise targeting of CXCL12 action to specific tissue (Table 1). Proper functioning of the immune system provides a balance between protection from pathogens and tissue damage, and between autoimmunity and cancer suppression. Disturbance of this balance might be beneficial in one process, while at the same time detrimental in another.

Conclusion

The role of CXCL12 in diabetes is very complex. For a better understanding of the biological effects of CXCL12, additional studies are needed to clarify several issues. As has already been mentioned, aside from CXCR4, CXCL12 also binds to its second receptor CXCR7, whose downstream signaling is elusive. The reported heterodimerization of these receptors has introduced additional complexity to CXCL12 signaling (55). CXCR7 also binds CXCL11 (56), while CXCR4 has been shown to be a receptor for the cytokine MIF and chemokine CXCL14 (57, 58). Therefore, the biological functions of CXCL12 must be considered in the

TABLE 1 | Yin-Yang nature of CXCL12 in diabetic complications.

Effects of activated CXCL12/CXCR4 axis (in a mouse model system)	Role of the CXCL12/ CXCR4 axis in diabetes	Reference
Accelerates wound healing in diabetes, improves angiogenesis		(45, 46)
Promotes diabetic retinopathy, contributes to angiogenesis via recruitment of EPCs to the site of vascular injury		(48)
Improves diabetes progression in NOD mice by sequestering Tregs in the bone marrow, which disturbs the balance in favor of autoreactive T cells		(10)
Prevents insulinitis and autoimmune diabetes via recruitment of Th2-type cells to the pancreas of NOD mice		(12)
Mediates kidney repair by homing of progenitor cells to the injured kidney in acute renal failure		(49)
Contributes to progression of diabetic nephropathy through involvement in glomerulosclerosis, podocyte loss and albuminuria		(50)
Induces M1 macrophage accumulation in adipose tissue which leads to secretion of pro-inflammatory cytokines in obesity, associated with insulin resistance		(26)

Balanced (☯) CXCL12 expression and distribution is indispensable for the final outcome of physiological action that can produce either protective (☰) or detrimental effects (☷) during diabetes onset and in diabetic complications.

context of a specific microenvironment, taking into account the site of CXCL12 expression, the expression of other chemokines, and all receptors on target cells. A complete understanding of the complex CXCL12 network is a prerequisite for the safe application of CXCL12-based therapy in diabetes.

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Role of macrophage migration inhibitory factor in obesity, insulin resistance, type 2 diabetes, and associated hepatic co-morbidities: a comprehensive review of human and rodent studies

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Obesity is associated with a chronic low-grade inflammatory state that drives the development of obesity-related co-morbidities such as insulin resistance/type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease. This metabolic inflammation is thought to originate in the adipose tissue, which becomes inflamed and insulin resistant when it is no longer able to expand in response to excess caloric and nutrient intake. The production of inflammatory mediators by dysfunctional adipose tissue is thought to drive the development of more complex forms of disease such as type 2 diabetes and NAFLD. An important factor that may contribute to metabolic inflammation is the cytokine macrophage migration inhibitory factor (MIF). Increasing evidence suggests that MIF is released by adipose tissue in obesity and that it is also involved in metabolic and inflammatory processes that underlie the development of obesity-related pathologies. This review provides a comprehensive summary of our current knowledge on the role of MIF in obesity, its production by adipose tissue, and its involvement in the development of insulin resistance, type 2 diabetes, and NAFLD. We discuss the main findings from recent clinical studies in obese subjects and weight-loss intervention studies as well as results from clinical studies in patients with insulin resistance and type 2 diabetes. Furthermore, we summarize findings from experimental disease models studying the contribution of MIF in obesity and insulin resistance, type 2 diabetes, and hepatic lipid accumulation and fibrosis. Although many of the findings support a pro-inflammatory role of MIF in disease development, recent reports also provide indications that MIF may exert protective effects under certain conditions.

Keywords: MIF, obesity, adipose tissue, insulin resistance, type 2 diabetes, non-alcoholic fatty liver disease

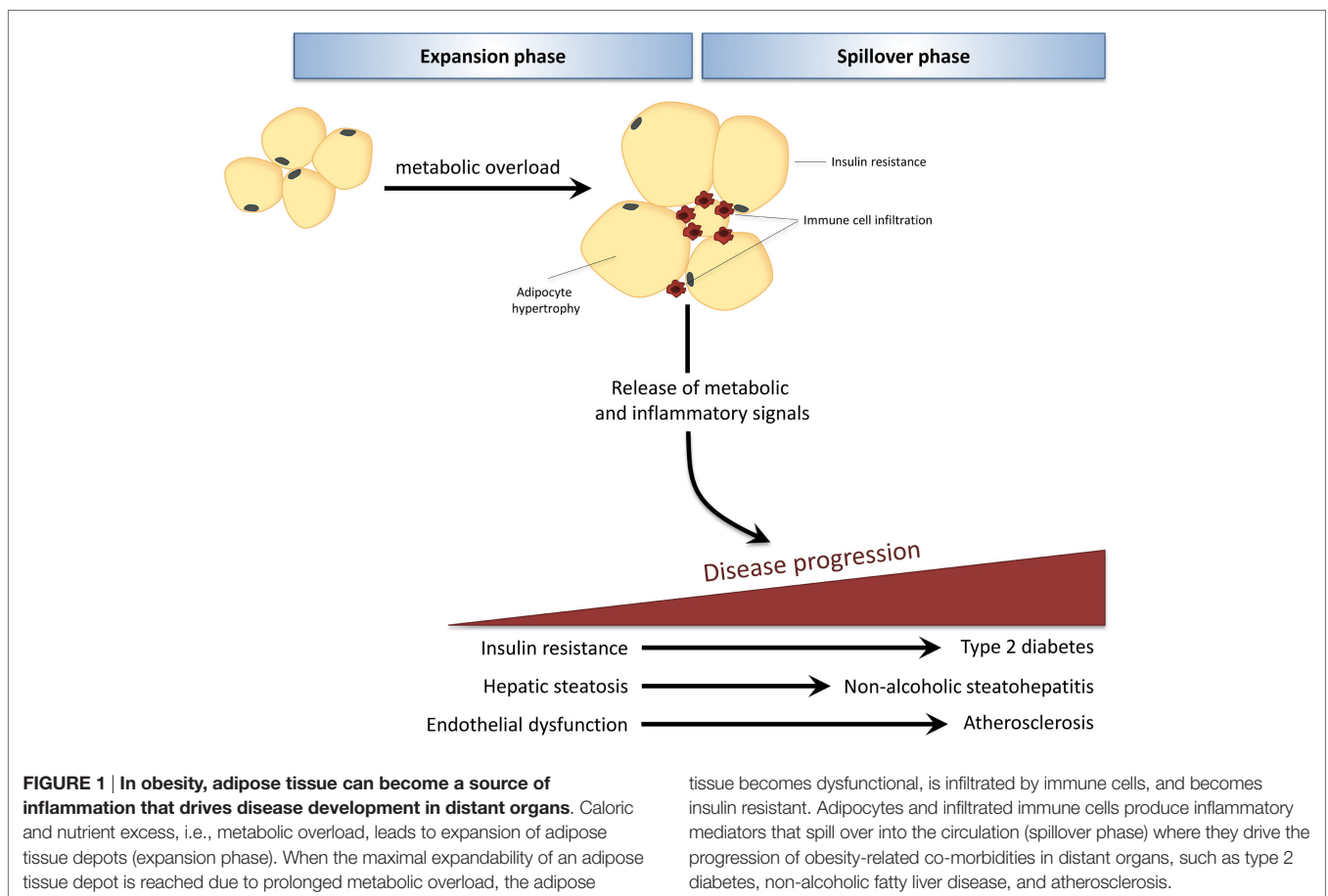
Abbreviations: ALAT, alanine transaminase; ASAT, aspartate transaminase; HFD, high-fat diet; HOMA, homeostatic model assessment; ipGTT, intraperitoneal glucose tolerance test; ipITT, intraperitoneal insulin tolerance test; MCD, methionine and choline deficient; MIF, macrophage migration inhibitory factor; MNC, mononuclear cell; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; T2D, type 2 diabetes.

Introduction

The development of obesity-associated co-morbidities such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease (CVD) is considered to be driven by the chronic low-grade inflammatory state that characterizes obesity (1). During obesity, excess caloric and nutrient intake, i.e., metabolic overload, leads to adipose tissue expansion and visceral adiposity. When the maximal expandability of an adipose tissue depot is reached (when adipocytes have maximally expanded) this leads to adipose tissue dysfunction and infiltration of immune cells, which is associated with insulin resistance of the adipose tissue itself (2, 3). Furthermore, adipocytes and infiltrated immune cells of this inflamed adipose tissue produce inflammatory mediators that can be released into the circulation (4). Thus, the adipose tissue becomes a source of inflammation that can drive pathogenesis in other tissues, leading to the development of T2D, NAFLD, and CVD (**Figure 1**).

Macrophage migration inhibitory factor (MIF) is a cytokine that is ubiquitously expressed, both by immune and non-immune cells. It is well known for its pro-inflammatory effects and is recognized as a negative regulator of the immunosuppressive actions of glucocorticoids (5). In line with this, MIF has been implicated in the development of many acute inflammatory and auto-immune diseases (6, 7), as well as chronic inflammatory

metabolic disorders (8–10). MIF can act via its receptor CD74 (11), and controls the recruitment of inflammatory cells via CXCR2 and CXCR4 signaling (8). Furthermore, MIF can exert pro-inflammatory effects through its enzymatic tautomerase and oxidoreductase activity (12, 13). Several lines of evidence provide indications that MIF, besides regulating inflammation, may also be linked to energy metabolism. It is expressed in metabolically active tissues such as the adipose tissue and the liver (9, 14). Its expression by adipocytes is regulated by glucose and insulin (15) and it has been shown to have catabolic effects in muscle (16). Furthermore, it co-localizes with insulin within the secretory granules of pancreatic beta cells and is a modulator of insulin release (17). Increasing evidence suggests that MIF may also control inflammatory and metabolic processes in the pathogenesis of obesity and associated disorders including insulin resistance, T2D, and NAFLD. Here, we provide a comprehensive review of the current knowledge on the role of MIF in obesity, MIF production by adipose tissue, and its role in the development of insulin resistance, type 2 diabetes, and NAFLD. Since epidemiological studies have shown both insulin resistance (18, 19) and NAFLD (20, 21) to be associated with future CVD risk, this may also have relevance for the role of MIF in vascular disease, which has been described elsewhere for rodents (22, 23) and humans (24, 25), and will not be addressed here. This survey aims to advance our understanding on the functions of



MIF in non-pathological and pathological processes connected to obesity and glucose homeostasis, with particular emphasis on adipose tissue and liver.

All values are mean \pm SEM. Where the original article stated mean \pm SD, SEM was calculated from SD by dividing SD by the square root of n .

Human Studies

Circulating MIF and Obesity

Several studies provide evidence for a positive association between obesity and circulating MIF levels (Table 1). Dandona and co-workers (26) reported that obese individuals (BMI 37.5 ± 4.9 kg/m²)

TABLE 1 | Relationship between (circulating) MIF and obesity and effects of weight loss thereupon.

Subjects	BMI	Gender		MIF (ng/ml)	Effect observed	Reference
		M	F			
Lean	22.6	26	14	1.2	Positive correlation plasma MIF with BMI. MNC <i>MIF</i> mRNA increased in obesity and positively correlated with BMI.	(26)
Obese	37.5	19	21	2.8		
Lean	22.6	–	16	1.3	Positive correlation plasma MIF with HOMA and BMI ($p = 0.10$). MNC <i>MIF</i> mRNA increased in obesity and positively correlated with BMI.	(27)
Obese	40.0	–	16	3.3		
Lean	20.6	–	20	0.5	Plasma MIF increased in obesity.	(30)
Obese	35.2	–	26	1.9		
Lean	22.1	–	6	5	Plasma MIF positively correlated with % body fat, % truncal fat, and fasting insulin.	(31)
Obese	35.8	–	6	16		
Lean boys	16.0	59	–	3.8	Plasma MIF higher in obese boys than in overweight boys. No effect in girls (population: pre-pubertal schoolchildren between 5 and 13 years, average 9 years).	(32)
Overweight boys	22.0	32	–	3.6		
Obese boys	25.7	70	–	4.2		
Lean girls	15.1	–	46	3.9		
Overweight girls	21.3	–	28	3.9		
Obese girls	25.1	–	70	4.3		
Waist circumference <90th percentile	20.7	41	–	Median 0.6	Positive correlation plasma MIF with weight, BMI, and waist circumference (population: Caucasian adolescents 13–17 years).	(33)
Waist circumference >90th percentile	31.9	38	–	Median 1.0		
Healthy controls	20.2	52	32	1.0	Plasma MIF increased in metabolic syndrome. No significant correlation with BMI.	(34)
Metabolic syndrome	27.2	62	26	1.4		
Lean	23.0	–	14	12.0	No difference in plasma MIF between lean and obese subjects.	(35)
Obese	32.7	–	33	13.5		
Lean	<27	–	ns	nd	MIF secretion from isolated (subcutaneous and omental) adipocytes is positively correlated with BMI. No difference between depots.	(38)
Obese	37	–	ns	nd		
Lean	23.1	9	–	nd	Subcutaneous abdominal adipose tissue <i>MIF</i> mRNA expression is increased in obesity and is positively correlated with waist circumference.	(39)
Obese	34.7	9	–	nd		
Overweight	29.3	–	17	nd	<i>MIF</i> mRNA expression in visceral adipose tissue twofold higher than in subcutaneous adipose tissue, and positively correlated with % body fat.	(40)
Obese	36	18	16	ns	Subcutaneous abdominal adipocyte <i>MIF</i> mRNA expression positively correlated with adipocyte diameter but not with plasma MIF.	(43)
Lean	24.2	21	4	Median 5.1	Plasma MIF higher in obese than in lean subjects. Weight loss (by diet and physical activity based weight management program) reduced plasma MIF levels.	(28)
Obese before weight loss	43.0	23	48	Median 8.4		
Obese after weight loss	38.3			Median 5.1		
Lean	19.9	–	10	5.0	Plasma MIF and mononuclear cell <i>MIF</i> mRNA are higher in obese subjects than in lean subjects and both are reduced by weight loss (12-week caloric restriction and light exercise-based weight loss program). Mononuclear cell <i>MIF</i> mRNA positively correlated with BMI.	(29)
Obese before weight loss	32.5	–	21	16.0		
Obese after weight loss	30.6			5.4		
Before intervention	27.6			nd	Weight loss did not affect subcutaneous adipose tissue <i>MIF</i> mRNA expression.	(44)
Control	27.3	5	6			
Caloric restriction	25.0	6	6			
Caloric restriction + exercise	24.8	5	7			
Obese before surgery	44.6	5	29	0.2	Serum MIF reduced 12 months after bariatric surgery. Positive correlation between reduction in serum MIF and body weight loss.	(45)
Obese after weight loss	35.2			0.02		
Obese before surgery	46.7	5	22	0.2	Plasma MIF levels increased after weight loss (at 24 months after bariatric surgery).	(46)
Obese after weight loss	33.0			0.7		

ns, not specified; nd, not determined.

had significantly higher plasma MIF levels (2.8 ± 2.0 ng/ml) than lean individuals (BMI 22.6 ± 3.4 kg/m²; plasma MIF 1.2 ± 0.6 ng/ml) and that there was a highly significant positive correlation between MIF levels and BMI. Results from another study by the same group (27) confirmed these results, reporting MIF levels of 3.3 ± 2.4 ng/ml in obese subjects (BMI 40.0 ± 4.4 kg/m²), which were significantly higher than the 1.3 ± 0.8 ng/ml observed in the lean controls (BMI 22.6 ± 1.9 kg/m²). Again, there was a positive correlation ($p = 0.10$) between MIF levels and BMI. Others have reported similar findings of increased circulating MIF in obese individuals compared with healthy lean controls (28–31), and showed associations with % body fat and % truncal fat (31). Studies in pre-pubescent schoolchildren (age 5–13 years) and male adolescents (age 13–17 years) demonstrate that these associations are not only observed in adults, reporting increased MIF levels in overweight and obesity in childhood and adolescence (32, 33), and showing positive correlations with waist circumference and BMI (33) in this study population as well.

A study by Kim et al. (34) compared healthy Korean subjects (BMI 20.2 kg/m²) without metabolic syndrome with patients with metabolic syndrome (BMI 27.2 kg/m²) and found higher levels in patients than controls (1.4 ± 0.1 and 1.0 ± 0.1 ng/ml, respectively), which was significant in women but not in men. N.B. there was no correlation between circulating MIF and BMI in this study, suggesting that other MIF-inducing factors play a role when overweight/obesity progresses toward a phenotype of metabolic disease.

Of note, plasma MIF concentrations tend to be higher in males than in females (26, 34), suggesting an inducing effect of male sex hormones. Indeed, circulating MIF levels are two to threefold higher in women with polycystic ovary syndrome (PCOS), an endocrine disorder characterized by elevated levels of androgens (31, 35). Within PCOS patients, the effect of obesity is not significant due to large variation within this population, and comparable plasma MIF levels were found in non-obese and obese patients [~ 35 and ~ 55 ng/ml, respectively, in Ref. (31); 35.2 and 48.6 ng/ml, respectively, in Ref. (35)].

Remarkably, there is a large variation in the absolute circulating MIF values in documented obesity studies, indicating that there may be underlying methodological differences causing these diverse values. A part of the variation may be explained by different analytical methods (e.g., different ELISA kits, multiplex technology) but even studies employing the same analytical tools report substantial differences [e.g., Ref. (26, 31)]. Hence, other technical or methodological issues may be responsible for these discrepancies. The MIF molecule itself and its oligomers are very hydrophobic (36, 37) and tend to precipitate or stick to plastic material and differences in sample preparation and/or pipetting can result in differences as reported in the human studies. Therefore, it is difficult to make comparisons between different studies. However, the direction of the effect (obese higher than lean) is mostly consistent and overall, these studies demonstrate a well-established association between obesity and circulating MIF that seems to transcend differences in age, sex, and ethnicity. The observed positive correlations with BMI and % body fat provide indication that the observed association may be related to the increased adipose tissue mass that is inherent

to obesity and hint at the adipose tissue as a contributor to the observed association.

Adipose Tissue MIF Production

Several groups have investigated adipose tissue as a potential source of circulating MIF in obesity (Table 1). Skurk and co-workers (38) demonstrated that primary human pre-adipocytes and mature adipocytes have the capacity to produce and secrete MIF and the secretion of MIF was found to increase in parallel with the (*in vitro*) differentiation of pre-adipocytes. There was a close positive correlation between MIF release from mature adipocytes and donor BMI, which was observed in both visceral and omental adipocytes, with no differences between these two depots. Others have shown similar effects on the gene expression level. González-Muniesa and colleagues (39) showed that *MIF* mRNA expression in subcutaneous abdominal adipose tissue is increased in obese individuals and correlates with waist circumference. A positive correlation was also reported by Alvehus and co-workers (40) for the visceral adipose tissue depot, which is an important source of inflammation and, in contrast to subcutaneous adipose tissue, is associated with inflammatory metabolic disorders such as NAFLD and CVD (41, 42). Alvehus et al. (40) showed that *MIF* mRNA expression in the visceral adipose tissue was higher than in the subcutaneous adipose tissue (~ 2 -fold) and was positively correlated with body fat percentage. Adipose tissue inflammation is considered to be a consequence of adipocyte hypertrophy and is thought to be triggered by maximal expansion of adipocytes (2, 3). In line with this notion, subcutaneous abdominal adipocyte diameter correlates with *MIF* mRNA expression (43).

If indeed the increased levels of circulating MIF in obesity are primarily the result of increased adipose tissue mass and adipocyte expansion, one would logically expect plasma MIF levels to decrease when obese subjects lose weight. Indeed, reduced MIF levels have been reported upon weight loss. Church and colleagues (28), for instance, observed significant reductions in plasma MIF levels in obese individuals after a diet and physical activity based weight-loss program. Participants' BMI after this 8.5-month program was reduced from 43.0 ± 8.6 to 38.3 ± 7.6 kg/m² (reflecting an average weight loss of 14.4 kg), which resulted in completely normalized plasma MIF levels comparable to those of lean controls (from median 8.4 to 5.1 ng/ml). Similar results were reported in a different study on the effects of weight loss (29) in which obese subjects participated in a 3.5-month diet and light exercise-induced weight-loss program. A reduction in BMI from 32.5 ± 1.2 to BMI 30.6 ± 1.6 kg/m² (reflecting a weight loss of 4.0 ± 0.4 kg) was accompanied by a reduction in serum MIF from 16.0 ± 4.0 to 5.4 ± 0.4 ng/ml, which again was comparable to lean control subjects. Remarkably, the observed weight loss in both these studies in obese subjects resulted in completely normalized MIF levels comparable to those observed in lean subjects, even though BMI was still considerably higher in the obese subjects (and subjects were still obese even after weight loss), thus indicating that other factors rather than fat mass *per se* (e.g., insulin sensitivity, hormonal changes) may determine circulating MIF levels, or that weight loss may normalize the state of a particular adipose tissue depot that is primarily responsible for MIF production and secretion. Furthermore, a relatively small

reduction in adipocyte size may already be sufficient to prevent further adipocyte damage and thereby attenuate the inflammatory process in adipose tissue.

Only a few studies have examined whether a specific adipose tissue depot is responsible for the release of MIF into the circulation. Support for the view that circulating MIF must be produced by adipose tissue depots other than the subcutaneous adipose tissue comes from studies by Koska et al. (43) who showed that subcutaneous adipose tissue *MIF* mRNA does not correlate with circulating MIF levels, and Tam et al. (44) who found no effect of weight loss on subcutaneous adipose tissue *MIF* mRNA expression. Identification of the underlying factors that determine circulating MIF in obesity warrants further investigation.

Two studies on the effects of bariatric surgery-induced weight loss in morbidly obese subjects report conflicting results in this specific population. Fenske et al. (45) showed that weight loss at 12 months after bariatric surgery (BMI from 44.6 ± 0.9 to 35.2 kg/m^2) resulted in reductions in serum MIF levels from 0.2 ng/ml before surgery to 0.02 ng/ml at 12 months after surgery. Van Dielen and colleagues (46), on the other hand, reported similarly low-plasma MIF levels in morbidly obese subjects before surgery (BMI $46.7 \pm 1.1 \text{ kg/m}^2$, plasma MIF 0.2 ng/ml) but observed increased MIF levels at 24 months after bariatric surgery (weight loss to BMI $33.0 \pm 0.9 \text{ kg/m}^2$, plasma MIF 0.7 ng/ml). To gain insight into the processes that determine circulating MIF levels in these patients, longitudinal analyses after bariatric surgery are needed and it is possible that co-variables such as impaired insulin secretion and diabetes may determine plasma MIF levels in this extreme group of metabolically deregulated subjects.

Overall, the above studies indicate that whole-body adiposity is not a key determinant of circulating MIF and suggest that MIF is produced by specific adipose tissue depots during a particular period of depot growth (i.e., adipocyte expansion within the depot) and resulting adipose tissue inflammation. Of note, circulating MIF may also be produced by other tissues and cells in obesity. Besides adipose tissue as a possible source of circulating MIF in obesity, there are some reports on MIF expression by mononuclear cells (MNCs), with reports of increased *MIF* mRNA expression by MNC from obese subjects (26, 27, 29). Although MNC *MIF* expression did not correlate with plasma MIF levels (26), a correlation with BMI was reported in two of these studies (26, 27) and weight loss (BMI from 32.5 ± 1.2 to $30.6 \pm 1.6 \text{ kg/m}^2$) significantly reduced MNC *MIF* mRNA expression (29).

Relationship (Circulating) MIF and Insulin Resistance

Results from observational studies have shown increased levels of circulating MIF in insulin resistant and T2D subjects (Table 2). Yabunaka et al. (47) reported increased serum MIF levels in Japanese type 2 diabetic subjects ($20.7 \pm 1.5 \text{ ng/ml}$) compared with healthy control subjects ($5.2 \pm 0.3 \text{ ng/ml}$); however, there was no correlation between MIF and fasting plasma glucose, HbA1c, or diabetes duration. Interestingly, BMI was comparable between T2D patients and controls in this study ($23.9 \pm 0.3 \text{ kg/m}^2$ in T2D vs. $23.8 \pm 0.4 \text{ kg/m}^2$ in controls) indicating that the observed difference was not attributable to a higher BMI in the diabetic subjects. (N.B. as the study was conducted in an Asian population absolute BMI values are lower than in Caucasian diabetes patients.)

TABLE 2 | Relationship between (plasma) MIF and insulin resistance/T2D.

Subjects	Gender		MIF (ng/ml)	Effect observed	Reference
	M	F			
Healthy controls	53	26	5.2	Serum MIF higher in T2D than in controls.	(47)
T2D	53	26	20.7		
Healthy controls	30		2.1	Plasma MIF higher in T2D than in healthy controls.	(48)
T2D	46		2.6		
Healthy controls	23	59	0.05	Serum MIF higher in T2D than in healthy controls, in both males and females.	(49)
T2D	27	46	0.2		
Normoglycemic controls	137	99	Median 4.97	Positive association plasma MIF with impaired glucose tolerance and T2D independent of plasma CRP and IL-6.	(50)
Impaired glucose tolerance	130	112	Median 7.95		
T2D	137	107	Median 10.96		
Non-diabetic Caucasians	24		<5.0 in 100%	Plasma MIF higher in Pima Indians and associated with insulin resistance.	(51)
Non-diabetic Pima Indians	28		>5.0 in 39%		
Non-case controls	859	773	Median 17.7	Baseline MIF concentrations higher in subjects that develop T2D than in non-case controls. Women with <i>MIF</i> genotype rs1007888CC have increased risk of T2D.	(52)
Cases (incident T2D)	293	209	Median 18.5		
Turkish adults	1093	1157	nd	In men, <i>MIF</i> genotype rs755622GC associated with baseline diabetes, and C-allele carriage tends to predict new-onset diabetes. No effect in women.	(53)
Healthy pregnant controls	–	40	5.3	Serum MIF higher in women with gestational diabetes.	(54)
Gestational diabetes	–	43	11.3		
Healthy pregnant controls	–	169	nd	<i>MIF</i> genotype rs1007888GG more common in gestational diabetes. GG genotype significantly associated with pre-pregnancy obesity and family history of diabetes, and twofold more frequent in women with metabolic syndrome.	(55)
Gestational diabetes	–	147	nd		

nd, not determined.

Similarly, Yu and colleagues (48) showed increased MIF levels in Chinese T2D patients (2.6 ± 0.1 ng/ml) compared with healthy controls (2.1 ± 0.1 ng/ml), which again were independent of BMI (23.9 ± 0.1 kg/m² in T2D vs. 22.9 ± 0.3 kg/m² in controls). In line with these findings, Sanchez-Zamora et al. reported increased MIF levels (~ 0.2 vs. ~ 0.05 ng/ml) in Mexican T2D patients relative to healthy controls (49), although BMI for the different groups was not stated in this study.

These associations with MIF have also been reported in earlier stages of T2D development (impaired glucose tolerance, insulin resistance), before overt T2D is present. Herder et al. (50) reported increased serum MIF concentrations in German individuals with impaired glucose tolerance (median MIF 7.95 vs. 4.97 ng/ml in healthy controls) and further increased levels in individuals with T2D (median MIF 10.96 ng/ml), suggesting a gradual increase of MIF with severity of the disease. In this study, BMI was higher in the insulin resistant and T2D subjects than in the healthy controls. In line with these results, a positive association between plasma MIF levels and insulin resistance was shown in a study in Pima Indians (51) and correlations with homeostatic model assessment (HOMA; a method used to quantify insulin resistance) (26, 27) and β -cell dysfunction (28) have also been reported. Furthermore, *MIF* mRNA expression in freshly isolated subcutaneous abdominal adipocytes was found to be negatively associated with donor peripheral and hepatic insulin action in another study in Pima Indians (43).

In a prospective case-cohort study (52), baseline levels of serum MIF were found to be higher in incident T2D cases than in non-case controls (mean follow-up 10.1 ± 0.1 years, only significant in women) and this association between elevated MIF serum levels and T2D risk was stronger in obese than in non-obese women. Furthermore, this study reported significant associations between serum MIF levels in women and *MIF* genotype rs1007888CC, and carriers of this allele had a 1.7-fold increased risk of T2D. A different *MIF* genotype (rs755622GC) was shown to be associated with baseline diabetes in men but not in women in a population-based cohort study in Turkish subjects, and carriage of the C-allele tended to predict new-onset diabetes in this population (53).

MIF has also been implicated in gestational diabetes, with higher serum MIF in women with gestational diabetes (11.2 ± 0.75 ng/ml) than in healthy pregnant controls (5.31 ± 0.64 ng/ml) (54). Furthermore, associations between *MIF* genotype Rs1007888GG and gestational diabetes as well as pre-pregnancy obesity and family history of diabetes have been reported (55). This specific genotype was also found to be twofold more frequent in women with metabolic syndrome (55).

Compared with the overweight/obesity studies, there are relatively few studies in insulin resistant/T2D subjects, and these have been conducted in very specific populations (e.g., Asians, Pima Indians). While there were intervention studies (weight loss, bariatric surgery) in overweight/obese subjects, no such reports exist in the case of T2D. Therefore, conclusions should be drawn carefully. Overall, circulating MIF seems to be associated with insulin resistance and overt T2D, and MIF levels rise with the severity of disease. This suggests that MIF-inducing processes unrelated to obesity/adiposity and adipose tissue expression may contribute to circulating MIF levels as overweight/obesity progresses to T2D.

Inflammatory processes in other tissues or in circulating immune cells may be of importance in this respect. The involvement of sources of inflammation other than adipose tissue may explain that the associations of MIF and metabolic disease (metabolic syndrome, insulin resistance, T2D) appear to be independent of obesity with increasing complexity of disease (Figure 2).

Relationship MIF and NAFLD

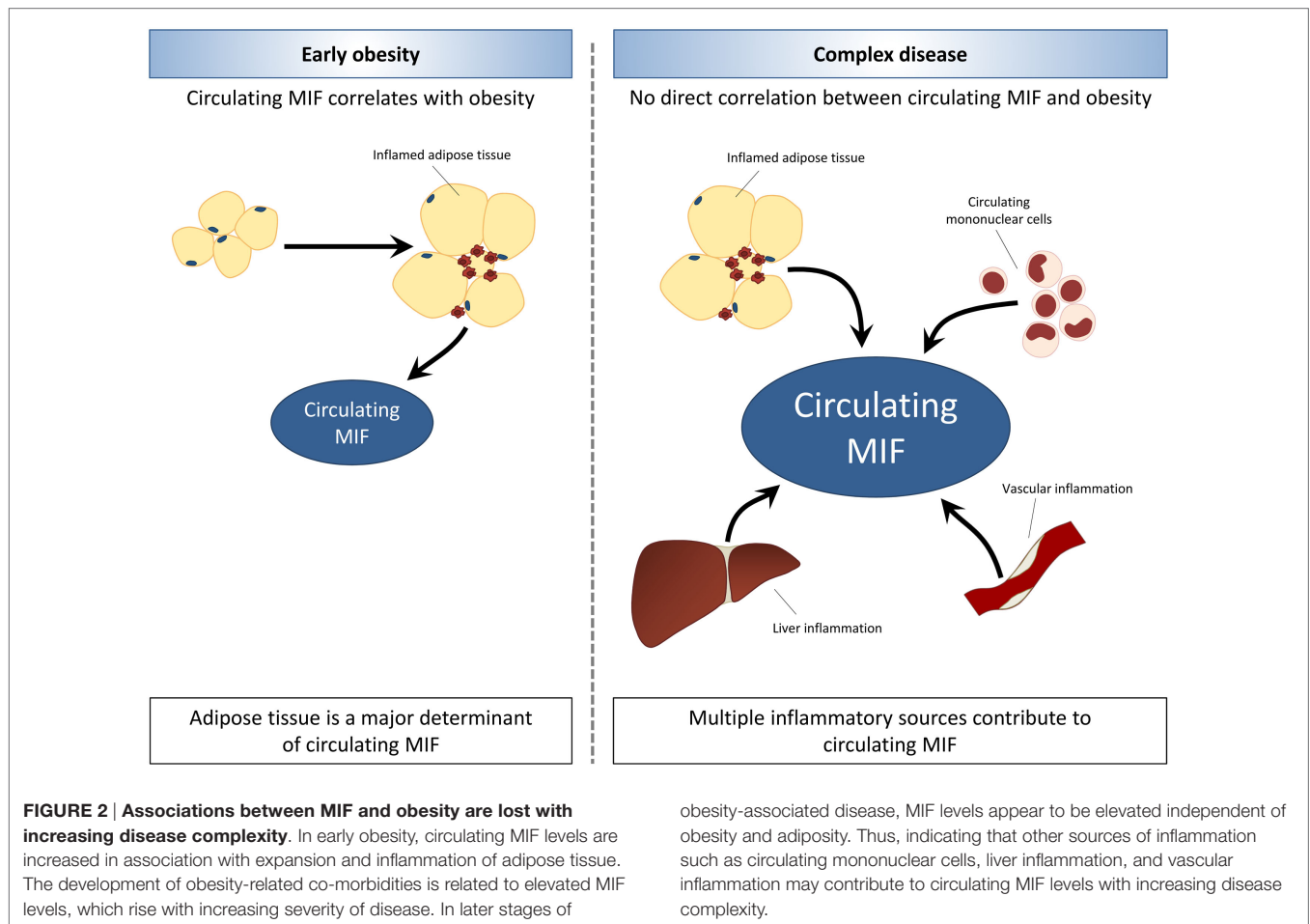
To date, only one study has examined the role of MIF in NAFLD patients. Akyildiz et al. (56) investigated the effect of MIF-173 G/C gene polymorphism in NAFLD and found no difference in genotype or C-allele frequency between healthy controls ($n = 104$) and NAFLD patients ($n = 91$). Furthermore, they did not find any differences in genotype or C-allele frequency within the NAFLD patient group when subjects were stratified for NAFLD score (steatosis/borderline NASH/NASH) or fibrosis stage. Immunohistological investigation of MIF expression in liver biopsies from the NAFLD patients in this study showed that MIF staining was increased in both hepatocytes and MNCs in NASH patients compared to those with only steatosis. While hepatocyte MIF expression was not associated with fibrosis stage, fibrosis stage was significantly higher in presence of MIF staining in MNCs and positive MNC MIF staining was associated with a 3.6-fold increased risk of fibrosis.

Animal Studies

Several studies in genetically engineered mouse models that lack the *Mif* gene have aimed to unravel the role of MIF in both non-pathological glucose homeostasis and in the obese/insulin resistant state. The results of these studies have been summarized in Table 3.

Role of MIF in Non-Pathological Glucose Homeostasis

Nikolic et al. (57) demonstrated that *Mif*-deficient mice (male, 12 weeks old, diet not specified, C57BL/6 background) have impaired insulin signaling (AKT phosphorylation) in liver and adipose tissue. This was reflected in their response to an intraperitoneal glucose tolerance test (ipGTT), during which *Mif*^{-/-} mice had a similar insulin response to wild-type mice, but showed impaired clearance of glucose. In this experiment, glucocorticoid receptor inhibition rescued the observed impairment of insulin signaling in liver and adipose tissue, and enhanced glucose-stimulated insulin release and glucose clearance during the ipGTT. A second study by the same group (58) confirmed that *Mif*^{-/-} mice (male, 8–12 weeks old, diet not specified, C57BL/6 background) show a comparable insulin response to an ipGTT to wild-type mice (however, the glucose response was not shown in this study). A rationale for these observations was provided by results from *in vitro* experiments (58) that showed that conditioned medium containing insulin secreted from *Mif*^{-/-} pancreatic islets had lower activity than conditioned medium with insulin produced by wild-type islets, as demonstrated by reduced insulin signaling (AKT phosphorylation) and glucose uptake in a human liver carcinoma cell line (Hep G2 cells). Furthermore, this study revealed that MIF physically interacts and binds with insulin in wild-type islets and affects the conformation of insulin, reducing the concentration of monomer/dimer insulin



and increasing hexamer formation of insulin, which may contribute to enabling insulin's full functionality. These effects were found to be independent of MIF's tautomerase activity.

Serre-Beinier and colleagues (59) investigated the role of MIF in carbohydrate homeostasis over time with aging. For this they used *Mif*^{-/-} C57BL/6 mice from a 129/Sv background (diet and sex not specified) and examined glucose and insulin tolerance over time. *Mif*^{-/-} mice had lower (13%) body weight at birth, but gained weight faster than wild-type mice. By 4 months of age, body weights were similar to those of wild-type mice, and at 12 months of age, they were 17% higher than in wild-type mice. While fasting blood glucose remained stable over time in both *Mif*-deficient and wild-type mice, fasting insulin increased over time in *Mif*^{-/-} mice but remained stable in wild-type mice. IpGTTs showed that *Mif*^{-/-} mice cleared glucose more rapidly than wild-type mice, but insulin levels were higher in the *Mif*-deficient mice, indicating that they required more insulin to achieve this effect. At 12 months of age, glucose clearance was impaired relative to wild-type mice, with comparable insulin levels. These results are in line with findings by Nikolic et al. (57) and Vujicic et al. (58) described above, which suggest that insulin produced in absence of MIF may have reduced activity/functionality. The response to exogenous insulin (during intraperitoneal insulin tolerance tests; ipITTs, and euglycemic-hyperinsulinemic clamps) was similar between genotypes at 4 and 12 months of age and there were no

differences in insulin-stimulated AKT phosphorylation in muscle, indicating that insulin sensitivity of this peripheral tissue is not affected by MIF.

While these studies indicate that MIF may contribute to insulin function in non-pathological glucose homeostasis (i.e., in absence of obesity or disease), the associations observed in human studies indicate that it may have a detrimental role in obesity and the development of obesity-associated insulin resistance and T2D, i.e. conditions of metabolic stress and disturbed metabolic homeostasis. Several groups have addressed this using experimental models of obesity and insulin resistance/T2D.

Role of MIF in (Diet-Induced) Obesity and Insulin Resistance

Several groups have investigated the role of MIF in obesity and associated development of insulin resistance/T2D using high-fat diet (HFD)-induced rodent models of disease (Table 3). Velickovic and colleagues (60) reported higher plasma MIF levels in fructose-fed Wistar rats than in controls (13.6 ± 1.7 vs. 11.0 ± 0.9 ng/ml), and showed that MIF levels correlated with visceral adipose tissue mass, consistent with observations made in humans. In line with this, Saksida et al. (61) showed that HFD feeding in C57BL/6 mice resulted in an obese phenotype, with increased fasting glycemia and serum insulin, which was accompanied by increased serum MIF

TABLE 3 | MIF in experimental models of obesity and insulin resistance/T2D.

Model	Diet	Sex	Effect observed	Reference
<i>Mif</i> ^{-/-} C57BL/6 mice	ns	Male	Glucose tolerance (ipGTT) is impaired in <i>Mif</i> -deficient mice.	(57)
<i>Mif</i> ^{-/-} C57BL/6 mice	ns	Male	Insulin secretion after ipGTT is similar in <i>Mif</i> -deficient and wild-type mice (glucose response is not specified).	(58)
<i>Mif</i> ^{-/-} C57BL/6 mice (from 129/Sv background)	ns	ns	Age-dependent impairment of glucose tolerance (ipGTT) in <i>Mif</i> -deficient mice.	(59)
Wistar rats	Standard diet + 10% fructose in drinking water	Male	Higher plasma MIF (tendency) in fructose-fed rats than controls. Correlation with visceral adipose tissue mass.	(60)
C57BL/6 mice	HFD (60% fat)	Male	Higher plasma MIF on HFD than on control diet.	(61)
<i>Mif</i> ^{-/-} C57BL/6J mice	HFD (45 en% from palm oil)	Male	<i>Mif</i> deficiency protects against development insulin resistance: lower ipGTT and ipITT.	(62)
<i>Ldlr</i> ^{-/-} <i>Mif</i> ^{-/-} mice (C57BL/6 background)	Chow	Male	<i>Mif</i> deficiency protects against development insulin resistance: fasting plasma glucose and insulin lower, improved ipGTT, ipITT, and euglycemic clamp.	(9)
<i>Mif</i> ^{-/-} C57BL/6 mice	HFD (60 en% fat)	ns	<i>Mif</i> deficiency increases fasting glucose and impairs glucose tolerance (ipGTT).	(63)
<i>Mif</i> ^{-/-} C57BL/6 mice (from <i>Mif</i> ^{+/+} Balb/c background)	HFD (60% fat)	Male	<i>Mif</i> deficiency does not affect development of glucose intolerance (ipGTT) or plasma insulin levels.	(64)
STZ- and L-NAME-induced impaired glucose tolerance in Sprague-Dawley rats	Chow	ns	Plasma MIF levels higher in impaired glucose tolerance rats than in controls.	(48)
STZ-induced T2D in <i>Mif</i> ^{-/-} Balb/c mice	ns	Female	<i>Mif</i> -deficient mice have lower STZ-induced blood glucose and better glucose tolerance (OGTT).	(49)
STZ-induced T2D in ICR mice + MIF antagonist (CPSI-1306)	ns	Female	MIF antagonism reduced STZ-induced blood glucose levels.	
C57BLKS/J db/db mice + MIF inhibitor (ISO-1)	Chow	Male	MIF inhibition normalized hyperglycemia and improved impaired glucose tolerance (ipGTT).	(65)

ns, not specified.

levels (~7 ng/ml compared with ~0 ng/ml in control diet-fed mice). This study also showed that *Mif* mRNA expression in pancreatic islets was increased in HFD-fed mice. Furthermore, they showed that in wild-type pancreatic islets, palmitic acid (a saturated fatty acid which is present in human diets as well as in experimental HFDs) induces MIF production and apoptosis, while pancreatic islets from *Mif*-deficient mice were entirely resistant to palmitic acid-induced apoptosis, which may be of importance in β -cell apoptosis in late-stage obesity-associated T2D.

Finucane and colleagues (62) also described protective effects of *Mif* deficiency in HFD-induced obesity/insulin resistance. In this study, *Mif*^{-/-} mice (male, 8–9 weeks old, C57BL/6J background) were fed a palm oil based (rich in palmitic acid) HFD (45 kcal% fat) for 16 weeks. *Mif* deficiency reduced HFD-induced body weight gain and fat mass and improved the response to ipGTT and ipITT. Glucose clearance during the ipGTT was improved compared with wild-type mice, with a reduced insulin response and similarly, the *Mif*^{-/-} mice showed a more efficient glucose clearance in the ipITT. Together these glucose and insulin tolerance tests show that the response to both endogenous insulin (produced during ipGTT) and exogenous insulin (injected in ipITT) is improved in absence of MIF, thus suggesting an improvement in peripheral insulin signaling in obese *Mif*^{-/-} mice. These effects were also observed when mice were weight-matched to exclude effects of body weight differences, suggesting that *Mif*

deficiency improves insulin signaling in peripheral tissues, such as white adipose tissue.

Support for this notion comes from studies that analyzed the effects of MIF on insulin signaling in adipose tissue. Finucane et al. (62) further showed that in wild-type mice, obesity increased MIF protein expression in the epididymal adipose tissue, for which the stromal vascular fraction rather than the adipocyte fraction was the primary cellular source. *Mif*^{-/-} mice were found to have reduced adipose tissue inflammation (reduced M1 macrophage infiltration and reduced *ex vivo* cytokine secretion) and this was accompanied by improved adipose tissue insulin signaling as demonstrated by increased insulin-stimulated AKT phosphorylation and *Glut4* expression *in vivo*, as well as increased insulin-stimulated glucose uptake into adipose tissue explants. Similarly, Verschuren et al. (9) used the hyperlipidemic *Ldlr*^{-/-} background to induce obesity and insulin resistance (male mice up to 52 weeks old, chow, C57BL/6 background) and showed that *Mif* deficiency protects against the development of insulin resistance. *Mif*-deficient mice had lower fasting glucose and insulin levels and showed improved glucose tolerance (assessed by ipGTT, with comparable insulin levels during the test) and improved insulin sensitivity (assessed by ipITT). Furthermore, a hyperinsulinemic–euglycemic clamp showed that insulin resistance was reduced in *Mif*-deficient mice. In line with the findings by Finucane et al. (62), analysis of adipose tissue in this study showed improvement of peripheral insulin signaling. This

study also revealed improvements in adipose tissue inflammation in absence of MIF, with smaller adipocytes, reduced macrophage infiltration and crown-like structure formation and a lower M1/M2 macrophage ratio. This was accompanied by improved insulin signaling in adipose tissue (increased insulin-stimulated Pi3-Kinase activity and AKT phosphorylation), which further corroborates the observed improvements in glucose handling and insulin sensitivity. In both these studies, *Mif* deficiency improved the inflammatory milieu in adipose tissue and resulted in improvement of insulin signaling.

While above studies used HFDs with a moderate fat content (translational to the human situation), two studies that used diets with a supraphysiological fat content (60 kcal%), made different observations; Heinrichs et al. (63) reported that *Mif* deficiency in HFD-fed C57BL/6 mice (age and sex not specified) promotes weight gain and has detrimental effects on glucose tolerance (impaired glucose clearance during ipGTT). Remarkably, adipose tissue inflammation was reduced in absence of *Mif* in this study, in line with observations in the more physiological diet-induced obesity studies. A study by Conine and co-workers (64) showed no difference between *Mif*^{-/-} mice (male, 6–8 weeks old, C57BL/6 mice from *Mif*^{+/-} Balb/c background) and wild-type mice after 15 weeks of HFD feeding. In this study, body weight gain and adipose tissue inflammation were similar between genotypes, and there was no difference in glucose intolerance (assessed by ipGTT) or fasting blood glucose and insulin levels.

Role of MIF in (STZ-Induced) Diabetes

Others have used streptozocin (STZ; a toxic glucose analog that preferentially accumulates in pancreatic beta cells but that can also be harmful to other tissues) to induce a (pre-)diabetic phenotype (severity depends on STZ dosage and regimen). Yu et al. (48) showed that plasma MIF levels were higher in a pre-diabetic rat model of impaired glucose tolerance (HFD-fed and STZ- and L-NAME-treated Sprague-Dawley rats) than in chow-fed controls (32.5 ± 1.9 vs. 24.8 ± 1.6 ng/ml). Sanchez-Zamora and co-workers (49) showed that *Mif* deficiency in Balb/c mice (female, age and diet not specified) lowered STZ-induced blood glucose levels and improved glucose tolerance in an oral glucose tolerance test (OGTT) without affecting insulin production by pancreatic β -cells. Similarly, MIF antagonism (with small molecule inhibitor CPSI-1306) reduced STZ-induced blood glucose levels in ICR outbred mice in the same study (49). Together, these studies in STZ-induced diabetes suggest an improvement of peripheral insulin sensitivity and/or more active insulin in *Mif*-deficient mice. The latter, however, would contradict findings by Nikolic et al. (57) and Vujicic et al. (58) discussed above.

Overall, these STZ studies lend further support to the notion that *Mif* deficiency protects from (pre)diabetes and are thus in line with most of the observations made in diet-induced models of obesity and metabolic disease. While in normal physiology, MIFs effects in glucose homeostasis were found to be independent of MIFs tautomerase activity (58), results from a study by Wang et al. (65) suggest that this activity may play a role under pathophysiological conditions. The authors showed that in diabetic db/db mice (male, 8 weeks old, chow, C57BLKS/J background), which

have an autosomal recessive mutation in the leptin receptor, treatment with the MIF tautomerase activity inhibitor ISO-1 reduced fasting glucose levels to those observed in non-diabetic controls (db/m mice). These effects were further supported by results from an ipGTT, which revealed significant improvement in glucose handling in ISO-1 treated mice. In the lean, non-diabetic control mice, ISO-1 treatment did not affect fasting blood glucose levels or ipGTT responses, consistent with the discussed observations made by Vujicic et al. (58).

Altogether, these studies indicate that while MIF seems to have a function for insulin conformation and functionality in non-pathological glucose homeostasis, its role is less clear when metabolic stress and pathological processes come into play. Under these conditions, *Mif* deficiency appears to be protective against white adipose tissue inflammation and may improve glucose metabolism and peripheral insulin resistance although results are partly conflicting. An important explanation for these discrepancies is likely to be the choice of rodent model in combination with the experimental conditions employed. Inbred mouse strains are known to differ greatly in their susceptibility to obesity as well as in glucose metabolism and insulin resistance (66–68). Differences in the genetic background of knockout mice (as well as successfulness of backcrossing onto a different strain) are therefore likely to have a large impact on study outcomes. A second factor that may influence study results is the choice of diet (i.e., percentage of fat, type of fat, palmitic acid content) and the age that mice are started on this dietary treatment [for instance, older *Ldlr*^{-/-} mice show a more aggravated disease phenotype in response to HFD feeding than younger mice (69)]. Although information on the mouse strain and dietary conditions used are critical for the interpretation and comparison of different studies, they are often inadequately described. Overall, the prevailing finding is that *Mif* deficiency is protective in insulin resistance, glucose tolerance, and STZ-induced diabetes, and reduced adipose tissue inflammation and improved insulin signaling in adipose tissue may play a role in these beneficial effects, in line with observations made in human studies. Besides driving the development of insulin resistance and T2D in obesity, metabolic inflammation is also believed to drive the development of NAFLD (Figure 1).

Role of MIF in Non-Alcoholic Fatty Liver Disease

Several studies provide indication that MIF may also play a role in the development of hepatic steatosis, inflammation and fibrosis (Table 4). Finucane and colleagues (62) showed that *Mif* deficiency alleviates HFD-induced hepatic steatosis (male, 8–9 weeks old, 16 weeks HFD 45 kcal% fat from palm oil, C57BL/6J background). In *Mif*^{-/-} mice, liver weight was reduced, and hepatic triglyceride content (as well as histologically observed steatosis) was reduced. *Mif*-deficient mice also had lower plasma alanine transaminase (ALT) levels. Hepatic gene expression analyses revealed that lipogenic gene expression (i.e., *Cd36*, *Dgat-1*, *Fasn*, *Srebp1-c*, *Pgc-a1*, *Lpl*, *Ppar γ*) was significantly lower in absence of MIF, providing a possible rationale for the observed reduction in hepatic steatosis. In line with these results, Heinrichs et al. (63) observed that hepatic steatosis was reduced in absence of MIF in both HFD-fed (60 kcal% fat, 16 weeks) and methionine and choline-deficient (MCD) diet-fed (8 weeks) *Mif*^{-/-} mice (sex and age not specified,

TABLE 4 | MIF in experimental models of NAFLD/liver fibrosis.

Model	Diet	Sex	Effect observed	Reference
<i>Mif</i> ^{-/-} C57BL/6J mice	HFD (45 en% from palm oil)	Male	Plasma ALT lower in <i>Mif</i> -deficient mice. Liver triglycerides, lipogenic gene expression, and pNFκB reduced in <i>Mif</i> -deficient mice.	(62)
<i>Mif</i> ^{-/-} C57BL/6 mice	HFD (60 en% fat)	ns	<i>Mif</i> deficiency increases hepatic steatosis and hepatic immune-cell infiltration	(63)
	MCD diet	ns	Liver triglycerides increased in <i>Mif</i> -deficient mice.	
<i>Mif</i> ^{-/-} C57BL/6 mice (from <i>Mif</i> ^{+/-} Balb/c background)	HFD (60% fat)	Male	<i>Mif</i> deficiency does not affect hepatic lipid accumulation.	(64)
<i>Mif</i> ^{-/-} C57BL/6 mice + CCl ₄	Chow (Teklad)	Male and female	Liver fibrosis in <i>Mif</i> -deficient mice similar to wild-type.	(70)
<i>Mif</i> ^{-/-} C57BL/6 mice + CCl ₄ or + TAA	ns	ns	Liver fibrosis more severe in <i>Mif</i> -deficient mice (both CCl ₄ - and TAA-induced).	(71)
C57BL/6 mice + CCl ₄ + rMIF	ns	ns	Treatment with rMIF reduced hepatic stellate cell activation and repressed expression of fibrosis-relevant genes.	

ns, not specified.

C57BL/6 background). In the HFD-fed mice, *Mif* deficiency increased liver weight while liver damage markers ALAT and aspartate transaminase (ASAT) were slightly reduced. The observed reduction in hepatic steatosis (analyzed by histological Oil Red O staining and biochemical measurement of hepatic triglycerides) was accompanied by increased lipogenic gene expression (i.e., transcription factors *Lxra* and *Srebp-1*, and enzymes *Acc*, *Fas*, *Dgat1*, *Dgat2*), which was also observed in the MCD diet-fed mice. Furthermore, HFD-feeding resulted in increased hepatic inflammatory cell infiltration (1.6-fold increase in F4/80⁺ macrophages) in *Mif*^{-/-} mice, which were skewed toward a M2 polarization. Mechanistic *in vitro* experiments in oleic acid/IL-β stimulated mouse hepatoma cells Hepa1-6 and primary murine hepatocytes showed that recombinant MIF reduced hepatocyte lipid content through a CD74 and AMPK-mediated pathway. In contrast to the above studies, Conine et al. (64) observed no difference in HFD-induced (60 kcal% fat) hepatic lipid accumulation in presence or absence of *Mif* (male, 6–8 weeks old, C57BL/6 mice from *Mif*^{+/-} Balb/c background). It is not clear whether absence of phenotypical differences in this study may be related to the relatively young age of the mice at the start of the study, the extreme (supraphysiological) dietary fat content or the genetic background of the mice.

While the above studies focused mainly on the lipid accumulation that is observed in NAFLD, others have investigated the role of MIF in the development of hepatic fibrosis, a characteristic of progressive NASH. Barnes and co-workers (70) studied effects of MIF in chemically induced liver fibrosis (male and female, 10–12 weeks old, C57BL/6 *Mif*^{-/-} mice). In wild-type mice, a single dose of CCl₄-induced mRNA expression of *Mif* and its cognate receptor *Cd74* (peaking between 4 and 8 h and resolving by 18 h), which was also observed in plasma with MIF protein levels increased as early as 2 h after injection (indicating a release of preformed pools of MIF). Circulating ALAT and ASAT were similarly increased in *Mif*^{-/-} and wild-type mice indicating comparative liver damage between genotypes. Assessment of hepatic stellate cell activation upon chronic CCl₄ treatment (2 i.p. injections per week for 5 weeks) revealed increased mRNA expression of *Acta2* (α-SMA) and *Col1a1* (Collagen type I, alpha 1) in male *Mif*^{-/-} mice compared with

wild-type, while expression of these genes was reduced in female *Mif*^{-/-} mice compared with wild-type mice. Despite these effects of *Mif* deficiency on hepatic stellate cell activation, hepatic collagen deposition (assessed by quantification of Sirius Red staining and hydroxyproline measurement) was comparable to wild-type mice in both male and female *Mif*^{-/-} mice. Authors then continued their studies in female mice only, to investigate a potential role of MIF on the progression of fibrosis via the regulation of extracellular matrix (ECM) degradation. They observed reduced infiltration of restorative macrophages in *Mif*^{-/-} mice, which was accompanied by reduced expression (mRNA, protein, and enzymatic activity) of the ECM-degrading metalloproteinase MMP13, suggesting a possible proresolution role for MIF during fibrosis.

Heinrichs and colleagues (71) also investigated the effects of *Mif* deficiency (age and sex not specified, C57BL/6 *Mif*^{-/-} mice) in two different models of chemically induced liver fibrosis. In this study, hepatic fibrosis (assessed histologically and by hydroxyproline measurement) was increased in absence of MIF in both CCl₄- and TAA-induced liver fibrosis. In line with this, *Mif*-deficient mice had increased mRNA expression of *Col1a1*, *Timp1*, *Mmp2*, and *Tgfb1*, as well as increased α-SMA protein expression. FACS analysis revealed no differences between genotypes in immune-cell subset infiltration, suggesting that differences in fibrosis development may be explained by effects on hepatic stellate cell activation rather than by effects on the intrahepatic immune response. *In vitro* studies in murine hepatic stellate cells showed that treatment with recombinant MIF inhibited PDGF-induced hepatic stellate cell activation, which was found to be mediated through CD74 and AMPK signaling. In line with these *in vitro* results, follow-up *in vivo* studies showed that CCl₄-induced hepatic fibrosis was increased in *Cd74*^{-/-} mice. Deficiency of *Cxcr4*, however, another MIF receptor expressed by hepatic stellate cells, did not affect fibrosis development. Therapeutic application of recombinant MIF in CCl₄-treated wild-type mice reduced hepatic stellate cell activation and repressed expression of fibrosis-relevant genes. However, the effects of this recombinant MIF treatment on fibrosis development are not mentioned.

Overall, very few studies to date have investigated the effects of MIF in the development of hepatic co-morbidities. MIF appears

to have detrimental effects in HFD-induced hepatic lipid accumulation through effects on lipogenic gene expression, providing further support for the notion that MIF may indeed not only have pro-inflammatory effects but may also be linked to metabolism. In chemically-induced liver fibrosis, it seems that MIF may have protective effects, possibly through an effect on the resolution of fibrosis. However, results from a clinical study in NAFLD patients indicate the opposite, showing that increased MIF expression in liver is associated with increased risk of fibrosis. This discrepancy may be explained by differences in disease etiology in chemically induced liver damage used in the experimental disease models, compared with the metabolically induced liver damage that is typical for human NAFLD development.

Conclusion and Future Directions

In early obesity, circulating MIF levels are increased in association with expansion and inflammation of adipose tissue. The development of insulin resistance and T2D in obesity is related to elevated MIF levels, which rise with increasing severity of disease. In later stages of obesity-associated T2D, MIF levels appear to be elevated independent of obesity and adiposity, indicating that with increasing disease complexity, other sources of inflammation may contribute to circulating MIF levels.

Mechanistic studies in experimental models of disease have shown that MIF contributes to insulin function in non-pathological glucose homeostasis (i.e., in absence of obesity or disease) but its role is less clear when metabolic stress and pathological processes come into play. Under these conditions, *Mif* deficiency appears to be protective against adipose tissue inflammation and may improve insulin resistance and T2D in both HFD- and STZ-induced models. Furthermore, there are some first indications that MIF may contribute to development of diet-induced hepatic steatosis in

NAFLD, while observations in advanced liver disease are conflicting, describing both pro- and anti-fibrotic effects of MIF.

To fully understand the intricacies of MIFs contribution to obesity-related disease development, future work is needed to decipher temporal (non-pathological – early disease – advanced complex disease) and spatial (tissue-specific) effects of MIF, as well as interorgan crosstalk that may be mediated by circulating MIF. For the interpretation of such studies, it is of paramount importance that they would be performed in translational, physiological (diet-induced) models under well-described experimental conditions. In addition, future studies assessing the complex interplay between metabolic and inflammatory balances will greatly enhance our understanding of the development of obesity-related diseases, and the role of MIF therein. Although most published data on MIF point toward a detrimental role in advanced stages of disease, the potential protective effects of this mediator under certain conditions deserve further investigation.

Author Contributions

MM and RK had substantial contributions to the conception and design of the work, drafted and revised the manuscript, had final approval of the version to be published and agree to be accountable for all aspects of the work.

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Macrophage Migration Inhibitory Factor in Clinical Kidney Disease

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Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine implicated in acute and chronic inflammatory conditions, including sepsis, autoimmune disease, atherogenesis, plaque instability, and pulmonary arterial hypertension. MIF in plasma and urine is significantly elevated in patients with acute kidney injury (AKI) and elevated MIF in serum is associated with markers of oxidative stress, endothelial dysfunction, arterial stiffness, and markers of myocardial damage in chronic kidney disease (CKD). Furthermore, MIF seems to be involved in vascular processes and cardiovascular disease associated with CKD, glomerulonephritis, autosomal dominant polycystic kidney disease, and possibly also in progression to renal failure. Moreover, in active anti-neutrophil cytoplasmic antibody-associated vasculitis, plasma MIF levels have been shown to be significantly elevated as compared with samples from patients in remission. A significant difference in the genotype frequency of high production MIF -173 G/C genotype has been found in end-stage renal disease, compared to controls. Inhibition of MIF in a diabetic nephropathy model ameliorated blood glucose and albuminuria and in a model of adult polycystic kidney disease cyst growth was delayed. Preclinical studies support a potential therapeutic role for MIF in AKI and in a number of CKDs, whereas these data in human disease are still observational. Future interventional studies are needed to delineate the role of MIF as a treatment target in clinical kidney disease.

Keywords: MIF, AKI, CKD, glomerulonephritis, vasculitis, MIF gene polymorphism, diabetic nephropathy, ADPKD

INTRODUCTION

Macrophage migration inhibitory factor (MIF) was one of the first cytokines that was identified after being isolated from the supernatants of T-lymphocytes, and initially described as a soluble factor with macrophage migration-inhibiting properties (1–3). It has later been shown that MIF is produced by a number of other cells, such as monocytes, macrophages, granulocytes, endocrine cells, epithelial cells, and endothelial cells (4, 5).

Migration inhibitory factor is a pleiotropic upstream proinflammatory integral mediator of the innate immune system, stimulating the release of multiple cytokines, including tumor necrosis factor (TNF)- α , with CD 74 being a binding receptor promoting the recruitment of leukocytes into inflammatory sites in a chemokine-like fashion (6). Three-dimensional X-ray crystallography has revealed that the MIF molecule contains a hydrophobic pocket, which has been identified as the proinflammatory active site of MIF (7) and compounds binding to this region decrease downstream MIF signaling (8, 9).

Migration inhibitory factor has been implicated in the pathogenesis of sepsis, autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and cardiovascular disease (CVD) (6, 10–13). In atherosclerosis animal models, aortic inflammation was reduced, and neointimal plaques were stabilized after administration of anti-MIF antibody (14, 15).

Chronic kidney disease (CKD) is a state of chronic inflammation with major implications for morbidity and mortality driven by a significant increased risk for CVD (16, 17). Delineating the role of inflammatory markers in atherosclerotic and inflammatory disease in CKD is therefore of considerable interest. Whether MIF has an important role in this area is not well known. This article therefore aims at reviewing available data on the role of MIF in acute kidney injury (AKI), CKD, diabetic nephropathy, inflammatory kidney disease, and genetic aspects of MIF and kidney disease.

MIF AND AKI

Urinary MIF has previously been reported to be increased, and associated with the severity of renal injury, in human glomerulonephritis and has also been suggested as a potential biomarker for acute kidney damage in acute pyelonephritis (18, 19). Similar findings have been demonstrated in kidney transplantation (20). Augmented plasma levels of MIF seem to be an early and predictive event of AKI in septic patients admitted to the ICU (21). In preclinical models, MIF stimulates leukocyte chemotaxis as well as tissue infiltration of leukocytes and induces multiorgan damage affecting both lungs and kidneys (6, 22–24). In a recent paper by Stefaniak et al., it was shown that increased plasma levels of MIF in patients undergoing liver transplantation was significantly more predictive than serum creatinine for AKI and the need for renal replacement therapy postoperatively (25).

MIF AND CKD, IMPLICATIONS FOR CARDIOVASCULAR DISEASE

The prevalence of CKD worldwide is 10–12% and its incidence is even greater in the elderly (26, 27). Systemic low-grade inflammation is associated with loss of renal function, and the uremic phenotype is also linked to premature aging and accelerated atherosclerosis (28, 29). CVD is a major challenge in this patient population in which mortality rates due to CVD are about 10–20 times higher in dialysis patients than those of the general population (30). A number of proinflammatory factors have been investigated, such as C-reactive protein (CRP), interleukin-6 (IL-6), TNF, and high-mobility group box-1 protein (HMGB1), and increased circulating levels have in most cases been shown to be associated with poor outcome (22–34). It has however been suggested that retention by reduced cytokine excretion or degradation in the kidney, not only increased production, may play a role for the elevated cytokine levels (35).

We have previously shown that circulating serum levels of MIF are significantly elevated in CKD stage 3–5 patients ($n = 257$), compared with controls ($n = 53$) in a cross-sectional study (36). MIF levels were also associated positively with markers of

oxidative stress and endothelial activation, such as 8-hydroxy-2-deoxyguanosine (8-OH-dG) levels and ICAM-1 levels, but not with inflammatory markers, such as CRP, IL-6, and TNF. However, in contrast to most previously described cytokines, we observed no correlation between MIF and glomerular filtration rate (GFR).

Ramos et al. recently showed that plasma MIF levels correlated negatively with endothelial function by flow-mediated dilation of the brachial artery, and positively with arterial stiffness indices using applanation tonometry in patients with end-stage renal disease (ESRD). In a multivariate regression model, MIF was an independent predictor for arterial stiffness. A correlation between high MIF and high-sensitive troponin I also suggested an association with myocardial injury in these patients (37). Taken together, these studies indicate that MIF may play a role in vascular disease associated with CKD, but further studies are needed.

MIF, PULMONARY ARTERIAL HYPERTENSION, AND CKD

Pulmonary arterial hypertension (PAH) is characterized by endothelial dysfunction, vasoconstriction, and pulmonary vascular remodeling. Haddad et al. have shown that AKI is relatively common in individuals with PAH and is a strong predictor of early death (38). Furthermore, in the study of Shah et al. (39), the severity of CKD in patients with PAH was directly related to the risk of death.

Recent studies have shown clear links among MIF, PAH, and the concomitant pulmonary vasoconstriction and vascular remodeling (40, 41). The release of MIF and the vascular changes are due, at least in part, to increased oxidative stress (42). Our study in CKD stage 3–5 patients showed that elevated serum MIF concentrations are associated with markers of oxidative stress (plasma 8-OH-dG levels) and endothelial activation (ICAM-1) (36). This further suggests possible links between MIF in CKD and the associated pulmonary vascular changes and cardiac changes.

MIF AND DIABETIC KIDNEY DISEASE

Diabetic nephropathy is one of the leading causes of CKD and dialysis dependency. Albuminuria and impaired GFR predominantly account for the increased mortality observed in type 2 diabetes (43). Accumulating evidence has revealed that immunological and inflammatory mechanisms may play a significant role in the development and progression of diabetic nephropathy, in addition to non-immunological factors (44, 45). Elevated MIF levels have been found in patients with impaired glucose tolerance and type 2 diabetes and have also been associated with coronary events in these patient populations (46–48). MIF protein expression and urinary MIF excretion, the latter preceding the onset of microalbuminuria, have been demonstrated in a diabetic mouse model (49). Glomerular and tubulointerstitial mRNA expressions of the MIF receptor CD74 were shown to be increased in Pima

Indians with type 2 diabetes and diabetic nephropathy (50). As shown by Wang et al., treatment of the diabetic db/db mice with the MIF inhibitor ISO-1 significantly decreased blood glucose levels and albuminuria in these mice, suggesting that MIF inhibition may be a potential therapeutic strategy in diabetic nephropathy (51).

MIF AND GLOMERULONEPHRITIS

It has been established that upregulation of renal MIF mRNA expression in the endothelium, glomerular, and tubular epithelial cells is closely related to macrophage accumulation and renal tissue lesions in experimental glomerulonephritis. By contrast, in the normal kidney, MIF mRNA and protein are largely restricted to tubular epithelial cells and some glomerular visceral and parietal epithelial cells (52). By using a neutralizing anti-MIF antibody, it was possible to partially reverse established crescentic glomerulonephritis (53). In human disease, a marked increase in both glomerular and tubular MIF mRNA and protein expression has been demonstrated in proliferative forms of GN, correlating with leukocyte infiltration, histologic damage, and renal function impairment (54). Elevated MIF concentrations have been measured in peripheral blood T cells and monocytes from patients with IgA nephropathy (IgAN), which is the most common form of primary glomerulonephritis and is characterized by IgAN immune complexes in the glomerular mesangium, proliferation of mesangial cells, infiltration of inflammatory cells, and progressive glomerular injury. MIF overproduction was also correlated with the intensity of acute exacerbation in these patients (55, 56). Polymeric IgAN isolated from patients with IgAN was able to induce MIF production in human mesangial cells, and anti-MIF treatment was shown ameliorate kidney injury and reduce glomerular TGF- β 1 expression in an experimental model of IgAN (57, 58).

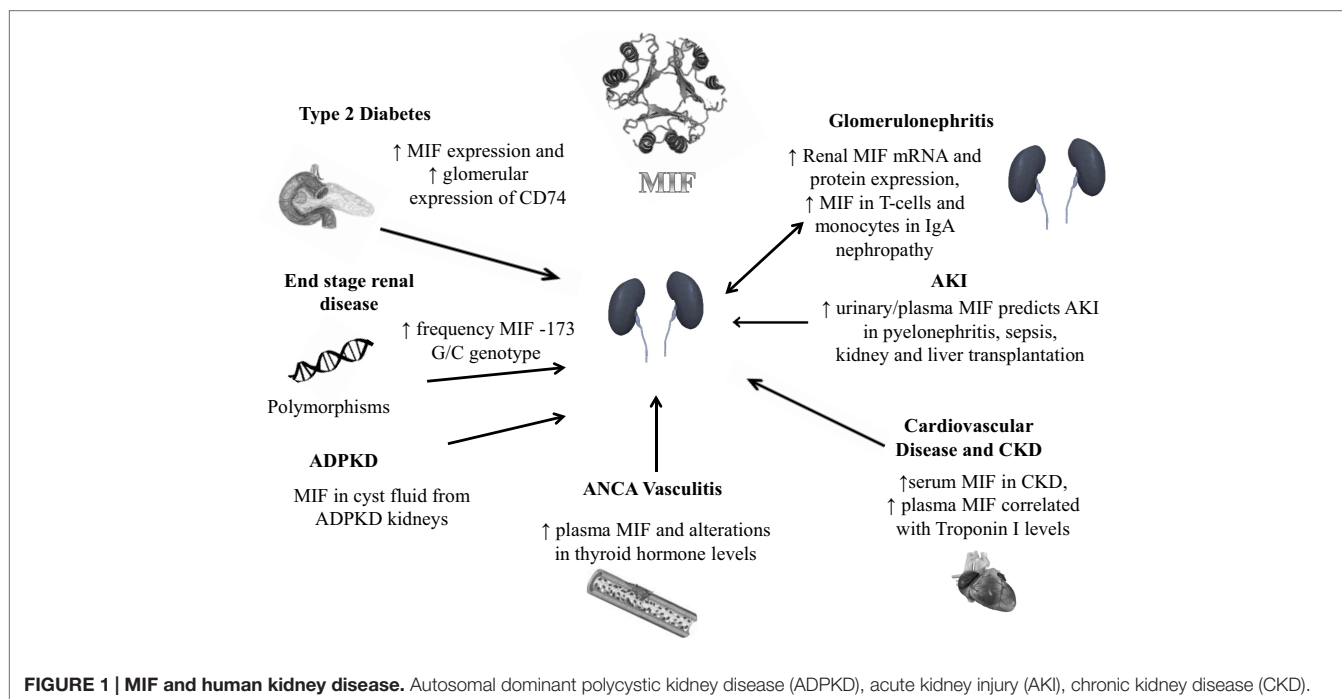
MIF AND VASCULITIS

Granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) are diseases characterized by systemic small vessel necrotizing inflammation, commonly affecting the kidneys and with a close association with the presence of anti-neutrophil cytoplasmatic antibodies (ANCAs), thus known as ANCA-associated vasculitides (AAV). A number of inflammatory cells and proinflammatory mediators have been implicated in the pathogenesis, which is believed to be initiated by priming of neutrophils and monocytes in the circulation by inflammatory stimuli (59). Increased levels of circulating MIF in serum have been found particularly in active AAV patients, but not in patients with other forms of vasculitis, such as giant cell arteritis and polyarteritis nodosa (60). We studied MIF in a prospective study of incident AAV patients at induction treatment, and at follow-up at 3 and 6 months and found elevated plasma levels at baseline. MIF decreased significantly during follow-up when most patients were in remission, but remained still elevated as compared with controls at all time points (61). MIF levels did not correlate with CRP, creatinine, or organ

involvement. MIF has intriguingly been shown to be induced from macrophages by low concentrations of glucocorticoids (GC), possibly acting as a counter-regulator for glucocorticoid action (62). However, we found no correlation between MIF levels and GC exposure possibly due to the unphysiologically high GC treatment doses at the time of sampling. Finally, since there is a known association between thyroid disease and AAV in addition to antithyroid drugs being associated with the development of ANCA and vasculitis (63, 64), we investigated the role of thyroid hormone activity and MIF in AAV. The thyroid hormone thyroxine (T4) and its dextrorotatory isomer (dextrothyroxine; D-T4), but not triiodothyronine (T3), bind to the hydrophobic pocket within the MIF molecule and has been shown to be a potent inhibitor of the inflammatory activity of MIF in a dose-dependent manner, which was clearly demonstrated by administration of exogenous D-T4 to mice with severe sepsis (65). Administration of the hormonally inactive D-T4 significantly improved survival, even in mice that had previously undergone thyroidectomy. In patients with severe sepsis, low plasma T4 concentrations were inversely correlated with plasma MIF concentrations (65). In our human study, there was a strong correlation over time between the baseline MIF/T4 ratio and the MIF/T4 ratio at 6 months in AAV patients in remission (61). Both the preclinical and clinical data therefore suggest that blocking the inflammatory active site of MIF may both reduce inflammatory responses and improve the availability of T4.

MIF AND AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited autosomal dominant disease characterized by renal cyst formation and is associated with renal interstitial inflammation and fibrosis. The most common is the PKD1 mutation that encodes for polycystin-1 (PC1), which frequently leads to loss of kidney function in mid-life (66). It was recently shown that MIF regulates cyst growth in a murine ADPKD model through several mechanisms and is also accumulated in cyst fluid of human ADPKD kidneys (67). In polycystin-1-deficient mice, recruitment and retention of renal macrophages were dependent on MIF, which promoted cyst expansion. By deleting MIF or by pharmacological inhibition, cyst growth was delayed in murine ADPKD models. Macrophage recruitment was associated with the upregulation of monocyte chemotactic protein 1 (MCP-1) and inflammatory cytokine TNF- α , which further induced MIF affecting renal epithelial cells and cyst development (68). These findings may potentially be very important as the therapeutic options in slowing ADPKD progression until recently have been limited. New drugs, such as tolvaptan, a vasopressin V(2)-receptor antagonist, have recently been approved after demonstrating an effect on the increased rate of total kidney volume and slowing down renal function decline. However, the long-term effect of tolvaptan is unclear and side effects may limit its use (68).



MIF AND GENETICS

Migration inhibitory factor corresponding gene has known polymorphisms in the -794 CATT₍₅₋₈₎ repeat and the single-nucleotide polymorphism (SNP) -173*G/C and is associated with increased susceptibility and severity of a number of inflammatory and autoimmune conditions (69, 70).

The frequency of high-producer MIF -173 G/C genotype was higher (10.1%) in ESRD than in controls (1.2%), suggesting that it may play a role in progression to renal failure. However, there was no clear association between the MIF genotype and type of kidney disease in ESRD (71). In children with idiopathic nephrotic syndrome, the high-producer MIF -173*C allele was significantly more common than in controls. Furthermore, this allele was more common in steroid-resistance cases and was also associated with significantly higher probability of ESRD compared with G/G homozygous patients within 5 years from onset (72). In a recent study, it was shown that patients with GPA have an increased frequency of high-expression MIF CATT, and higher plasma MIF levels. In a murine model of granulomatous vasculitis, higher MIF expression increased mortality and pulmonary granulomas while injection of anti-MIF mAb protected mice from dying suggesting a role for MIF in the pathogenesis of GPA (73).

CONCLUSION

We have here described the current evidence of MIF being a mediator in a number of diseases and conditions associated with kidney disease (Figure 1). Both MIF and its receptor CD74 may be potential biomarkers in these disorders and possible targets

for pharmacological modulation. However, since MIF is also constitutively expressed it may be problematical to interfere with MIF activity in an interventional setting. Also, while we have discussed the possible detrimental effects of MIF, especially those associated with its inflammatory active site, the molecule can also be protective in certain circumstances. In particular, in addition to the inflammatory site, MIF also has an intrinsic thiol protein oxidoreductase activity. We, and others, have shown that this enzyme activity is protective against oxidative injury induced by ischemia reperfusion injury (74, 75).

Recently, Thiele et al. demonstrated that there are two redox-dependent conformational MIF isoforms. Oxidized MIF (oxMIF) is selectively expressed in the plasma and on the cell surface of immune cells of patients with different inflammatory diseases, but not in healthy individuals and is specifically recognized by three monoclonal antibodies (mAbs) directed against MIF. The authors also found a clear correlation between disease severity and the oxMIF/Cr ratio in the urine in patients with acute lupus nephritis, but not in patients with SLE without renal manifestations or in remission. Anti-oxMIF mAbs alleviated disease severity in a rat model of crescentic glomerulonephritis, interestingly with further improvement in synergy with GC (76). However, while there is much evidence, from preclinical studies, for the participatory role of MIF in the pathogenesis of several conditions few clinical trials of anti-MIF agents have been recorded. Anti-MIF mAb therapy is currently in phase I trials both for solid tumors (NCT01765790) and for lupus nephritis (NCT01541670). Whether inhibition of MIF or oxMIF may offer promising therapies in clinical conditions, such as AKI, CKD, diabetic nephropathy, inflammatory kidney diseases, and ADPKD, needs to be elaborated in future interventional studies.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CD74 in kidney disease

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CD74 (invariant MHC class II) regulates protein trafficking and is a receptor for macrophage migration inhibitory factor (MIF) and D-dopachrome tautomerase (D-DT/MIF-2). CD74 expression is increased in tubular cells and/or glomerular podocytes and parietal cells in human metabolic nephropathies, polycystic kidney disease, graft rejection and kidney cancer and in experimental diabetic nephropathy and glomerulonephritis. Stressors like abnormal metabolite (glucose, lyso-Gb3) levels and inflammatory cytokines increase kidney cell CD74. MIF activates CD74 to increase inflammatory cytokines in podocytes and tubular cells and proliferation in glomerular parietal epithelial cells and cyst cells. MIF overexpression promotes while MIF targeting protects from experimental glomerular injury and kidney cysts, and interference with MIF/CD74 signaling or CD74 deficiency protected from crescentic glomerulonephritis. However, CD74 may protect from interstitial kidney fibrosis. Furthermore, CD74 expression by stressed kidney cells raises questions about the kidney safety of cancer therapy strategies delivering lethal immunoconjugates to CD74-expressing cells. Thus, understanding CD74 biology in kidney cells is relevant for kidney therapeutics.

Keywords: kidney, CD74, D-dopachrome tautomerase (D-DT/MIF-2), macrophage inhibitory factor, inflammation, Fabry, diabetes, polycystic kidney disease

CD74

CD74 (MHC class II invariant chain, Ii) is a transmembrane glycoprotein that regulates protein trafficking and is a cell surface receptor for the cytokines macrophage migration inhibitory factor (MIF) and D-dopachrome tautomerase (D-DT/MIF-2) (1). During kidney injury, leukocytes and intrinsic renal cells express CD74 (2). In the kidneys, MIF promotes experimental glomerular injury and cystogenesis, while recent reports suggest a protective role in interstitial fibrosis (2–4). However, CD74 is a multifunctional protein. Thus, the consequences of therapeutically targeting CD74 may differ from the consequences of targeting MIF. We, now, summarize the function of CD74 and review its expression and role in kidney injury, highlighting open questions. The detailed description of the role of CD74 in the immune system is beyond the scope of this review.

CD74 Functions

CD74 regulates intracellular trafficking and functions as a chaperone and a cell membrane receptor, modulating B, T, and dendritic cell responses (1, 5) and promoting tumor growth by increasing tumor cell survival or proliferation. Thus, CD74 is considered a therapeutic target in malignancy. In addition, CD74 regulates proliferation, survival, and secretion of inflammatory and fibrosis mediators in non-immune and non-tumor cells (1, 2). Thus, CD74 may modulate tissue injury and homeostasis beyond its effect on immune regulation.

Protein Trafficking

CD74 interacts with MHC class I and II proteins, contributing to antigen presentation. CD74 directs transport of MHC class II α and β chains from the endoplasmic reticulum (ER) or the cell surface to endosomes (6). As a chaperone, CD74 contributes to peptide editing in the MHC class II compartment. In endosomes, proteases degrade CD74, releasing MHC class II molecules. Prevention of CD74 degradation promotes the cell surface localization of MHC II. Extracellular CD74/cathepsin L complexes are found in human kidneys (1, 7, 8). CD74 also associates with angiotensin II type I receptors (AT1), leading to AT1 proteasomal degradation (9).

Cell Surface Receptor

Only a small amount of CD74 (2–5%) is expressed at the cell surface (10, 11). Cell surface CD74 is a receptor for MIF and MIF-2. CD74 lacks signaling motifs, but may generate fragments with transcription factor activity and binds to signaling proteins, such as CD44. Ligand binding to CD74 leads to CD74 phosphorylation, endocytosis, regulated intramembrane proteolysis (RIP), and release of CD74 intracellular domain (CD74-ICD) into the cytosol that translocates to the nucleus and activates NF- κ B (10, 12–14). RIP might occur in cultured vascular smooth muscle cells since γ -secretase activity was required for MCP-1 expression induced by activating anti-CD74 antibodies. However, the pathophysiological implications remain unclear since MIF did not increase MCP-1 in these cells (15).

Migration inhibitory factor trimers activate CD74, and CD74 alone is required for MIF binding (16). In addition, MIF can also engage chemokine receptors CXCR2, CXCR4, and CXCR7 (10, 17). Cells that express two receptors, e.g., CD74 and CXCR2, may be more responsive to MIF (17). MIF-induced CD74 signaling complexes with CXCR2, CXCR4, or CXCR7 promote chemokine expression and chemotaxis (17–19). CD44 is the signaling component of the MIF-CD74 receptor complex and recruits the Src tyrosine kinase to activate ERK1/2 in macrophages and Syk, Akt, and NF κ B in B cells (20, 21). Thus, the receptor complex activates kinase cascades and transcription factors. Interestingly MIF, itself increases CD44 expression (22).

Migration inhibitory factor-2 is 30% homologous to MIF, also activates CD74 (23, 24) and is responsible for residual CD74-dependent chemotactic activity in MIF^{-/-} mice (17). MIF-2 binding to CD74/CD44 activates kinases, downstream proinflammatory pathways, and β -catenin (25). However, MIF-2 lacks the pseudo(E)LR motif present in MIF that mediates interaction with CXCR2 and CXCR4, and thus, it is a more selective CD74 agonist than MIF. Circulating MIF-2 levels correlate with severity of sepsis, a cause of acute kidney injury, and MIF or MIF-2 blockade reduced systemic inflammation, protecting mice from lethal endotoxemia.

Migration inhibitory factor or MIF-2 activation of CD74 regulates cell survival and proliferation of B cells (21) and epithelial cells, including gastric epithelial cells and type II alveolar epithelial cells (26, 27). MIF or MIF-2 binding to CD74 protects the heart and liver from injury, including from ischemia–reperfusion (28–30). In the heart, CD74 promotes phosphorylation of the AMPK catalytic alpha subunit in response to increased

intracellular calcium and activation of Ca²⁺/calmodulin-activated kinase kinase-2 (CaMKK-2) (29). Interestingly, liver expression of CD74 protected mice from lethality induced by agonistic anti-Fas antibodies as CD74 interfered with immediate early steps in Fas signaling at the plasma membrane, and the anti-CD74 antibody milatuzumab sensitized BJAB cells to Fas-mediated apoptosis (31). Fas ligand and Fas have long been known to promote kidney injury (32). In fact, agonistic anti-Fas antibodies induced glomerular cell apoptosis *in vivo* (33). Thus, CD74 interference with Fas signaling should be explored in kidney cells. The MIF/CD74/AMPK pathway also protects hepatocytes in metabolic liver injury, such as non-alcoholic steatohepatitis (30). In this regard, liver fibrosis was increased in MIF^{-/-} or CD74^{-/-} mice suggesting an antifibrotic effect of MIF/CD74 (34). Enhanced fibrosis was thought to result from the release of MIF inhibition of PDGF-induced migration and proliferation of hepatic stellate cells. MIF/CD74 also protects the lungs. Both MIF^{-/-} and CD74^{-/-} mice developed spontaneous emphysema by 6 months of age (35). However, CD74 may also contribute to disease, as discussed below for glomerulonephritis and kidney cysts. In this regard, CD74 deficiency reduced atherosclerosis in low-density lipoprotein receptor-deficient LDLR^{-/-} mice (36) and protected NOD mice from development of diabetes, probably by enhancing T regulatory cell number and impairing antigen presentation (37).

Among kidney cells, MIF induced proliferation in parietal epithelial cells but not in podocytes (4) (**Figure 1**). Absence of CD44 or the terminal differentiation state of podocytes may account for the differences. MIF, MIF-2, CD74, and CD44 promote clear cell renal cell carcinoma, cell proliferation, and HIF-activation (38, 39). While MIF and MIF-2 overlap in controlling cell survival and tumor formation, MIF-2 plays a dominant role in renal cancer tumor growth *in vivo* (40). MIF also confers resistance to senescence and cell death in mesenchymal stem cells through CD74-dependent AMPK-FOXO3a signaling and c-Met activation (41).

In renal tubular epithelial cells and podocytes, MIF binding to CD74 leads to persistent activation of p38 and ERK1/2 MAPK and expression of inflammatory mediators (e.g., TRAIL and MCP-1) (11, 42). MIF upregulation of inflammatory mediators was a late event, observed at 24 h (11). Thus, it was delayed as compared to responses elicited by the inflammatory cytokines TNF or TWEAK or metabolites, such as lyso-Gb3 (43, 44).

In summary, MIF-2 and MIF have an overlapping spectrum of activities mediated by CD74 activation and may cooperate, additively inducing chemokine secretion or survival in non-renal cells and proliferation in kidney cancer cells (45).

Regulation of CD74 Expression

CD74 expression is increased during tissue injury in diverse organs and in malignancies, including kidney cancer (2, 15, 28, 34, 46–48). There is limited information on the regulation of CD74 expression in renal cells. In normal mouse and human kidneys, tubular but not glomerular epithelium expresses low levels of CD74 (4, 11). By contrast, cultured human podocytes and proximal tubular cells and murine glomerular parietal epithelial cells express CD74 (4, 11). Abnormally high concentrations of certain metabolites (e.g., glucose and lyso-Gb3) and

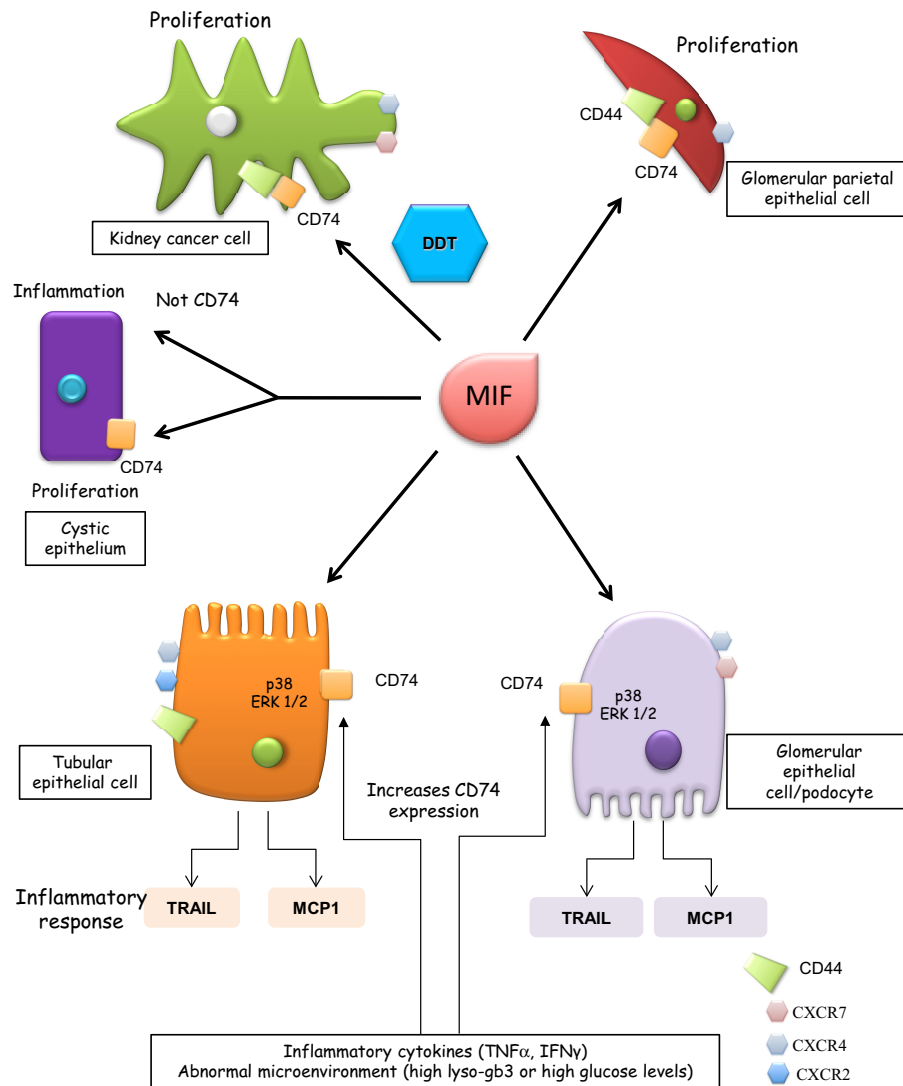


FIGURE 1 | CD74 functions in renal cells. Glomerular parietal epithelial cells express CD44 when activated and it is thought that CD44 contributes to the proliferative response. CD44 is not expressed by podocytes and its role of CD74 signaling in tubular cells has not been characterized. Thus, in tubular cells, CD44 is not depicted as part of the CD74 signaling complex. Cells expressing CXCR2, CXCR4, and CXCR7 are also indicated, although these receptors are depicted away from MIF when in that specific cell type, there is no information on their involvement in MIF signaling. In tubular epithelium with genetic defects in PKD1, MIF promotes tubular cell proliferation and cystogenesis and a CD74 antibody blocked the MIF-induced phosphorylation of ERK but not inflammatory responses.

inflammatory cytokines, such as TNF, increase CD74 expression in podocytes and/or tubular cells (11, 49). IFN- γ increases CD74 expression *in vivo* in kidney tubular epithelium and in endothelial cells of larger kidney vessels (50). The factors known to upregulate CD74 expression in kidney cells may be relevant for diabetic nephropathy, Fabry disease, and inflammatory conditions.

Regulation of CD74 Interaction with MIF

Endogenous factors or drugs interfere with MIF binding to CD74 or downregulate CD74 expression and a better understanding of these interactions may provide therapeutic tools to manipulate the system. Some agents targeting MIF prevent MIF binding to CD74. These include antibodies, RPS19 (ribosomal

protein S19), a component of the 40S small ribosomal subunit that binds MIF and behaves as an endogenous blocker of MIF binding to CD74 (51) and the small molecule MIF antagonist 3-(3-hydroxybenzyl)-5-methylbenzoxazol-2-one (MIF098) that decreases tautomerase activity and MIF-CD74 binding. MIF098 attenuated MIF-dependent ERK1/2 phosphorylation in human synovial fibroblasts (52) and promoted hyperoxia-induced lung injury *in vivo* (53), supporting the tissue-protective properties of MIF/CD74. Other compounds bind to CD74 or interfere with CD74 processing. The HLA-DR α 1 domain binds to and down-regulates CD74 on monocytes, directly inhibiting MIF binding to CD74 and blocking downstream inflammation in murine autoimmune encephalomyelitis. Adding a peptide extension [myelin

oligodendrocyte glycoprotein (MOG)-35–55 peptide] that modified the secondary structure, enhanced the potency of the DR α 1 domain in downregulating CD74 cell surface expression (54). Binding of partial MHC class II complexes comprised of linked β 1 α 1 domains with covalently attached antigenic peptides [recombinant T-cell receptor ligands (RTLs)] to CD74 blocks the accessibility and availability of CD74 for MIF binding and downstream inflammation in monocytes (55).

The intramembrane protease presenilin homolog signal-peptide-peptidase-like 2a (SPPL2a) cleaves CD74. SPPL2a^{-/-} mice accumulate N-terminal fragments of CD74 that impair membrane traffic within the endocytic system and alter B cell biology (56, 57). Since in SPPL2a^{-/-} mice, CD74 signaling is inhibited, SPPL2a inhibitors may offer new pathways to inhibit CD74 signaling (58). Despite these recent advances, there is little or no information on the potential therapeutic or adverse effects of CD74 targeting strategies for kidney diseases and the available information is

mainly derived from the study of CD74^{-/-} mice, which may have developed adaptive mechanisms over development.

The role of soluble CD74 is unclear. Circulating soluble CD74 with MIF neutralizing activity was increased in primary biliary cirrhosis and was hypothesized to contribute to liver fibrosis (59). Indeed, soluble CD74 ectodomain prevents binding of MIF to cell surface receptors (10). However, MIF/sCD74 complexes were found to enhance MIF antioxidant activity (60). Autoantibodies against CD74 (anti-HLA class II-associated invariant chain peptide, CLIP) were found in 67% of ankylosing spondylitis patients, in 15% of systemic lupus erythematosus patients, and only in 0.8% of blood donors (61, 62).

CD74 and Kidney Injury

There is a growing body of evidence linking CD74 to promotion or protection from kidney injury (**Figure 2**).

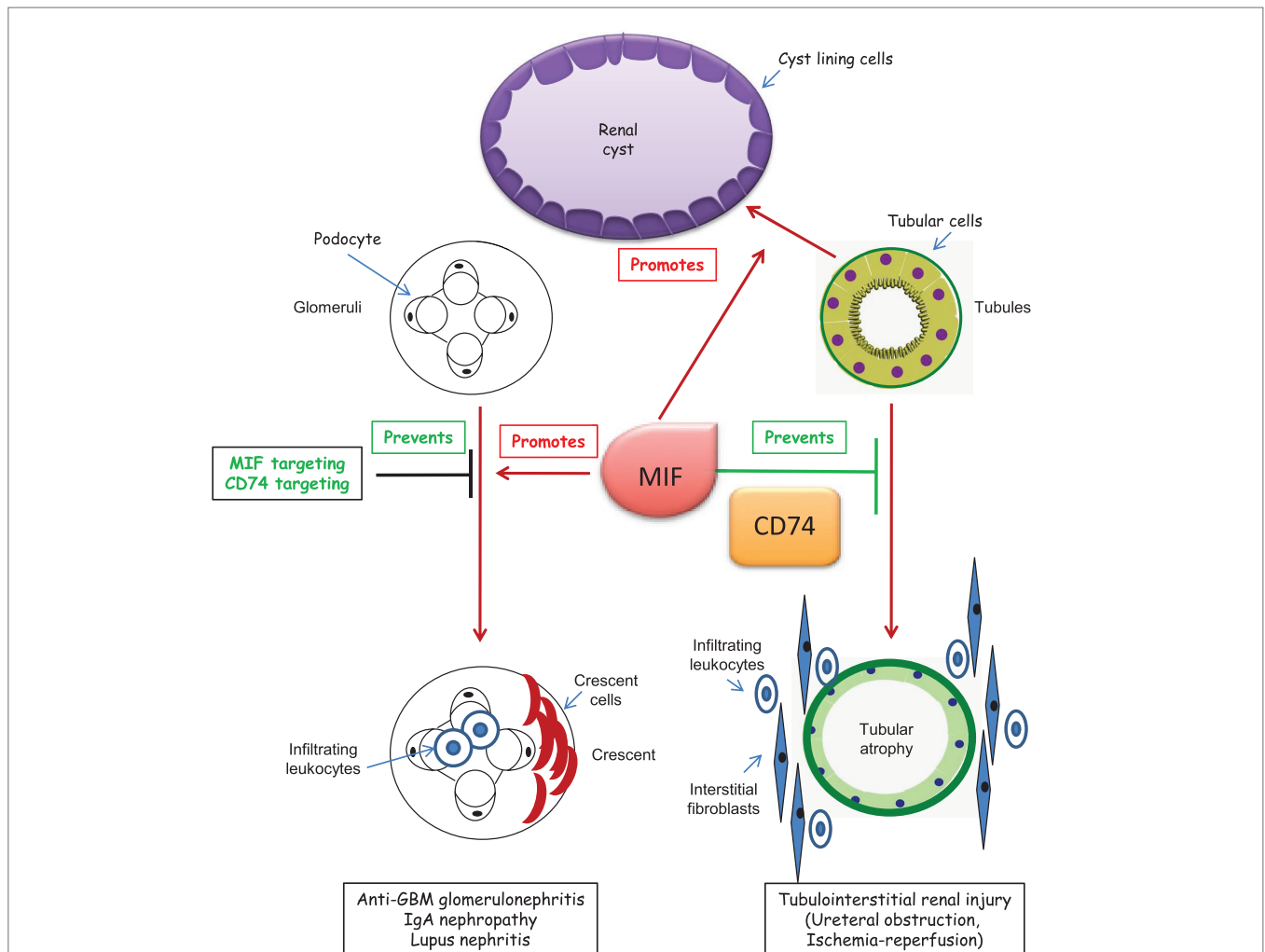


FIGURE 2 | CD74 in kidney disease. Studies in CD74-deficient mice with kidney disease have only been published in abstract form. Thus, potential roles of CD74 in kidney disease have been mainly derived from abstracts or studies in which MIF was targeted in cultured cells or experimental animals. A putative effect of CD74 targeting on glomerular injury is only hypothetical and based on studies in which MIF was targeted. In polycystic kidney disease, MIF promotes cystogenesis. The role of CD74 is unclear, but CD74 expression is increased in cystic epithelium.

Expression of CD74 and Related Molecules in Kidney Injury

MIF has long been known to be upregulated in kidney injury (2). However, reports of increased CD74 expression are more recent and there is very little information on MIF-2.

Low CD74 expression may limit inflammatory and proliferative cell responses to MIF, and the increased renal cell CD74 expression observed during kidney injury (discussed below) may contribute, together with increased ligand availability, to elicit biological responses during kidney disease. Thus, overexpression of CD74 led to upregulation of NF- κ B-dependent genes encoding cytokines in macrophages (63) and to NF κ B activation and proliferation in human embryonic kidney cells (64).

CD74 is upregulated in tubular epithelial cells in at least some forms of chronic kidney disease (CKD), such as kidney graft rejection, diabetic and Fabry nephropathy and autosomal dominant polycystic kidney disease (ADPKD), and in podocytes in human diabetic nephropathy and Fabry nephropathy (11, 65, 66). A transcriptomics analysis also revealed increased kidney CD74 mRNA expression in human hypertensive nephropathy (11). CD74 is also increased in human clear cell renal cell carcinoma (67), in B cells, and kidney of lupus mice in parallel to progression of inflammation (68) and in the tubulointerstitium in anti-GBM nephritis, where it was expressed *de novo* in glomerular parietal epithelial cells and crescents (51). Interestingly, kidney cancer may be a complication of CKD-related acquired polycystic kidney disease (69). There is much less information on the expression of CD74 in acute kidney injury.

Migration inhibitory factor was recently identified as an important regulator of cyst growth and therapeutic target in ADPKD (3). MIF accumulated in cyst fluid of human ADPKD, promoted cystic epithelial cell proliferation and regulated apoptosis. In experimental murine polycystic kidney disease, MIF was required for renal inflammation and cyst expansion. CD74 expression was increased in *Pkd1* mutant renal epithelial cells and ADPKD kidneys, suggesting that MIF actions may imply CD74 activation. However, a CD74 antibody blocked the MIF-induced phosphorylation of ERK but did not modulate the MIF-induced inflammatory response of increased MCP-1 expression in culture (3).

The CD74 signaling machinery may also be upregulated in kidney disease. CD44 is rapidly upregulated after injury in acute and chronic experimental and human kidney disease including glomerulonephritis, diabetic nephropathy, cyclosporine nephrotoxicity, urate nephropathy, and ischemia-reperfusion kidney injury (70–76). Increased CD44 expression was localized to tubules, glomerular parietal epithelial cells, mesangial cells and infiltrating macrophages, T cells, and neutrophils (70, 72, 75–78).

Finally, the expression of at least some CD74 ligands is also increased in kidney injury. MIF expression is upregulated in acute and chronic, human, and experimental kidney diseases (2). However, there is no information on MIF-2 expression and kidney disease.

CD74 Targeting and Kidney Injury

Neutralizing anti-MIF antibodies, small molecules or endogenous inhibitors or MIF^{-/-} mice have shown that MIF aggravates anti-GBM glomerulonephritis (69–71), experimental IgAN

(79), and lupus nephritis (80). More recently, the inhibitor of MIF/CD74 interactions RPS19 prevented the development of glomerular crescents, necrosis, inflammation, renal dysfunction, proteinuria, and the upregulation of MIF and CD74 in anti-GBM glomerulonephritis (51). However, MIF absence from mice did not protect from renal allograft rejection (81) or ureteral obstruction-induced kidney injury (82).

There is limited experimental and no clinical data on CD74 targeting in kidney injury. Given the multiple MIF and CD74 functions, studies are needed that explore whether targeting CD74 is therapeutic in models in which targeting MIF was beneficial. Information on the role of CD74 as a potential therapeutic target in tissue injury is mainly derived from CD74^{-/-} mice. As discussed above, CD74 deficiency may be beneficial for some non-renal diseases, such as liver and heart disease, and deleterious in others, such as vascular injury. Specifically, liver fibrosis was increased in CD74^{-/-} mice (34). An initial report found no protection from ureteral obstruction-induced kidney inflammation or fibrosis (82), but an abstract indicated that MIF targeting promoted interstitial fibrosis and inflammation following ureteral obstruction, whereas recombinant MIF reduced fibrosis (4). According to abstract reports, CD74 deficiency was also associated with increased interstitial fibrosis and inflammation following ureteral obstruction (day 5) and ischemia-reperfusion (day 21) (4). By contrast, CD74^{-/-} mice are protected from glomerular injury induced by anti-GBM antiserum (Djudjaj JASN2015).

Since MIF or MIF-2 activation of CD74 is tissue protective in heart ischemia and liver injury induced by activation of the Fas receptor or metabolic disorders, a similar protective effect maybe hypothesized in ischemic acute kidney injury or metabolic kidney diseases characterized by increased CD74 expression, such as diabetic nephropathy or Fabry disease. Interventional studies should test these hypotheses and evaluate whether changes in fibrosis after kidney ischemia-reperfusion is secondary to an improved initial kidney injury or to a specific action of fibrosis mechanisms. These studies should differentiate between complete abrogation of CD74 expression (CD74^{-/-}) and therapeutic downregulation of CD74. Incomplete blockade of the system may have different consequences than complete CD74 absence, given the potential proinflammatory effects of excess CD74 activation.

Kidney Safety of Lethal Anti-CD74 Immunoconjugates

The anti-CD74 antibody hLL1 milatuzumab, alone or as an immunoconjugate, is undergoing clinical trials to treat malignancy (83, 84). Milatuzumab binds to CD74 and promotes internalization of the antibody-CD74 complex, thus delivering conjugated antitumoral agents inside tumor cells with high CD74 expression, but not to normal cells with low CD74 levels (85). Since CD74 expression is also increased in renal cells from injured kidneys, nephrotoxicity is a potential complication of antitumoral anti-CD74 therapy, especially in patients with prior kidney disease since the active chemotherapeutic agent may be released inside already injured, CD74-expressing renal cells. This may be of special concern for one of the indications under study, multiple myeloma, which frequently causes kidney disease.

Summary and Conclusions

The role of CD74 in kidney injury has barely been explored and the scarce information available is derived from CD74-deficient mice that may not recapitulate the findings of targeting CD74 *de novo* in an adult. CD74 may contribute to or protect from tissue injury in a disease-specific manner. Thus, like MIF, CD74 may protect from experimental kidney interstitial fibrosis but promotes glomerular injury, while MIF (and potentially CD74) also promotes polycystic kidney disease. A few years ago, the situation was less complex. Given the longstanding preclinical evidence for a pathogenic role of MIF in glomerular kidney disease, anti-MIF strategies were tested in renal disease, although the sponsor decided to terminate a phase 1 trial of the anti-MIF monoclonal antibody imalumab in lupus nephritis (NCT01541670). However, MIF, MIF-2, and CD74 may be tissue protective or promote injury in an organ- and disease-specific manner and different forms of therapeutic manipulation of the system may be envisioned for different indications, from inhibiting to actually activating CD74 signaling. The potential impact of future intervention on CD74 for kidney disease is double. On one hand, therapeutic modulation of the system may be used to treat kidney disease. On the other, the kidney may suffer adverse effects from the therapeutic targeting on non-kidney diseases. Therapeutic approaches blocking MIF or CD74 signaling for non-renal indications may theoretically promote kidney fibrosis as an adverse effect, while therapeutic approaches activating

the system may cause or aggravate glomerular injury. Thus, a better understanding of CD74 and the kidney is required for nephrologists and non-nephrologists. Future areas of research include the potential therapeutic interest of molecules aimed at increasing or decreasing CD74 activity for different forms of kidney disease, including acute and chronic kidney injury, the potential renal adverse effects of these approaches when used for non-renal indications, the impact on kidney disease of naturally occurring circulating soluble CD74 or CLIP and the kidney safety of antitumor therapeutic strategies delivering toxins into CD74-expressing cells or targeting CD74. Additional unknowns to be solved include the drivers and consequences of CD74 RIP in kidney cells, the significance for kidney disease of MIF-2 and of CD74 actions on mesenchymal stem cells, and whether AMPK is activated by CD74 in renal cells since, contrary to the heart, no difference in AMPK activation by acute ischemia was observed between MIF^{-/-} and wild-type mice in the kidney, and this was attributed to lower CD74 expression in the kidney (86).

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The dipeptidyl peptidase family, prolyl oligopeptidase, and prolyl carboxypeptidase in the immune system and inflammatory disease, including atherosclerosis

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Research from over the past 20 years has implicated dipeptidyl peptidase (DPP) IV and its family members in many processes and different pathologies of the immune system. Most research has been focused on either DPPIV or just a few of its family members. It is, however, essential to consider the entire DPP family when discussing any one of its members. There is a substantial overlap between family members in their substrate specificity, inhibitors, and functions. In this review, we provide a comprehensive discussion on the role of prolyl-specific peptidases DPPIV, FAP, DPP8, DPP9, dipeptidyl peptidase II, prolyl carboxypeptidase, and prolyl oligopeptidase in the immune system and its diseases. We highlight possible therapeutic targets for the prevention and treatment of atherosclerosis, a condition that lies at the frontier between inflammation and cardiovascular disease.

Keywords: dipeptidyl peptidase, prolyl oligopeptidase, fibroblast activation protein α , prolyl carboxypeptidase, inflammation, immunophysiology, atherosclerosis

Introduction

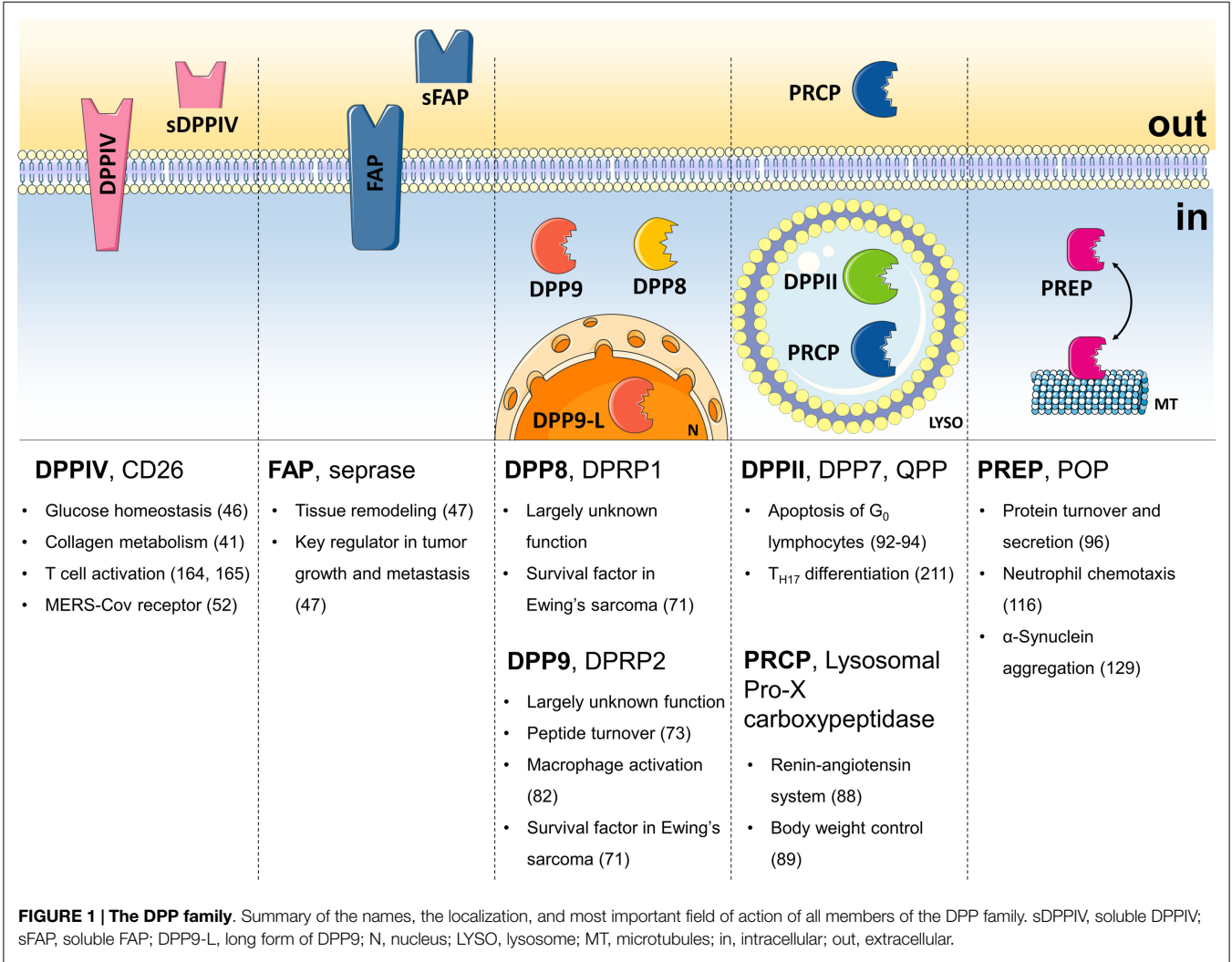
Research from over the past 20 years has implicated the dipeptidyl peptidase (DPP) family in various physiological processes and pathologies of the immune system. Usually only four prolyl-specific peptidases are considered: DPPIV (EC 3.4.14.5), fibroblast activation protein α (FAP; EC 3.4.21.B28), and the more recently discovered DPP8 and DPP9 (EC 3.4.14). However, due to similarities in substrate specificity and structural homology, it is more relevant to consider a broader family that also includes prolyl oligopeptidase (PREP; EC 3.4.21.26), dipeptidyl peptidase II (DPPII) (EC 3.4.14.2), and prolyl carboxypeptidase (PRCP; EC 3.4.16.2). First, DPPII and PRCP share the α/β hydrolase fold with the other DPPs and the catalytic triad is completely conserved in both enzymes (2). Moreover, DPPII can cleave several DPPIV substrates *in vitro* (3). Conversely, due to its substrate preference for tripeptides (4), DPPII could actually be considered as a prolyl carboxytripeptidase, emphasizing its similarities to PRCP. Another argument for considering a broader family stems from the fact that functional studies on the role of peptidases rely heavily on the use of enzyme inhibitors and many of the inhibitors used in earlier studies are now known to inhibit more than one family member. For example, early studies on DPPIV used inhibitors which we now know also inhibit DPPII, DPP8, DPP9, FAP, and/or PREP due to their sequential and/or structural similarity [e.g., Ref. (5–9)]. PRCP is known to be inhibited by KYP-2047 and Z-Pro-Prolinal at higher concentrations,

which have often been used for the functional study of PREP [e.g., Ref. (10–12)]. **Table 1** summarizes the most commonly used DPP inhibitors and their selectivity compared to DPPIV. In view of the aforementioned reasons and for the sake of simplicity, we will use “DPP family” as a blanket term, which includes DPPII, PRCP, and PREP even though strictly speaking they are not DPPs. **Figure 1** provides a general overview of this broadly defined DPP family. The roles of various family

TABLE 1 | Overview of commonly used inhibitors within the DPP family and the ratio of inhibitor needed to inhibit the respective DPP family member compared to what is needed to inhibit DPPIV.

Inhibitors		DPPII	DPP8	DPP9	FAP	PREP	PRCP	Reference
Clinical	<i>Alogliptin</i>	>14,000	>14,000	>14,000	>14,000	>14,000	ND	(16)
	<i>Linagliptin</i>	>100,000	40,000	>10,000	89	>100,000	ND	(17)
	<i>Saxagliptin</i>	>50,000	390	77	>4,000	ND	ND	(18)
	<i>Sitagliptin</i>	>5,550	>5,550	>2,660	>5,550	>5,550	ND	(19)
	<i>Talabostat</i>	4	8	4	3	44	ND	
	<i>Vildagliptin</i>	>100,000	270	32	285	60,000	ND	(20, 21)
Experimental	<i>1G244</i>	1	<10 ^{−3}	<10 ^{−3}	1	ND	ND	(21)
	<i>KYP2047</i>	1	ND	1	1	<10 ^{−4}	1	(12, 22)
	<i>UAMC01110</i>	1	ND	0.5	<10 ^{−4}	0.1	ND	(23)
	<i>UAMC00039</i>	<10 ^{−5}	1	2	>10	ND	ND	(24, 25)
	<i>Z-Pro-Prolinal</i>	ND	ND	ND	ND	<10 ^{−2}	*	(26, 27)

Partly adapted from Deacon (28).
*Z-Pro-Prolinal is currently used as an inhibitor for PRCP although it is in vitro 10³ times more selective toward PREP.



members in certain aspects of the immune system or immune dysfunction have been reviewed in the past [e.g., Ref. (13–15)]. In this review, we provide a comprehensive discussion and update on the roles of DPPIV, DPPII, DPP8, DPP9, FAP, PREP, and PRCP in the immune system and inflammatory disease. We highlight the role of these enzymes in atherosclerosis, a condition that lies at the frontier between inflammation and cardiovascular disease, as the DPP family encompasses possible therapeutic targets for the prevention and treatment of this disease.

A Brief Guide to the DPP Family

Dipeptidyl Peptidase IV

The prototypical DPP, DPPIV (often DPP4 in medical jargon) cleaves off an N-terminal dipeptide from peptides with Pro or Ala on the penultimate position. Its localization as a soluble enzyme in body fluids, or anchored in the plasma membrane of cells provides it with the necessary access to cleave a wide range of bioactive peptides. As such, it can modify their biological activity. Glucagon-like peptide (GLP)-1 and -2, and glucose-dependent insulinotropic peptide (GIP) (29, 30), substance P (31), neuropeptide Y (NPY) (32), stromal cell-derived factor-1 α/β (SDF-1 α/β or CXCL12) (33), granulocyte macrophage colony-stimulating factor (GM-CSF) (1), CXCL10 (34–36), and high-mobility group box 1 (HMGB1) (37) have been identified as physiological substrates, while others, such as RANTES, have been proposed based on *in vitro* experiments [e.g., Ref. (38)]. DPPIV also performs many of its physiological functions through interactions with other proteins, such as collagen, fibronectin, adenosine deaminase (ADA), caveolin-1, and the mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFIR) (39–41). Some of those will be discussed in more detail below.

Dipeptidyl peptidase IV is well known for its role in glucose homeostasis. It has become a validated therapeutic target for the treatment of type 2 diabetes (T2D) (46). DPPIV inhibitors reduce the rate of GLP-1 inactivation (Boxes 1 and 2). It has also been shown to be involved in cancer biology. The role of the DPP family in cancer has been addressed in several other reviews (39, 47–51). Finally, DPPIV has recently come back into the center of attention as the receptor for the MERS coronavirus (52).

BOX 1 | Incretins.

The incretins are a group of glucose-lowering molecules produced by the intestines. The best known incretin is glucagon-like peptide-1 (GLP-1). This incretin is derived from proglucagon and secreted after a meal from L-cells in the distal ileum and colon. In the pancreas, it induces insulin secretion and biosynthesis while lowering glucagon secretion. In addition, GLP-1 increases the β -cell mass, thereby restoring insulin production. It is clear that GLP-1 also has functions outside glucose metabolism. Its receptor, GLP-1-R, is not only found in the pancreas but also expressed in brain, lung, kidney, stomach, and heart (42, 43). Recently, it was shown that stimulation after myocardial infarction reduces the infarct size (44, 45). Currently, GLP-1 agonists are approved for the treatment of type 2 diabetes. These incretin mimetics seem to have a slightly better efficacy as DPPIV inhibitors and lead more frequently to weight loss. Unfortunately, an important drawback for their therapeutic use is that they can only be administered by subcutaneous injection (46).

Fibroblast Activation Protein α

Fibroblast activation protein α , also known as seprase can present itself as a type II transmembrane protein or as a shedded plasma protease (57). In the latter case, it is also known as antiplasmin-cleaving enzyme, which converts α 2-antiplasmin into a more active form, suppressing fibrinolysis (58). Some of the known DPPIV substrates were later found to be cleaved *in vitro* by FAP as well (59), though any physiological relevance remains unclear.

Unlike DPPIV, FAP also possesses a gelatinase activity. This enables FAP to degrade proteins of the extracellular matrix (60). This is of particular interest with regard to its involvement in a number of pathological processes (47). FAP is highly induced during inflammation, activation of hepatic stellate cells in liver cirrhosis and strongly expressed by mesenchymal cells of remodeling tissue (47, 61). FAP is also a key regulator during tumor growth and metastasis (47). As all these processes require degradation of the extracellular matrix, FAP's involvement in these pathologies is most likely associated with its gelatinase activity (51). Its role in cancer biology has been reviewed before (47, 62). It is interesting to note that, so far, in clinical trials Talabostat has shown minimal or no clinical benefit for the treatment of metastatic colorectal cancer, advanced non-small cell lung cancer, or stage IV melanoma (63–65). It should be mentioned, however, that Talabostat is a broad-range inhibitor also targeting DPPIV, DPP8, and DPP9.

Dipeptidyl Peptidases 8 and 9

Dipeptidyl peptidases 8 and DPP9 show DPPIV-like activity and share a very high-sequence similarity to each other (77% aa similarity, 57% aa identity) (24). These cytoplasmic enzymes have several isoforms. It has been a matter of debate whether all are expressed as protein in cells and, if so, whether they are active (66–69). Interestingly, the N-terminal extension of the longer DPP9 variant contains a nuclear localization signal and, indeed, this form localizes to the nucleus (69). DPP8 has been shown to cleave a number of DPPIV chemokine substrates *in vitro* (70). Another DPPIV substrate, NPY, has indirectly been shown to be

BOX 2 | DPPIV inhibitors.

Dipeptidyl peptidase IV inhibitors prolong the biological half-life of the incretins and are therefore used for the treatment of type 2 diabetes. Sitagliptin, vildagliptin, saxagliptin, linagliptin, and alogliptin are DPPIV inhibitors currently available on the market for treatment of type 2 diabetes. Sitagliptin and alogliptin are highly selective toward DPPIV *in vitro*, whereas vildagliptin and saxagliptin are less selective with regard to DPP8 and 9, and linagliptin with regard to FAP (28). Their clinical efficacy and safety in the use of type 2 diabetes seem comparable as far as can be judged from the data available.

There is a growing interest toward a use outside type 2 diabetes as it has become clear that DPPIV inhibitors have pleiotropic effects. While negative effects have been found in heart failure (53), some studies suggest them as a possible therapeutic strategy in cardiovascular pathologies (28, 54). The SITAGRAMI trial and follow-up studies revealed that the combination of a DPPIV inhibitor with granulocyte-colony-stimulating factor or in monotherapy presents a therapeutic option after myocardial infarction (55, 56). As stated above, the mechanism is not yet clear but may be explained by a longer biological half-life of DPPIV substrates, glucagon-like peptide-1, B-type natriuretic peptide, and stromal cell-derived factor-1 α/β . All three peptides have a cardioprotective effect that is abolished by DPPIV-mediated cleavage. For an extensive review of the involved substrates, see Matheeußen et al. (43).

a DPP8 and DPP9 substrate as well (71). Efforts have been made to find intracellular DPP8 and 9 substrates using a peptidomic approach (72), but so far it has been hard to attribute physiological relevance to the possible substrates beyond the role of DPP8 and 9 in intracellular peptide turnover (73).

The physiological functions of DPP8 and DPP9 are still not properly understood. Mainly, a lack of available knockout animals, specific inhibitors, and substrates has hampered progress (24). A mouse model has been established with a targeted inactivation of DPP9 enzymatic activity (74), but homozygous DPP9-inactive neonates die within 8–24 h after birth. Despite these limitations, some indications toward their role are surfacing. Using immunohistochemistry, DPP8 and 9 were found associated with spermatozooids and spermatids and the short mRNA of DPP8 is predominantly expressed in testes (75, 76), suggesting a role in spermatogenesis and male fertility. Recent work has found SUMO1 to be an allosteric activator of DPP9 (77), whereas a small peptide corresponding to the interaction surface of SUMO1 is a non-competitive inhibitor of DPP8 and DPP9 (78). A genome-wide association study has linked DPP9 to idiopathic pulmonary fibrosis (79).

Finally, a number of studies have shown a role for DPP8 and DPP9 in apoptosis (71, 80–83). Two studies showed that overexpression enhanced induced apoptosis and impaired cell adhesion and migration (80, 81). Conversely, DPP8/9 inhibition in tumor cells decreased the number of viable cells because of a decreased cleavage of pro-apoptotic NPY (71). In macrophages, inhibition caused a marginal, yet significant increase in apoptosis, independent of NPY cleavage (82). Interestingly, vildagliptin, a DPPIV inhibitor already on the market to treat type 2 diabetes, but with poorer selectivity toward DPP8 and 9, was shown to enhance parthenolide's anti-leukemic activity through its inhibition of DPP8 and 9, and not DPPIV (83).

Dipeptidyl Peptidase II and Prolyl Carboxypeptidase

Prolyl carboxypeptidase, also called angiotensinase C or lysosomal Pro-X carboxypeptidase, is a lysosomal carboxypeptidase sharing strong sequence homology with the likewise lysosomal DPPII (4, 84). PRCP preferentially cleaves off the C-terminal amino acid when Ala or Pro is in the penultimate position, while DPPII targets N-terminal X-Pro or X-Ala dipeptides (85, 86). In addition to a structural similarity, PRCP and DPPII have partially overlapping substrate specificities due to DPPII's preference for tripeptide substrates (4). Perhaps surprisingly, Gly-Pro-pNA and Ala-Pro-pNA, two typical synthetic DPP substrates, have actually been used to perform PRCP activity measurements (87).

Prolyl carboxypeptidase is particularly known as one of the key enzymes of the renin–angiotensin system (RAS). It inactivates the vasoactive peptides angiotensin II and angiotensin III by cleaving off the C-terminal Phe (88). α -Melanocort-stimulating hormone 1–13, an anorexigenic neuromodulator, is inactivated by PRCP, implying a role in body weight control (89). Based on the involvement of PRCP in the conversion of these peptide hormones, the enzyme has also been associated with diseases, such as hypertension, diabetes mellitus, obesity, inflammation, and cardiovascular dysfunction (90, 91).

Dipeptidyl peptidase II has no known natural substrates. The DPPIV substrate substance P has been shown to be cleaved by DPPII *in vitro* (3), but much less efficiently, casting doubt over any physiological relevance. It has been shown that inhibition or silencing of DPPII causes apoptosis of quiescent G₀ lymphocytes (92–94). On the other hand, a highly specific DPPII inhibitor, UAMC00039, did not induce apoptosis, autophagy, or necrosis in human leukocytes (25, 95), but this study did not specifically look at quiescent cells or lymphocytes. Finally, changes in DPPII activity levels have been observed in a number of pathologies, such as neurodegenerative disorders, myopathies, cancer, and gastro-intestinal disorders (4).

Prolyl Oligopeptidase

Prolyl oligopeptidase is an oligopeptidase with endopeptidase activity. It has been shown to be localized in the cytoplasm (96–99), but given its ability to inactivate several neuropeptides *in vitro* by limited proteolysis (100–115), its involvement in the *in vivo* generation of immunoactive peptides N-acetyl-prolyl-glycyl-proline and N-acetyl-seryl-aspartyl-lysyl-proline (116, 117), and its presence in plasma (118, 119), it most likely also has an extracellular role.

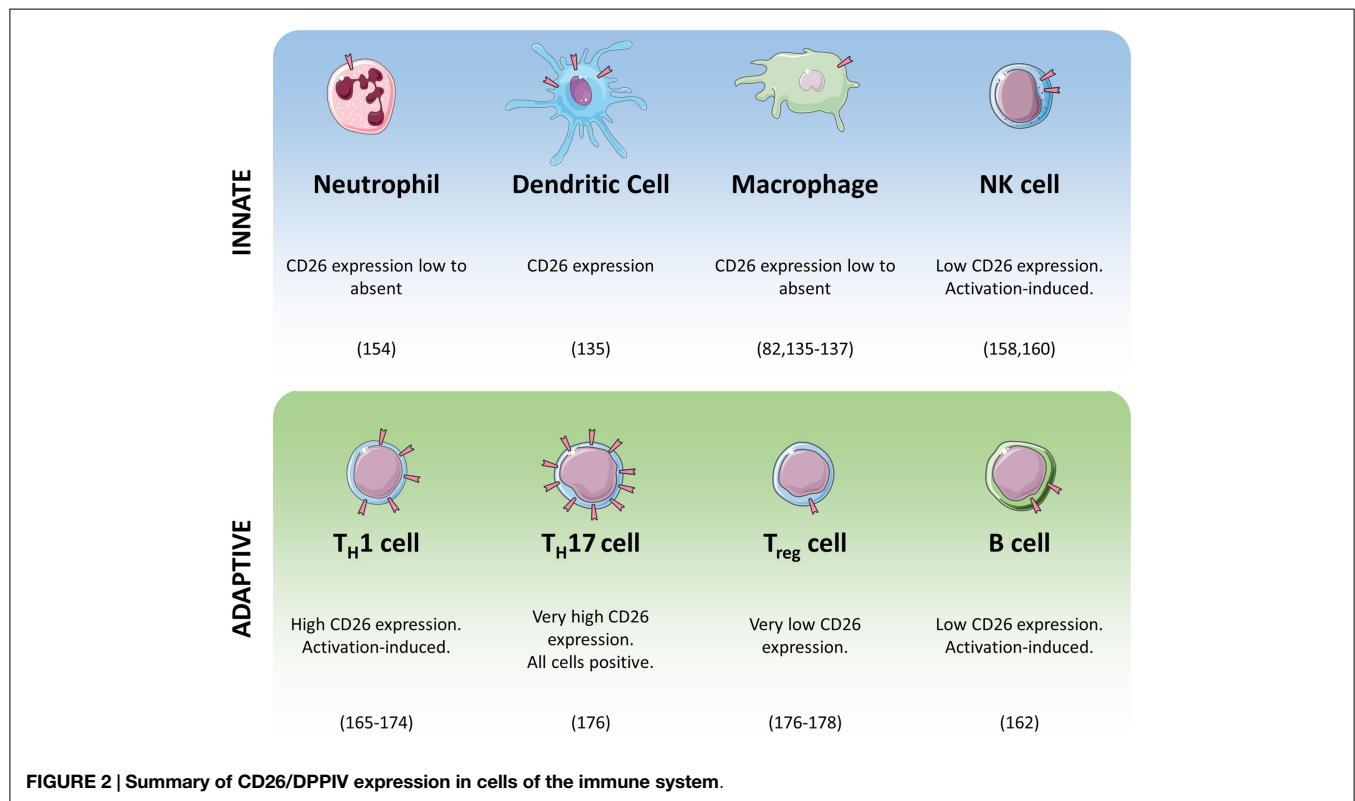
Initial interest for PREP derived from the positive effects of PREP inhibitors on scopolamine-induced amnesia in rats (120–123). PREP inhibition was also found to promote neuronal survival and neurite outgrowth of cerebellar granule cells (124). However, a recent study in mice shows that the lack of PREP *in vivo* causes a reduction of synaptic spine density in the hippocampal region along with reduced long-term potentiation and memory functions (125).

Many of PREP's functions are mediated through its interactions with other proteins. PREP is known to interact with GAP-43 (126, 127), α -tubulin (96), and GADPH (128). Its most studied interaction is with α -synuclein (126), reviewed in Ref. (129). PREP and α -synuclein have been shown to co-localize in cell models of stress and in the substantia nigra of post-mortem Parkinson's disease brain (11, 130). *In vitro*, the aggregation rate of α -synuclein increases in the presence of high concentrations of PREP, which is abolished through active site inhibitors of PREP and absent with a catalytically impaired PREP mutant (131). *In vivo*, PREP inhibition reduces α -synuclein aggregates in a cellular and animal model for Parkinson's disease (11).

The DPP Family in the Immune System

The DPP Family in the Innate Immune System DPP Family Members in Monocytes and Macrophages

The role of DPPIV in monocytes and macrophages has been somewhat contested. Whereas DPPIV's presence on monocytes and macrophages has been shown repeatedly in mice and rats (132–134), its expression in human monocytes and macrophages is less obvious. **Figure 2** shows an overview of the expression of DPPIV throughout the immune system. In visceral obesity, DPPIV expression is low on peripheral blood monocytes, macrophages, and dendritic cells, but it is upregulated *in vitro*



after differentiation and activation of isolated monocytes into macrophages or dendritic cells, and *in vivo* locally in adipose tissue (135). Interestingly, the authors showed that macrophage- or dendritic cell-associated DPPIV most likely binds ADA, promoting local degradation of adenosine, a T-cell proliferation suppressor, thereby inducing T-cell proliferation (135). Three other studies also found no to low DPPIV expression or activity associated with human monocytes and/or macrophages (82, 136–138). Others have investigated DPPIV in monocyte- or macrophage-like cell lines (136, 137, 139–144). In HL-60 cells, its expression has been found to be regulated by differentiation into macrophage-like cells (139). DPPIV inhibitor alogliptin can affect ERK activation, MMP1 and IL-6 secretion in U937 cells (140, 141). However, these studies employed alogliptin at concentrations lower than its IC_{50} for DPPIV. It is therefore questionable whether the observed effects were mediated by DPPIV at all. On the other hand, proliferation is reduced in the presence of a DPP inhibitor in U937 cells expressing high levels of DPPIV, but not in the same cell type expressing low levels of DPPIV (144). Moreover, the same inhibitor causes the former cells to secrete lower amounts of IL-1 β , but higher amounts of TNF α (144). It could be that inhibition merely increases TNF α 's half-life, as DPPIV has been implicated in its degradation in U937 cells (137). In THP-1 cells, DPPIV inhibitors alogliptin and sitagliptin both reduced these cells' chemotactic potential (142). DPPIV inhibitors sitagliptin and NVPDPP728 also reduced NLRP3, TLR4, and IL-1 β expression and increased GLP-1R expression in THP-1 cells and this effect was blocked through PMA differentiation (143). Importantly, such cell lines have been derived from different types of myeloid leukemia, and as it is known that DPPIV

expression is often dysregulated in cancer (47–51), the physiological relevance of these findings remains uncertain. FAP has been shown on tumor-associated macrophages in human breast cancer (145).

Dipeptidyl peptidase 8/9 activity has been found in human monocytes and U937 cells (136). DPP8 was found associated with activated microglia/macrophages in a rat model of cerebral ischemia (146). DPP8 and 9 are abundantly present in macrophage-rich regions of atherosclerotic plaques (82). Interestingly, DPP9 is upregulated after *in vitro* monocyte-to-macrophage differentiation. Moreover, inhibition or RNA silencing of DPP9 attenuates pro-inflammatory M1, but not M2, macrophage activation (82).

In rats, DPPII is expressed in tissue-resident macrophages (147, 148). Humans show DPPII activity in monocytes as well as U937 cells (25, 136). Human blood derived alveolar macrophages show high-PRCP activity (138, 149). Interestingly, in a mouse *in vivo* angiogenesis assay, macrophage infiltration into the wound was increased in mice with a PRCP deletion (150).

Prolyl oligopeptidase activity has been shown in mouse and rat peritoneal macrophages and in rat pulmonary macrophages (134, 151, 152). Its activity in mouse peritoneal macrophages is increased after thioglycollate elicitation (134). In addition, PREP has been identified as a neurotoxic component in the supernatant of activated THP-1 cells, which are monocyte-like cells (153). Apparently, these cells secrete PREP upon activation with IFN γ and LPS and partly because of this, their supernatant is toxic to neuroblastoma SH-SY5Y cells, as shown through the use of PREP-specific inhibitors (153). PREP's mode of action in this remains unclear.

DPP Family Members in Granulocytes

Recently, a study showed that DPPIV acts as a chemorepellent for human and murine neutrophils (154). Adding recombinant DPPIV to purified human neutrophils in an Insall chamber causes the neutrophils to migrate away from the higher concentration of DPPIV. This effect is blocked by DPPIV inhibitors, meaning that the effect is mediated through DPPIV's enzymatic activity, although a candidate substrate is not obvious. Moreover, in a mouse model of acute respiratory distress syndrome, oropharyngeal aspiration of DPPIV prevented accumulation of neutrophils in the lung (154). By contrast, PREP is involved in the generation of prolyl-glycyl-proline, a collagen fragment that is an efficient neutrophil chemoattractant (155). Human peripheral blood neutrophils contain PREP activity and are themselves capable of generating prolyl-glycyl-proline after LPS-activation, alluding to a self-sustaining pathway of neutrophil inflammation (116). PRCP is also abundantly expressed in human neutrophils (90).

The recruitment of eosinophils is affected by DPPIV activity. CCL11, also known as eotaxin, is a DPPIV substrate and cleavage by DPPIV prevents the activation of its receptor CCR3 (156). In rats, it was shown that administration of CCL11 results in eosinophil recruitment and this recruitment is significantly more effective in DPPIV-deficient F344 mutants (156).

Finally, DPPII activity has been reported in the granules of mast cells in several publications (147, 148, 157). It is released from peritoneal mast cells upon degranulation and is apparently inhibited by histamine and Zn^{2+} at concentrations present in the granules of mast cells (157).

DPP Family Members in Natural Killer Cells

Dipeptidyl peptidase 4 is present in low amounts on freshly isolated human NK cells and its expression is only upregulated in a small subpopulation after IL-2 stimulation (158). In that study, it was also shown that DPPIV inhibition suppresses DNA synthesis and cell cycle progression of NK cells, but these effects may be DPP8/9 mediated as the inhibitors used in that study are now known to also inhibit DPP8/9 activity (159). Another study shows that DPPIV is actually only expressed by a small subpopulation of peripheral NK cells (160). The natural cytotoxicity of NK cells is not influenced by the presence or absence of DPPIV on their cell surface (158, 160). However, DPPIV-negative NK cells show significantly less CD16-dependent lysis than DPPIV-positive NK cells (160). Interestingly, NK cytolytic function against tumor cells was diminished in DPPIV-deficient rats in a model for lung metastasis (161).

Figure 3 shows an overview of published data on the DPP family in the innate immune system.

The DPP Family in the Adaptive Immune Response

DPP Family Members in Humoral Immunity

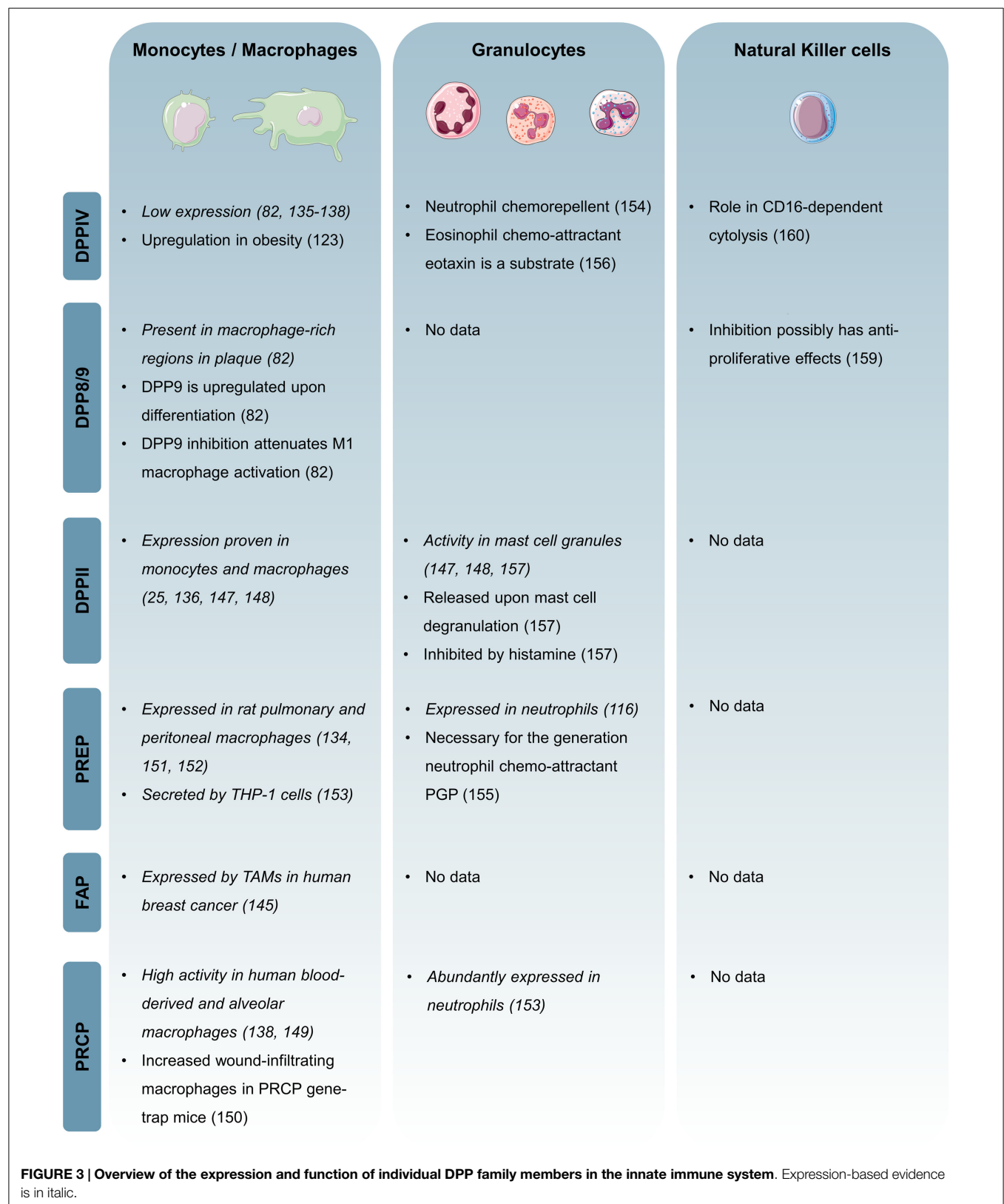
Only about 5% of freshly isolated CD20-positive B cells express DPPIV, but this fraction grows significantly upon pokeweed mitogen (PWM) or *S. aureus* protein stimulation (162). Similar to NK cells, DPPIV inhibitors significantly suppress DNA synthesis in B-lymphocytes (162), but again these inhibitors are now known to also inhibit DPP8 and 9 (159). Mouse spleen-derived

B-lymphocytes only express low amounts of DPPIV mRNA (163). DPP8 and 9 mRNA, on the other hand, are expressed at much greater levels in these cells, and they are upregulated in Raji cells, a B-lymphocyte-like cell line, after PWM, LPS stimulation or mitomycin c treatment, and downregulated after DTT treatment (163). DPP8 and 9 have also been shown immunohistochemically in human lymph follicular lymphocytes (164). DPPII activity has also been shown in human B-lymphocytes (25).

DPP Family Members in Cell-Mediated Immunity

Dipeptidyl peptidase IV was originally described as a surface marker for T-lymphocytes, in which case it is better known as CD26, and later more specifically for a subset of CD4-positive memory cells, $CD4^+ CD45RO^+ CD29^+$ cells, which respond maximally to recall antigen tetanus toxoid and induce B-cell IgG synthesis (165, 166). Indeed, CD26 surface expression is augmented along with the antigen sensitivity of a particular $CD4^+$ T-cell clone (167). $CD26^{high} CD8^+$ T-cells belong to the early effector memory T-cell subset (168). CD26 is also a marker for T-cell activation (165, 169–171). CD26 expression on $CD4^+$ T-cells correlates with T_H1 responses. Stimuli that typically induce a T_H1 phenotype tend to induce CD26 expression (172). Additionally, the $CD4^+$ T cells capable of transendothelial migration *in vitro* are characterized by a bright expression of CD26 (173, 174), but CD26 does not seem to be actually involved in T-cell adhesion to endothelial cells or fibroblasts (175). Recently, it was shown that up to 98% of all T_H17 cells show very high CD26 expression, with mean fluorescent intensity on these cells almost twice as high as on T_H1 or T_H2 cells. Therefore, the authors of this study suggest CD26 as a marker for T_H17 cells (176). Conversely, CD26 has been proposed as a negative marker for the selection T_{reg} cells due to its very low-surface expression on these cells (176–178).

CD26 is also a costimulatory molecule for T-cell activation. Crosslinking of CD26, along with CD3, stimulates T-cell activation and proliferation (168, 179, 180). CD26 can also directly activate T-cells in an alternative activation pathway, but this requires the presence of the TCR/CD3 complex (181–183). During costimulation, CD26 is mannose-6 phosphorylated and internalized, the latter of which is mediated in part by its interaction with M6P/IGFIR (184). It then localizes to lipid rafts where it might interact with CD45, required for TCR signaling, facilitating colocalization of this molecule with TCR signaling molecules (185, 186). A number of candidate binding partners for costimulation have been proposed. ADA and CD26 are known binding partners (187). Even though ADA binding to CD26 does not seem to be essential for immune functions in humans (188), the nanomolar affinity of this interaction probably reflects its importance (189). Indeed, association with free ADA or ADA presented by ADA-anchoring proteins on dendritic cells seems to costimulate T-cells through CD26 binding (190, 191). On the other hand, it has been shown that soluble DPPIV enhances T-cell proliferation independent of its enzyme activity or ADA-binding capability (192). Interestingly, the ADA-CD26 interaction can be inhibited by HIV-1 external envelope protein gp120 and this requires interaction of gp120 with CXCR4 (189). In fact, evidence suggests a physical association between CXCR4 and CD26



on peripheral blood lymphocytes (193). Fibronectin is another known binding partner of CD26 involved in T-cell costimulation (194–196). Finally, CD26 interacts with caveolin-1 on monocytes.

This interaction causes an upregulation of CD86 on these cells, which potentiates antigen-specific T-cell activation (197). Most studies seem to find no need for DPPIV's enzymatic activity for

successful costimulation, as evidenced through the use of inhibitors and catalytically impaired DPPIV mutants (198–201).

Dipeptidyl peptidase 8 and 9 are present in baboon spleen interfollicular T-lymphocytes and Jurkat T cells (164). They are upregulated in the latter after PWM and LPS, but not PHA, stimulation (163, 202, 203). Activation of PWM-stimulated T-cells is suppressed after DPPIV/8/9 inhibition. Moreover, DNA synthesis and T-cell proliferation are reduced, as well as production of IL-2, -10, -12, and IFN- γ . This is due to an induction of TGF- β secretion (159, 204–207). Inhibition also upregulates CTLA-4 and down-regulates DPPIV expression (206, 208). These observations might be physiologically relevant as endogenous inhibitors of DPPs are known which have similar effects in cell-based experiments as the synthetic inhibitors (209, 210).

Dipeptidyl peptidase II activity is higher in T-lymphocytes than in B-lymphocytes (25) and absence of DPPII steers T-lymphocytes toward a T_H17 phenotype. T-lymphocytes of DPPII KO mice

hyperproliferate and secrete IL-17 after CD3 crosslinking or after *in vivo* priming and *in vitro* antigen-specific restimulation (211). PREP activity has also been shown in mouse T-lymphocytes (212). Its activity is significantly higher in immature, double-positive thymocytes compared to mature, single-positive thymocytes, or peripheral T-cells. T-cells stimulated with Con A followed by IL-2 show a time-dependent increase in PREP activity and pre-treatment of cells with a PREP inhibitor renders them resistant to activation-induced cell death (212).

Figure 4 shows an overview of *in vitro* data on DPP involvement in primary human T cell activation.

The DPP Family in Inflammatory Disease

The DPP family has been reported to be dysregulated or even involved in a number of inflammatory disorders. Expression levels of a number of family members are modulated in rheumatoid

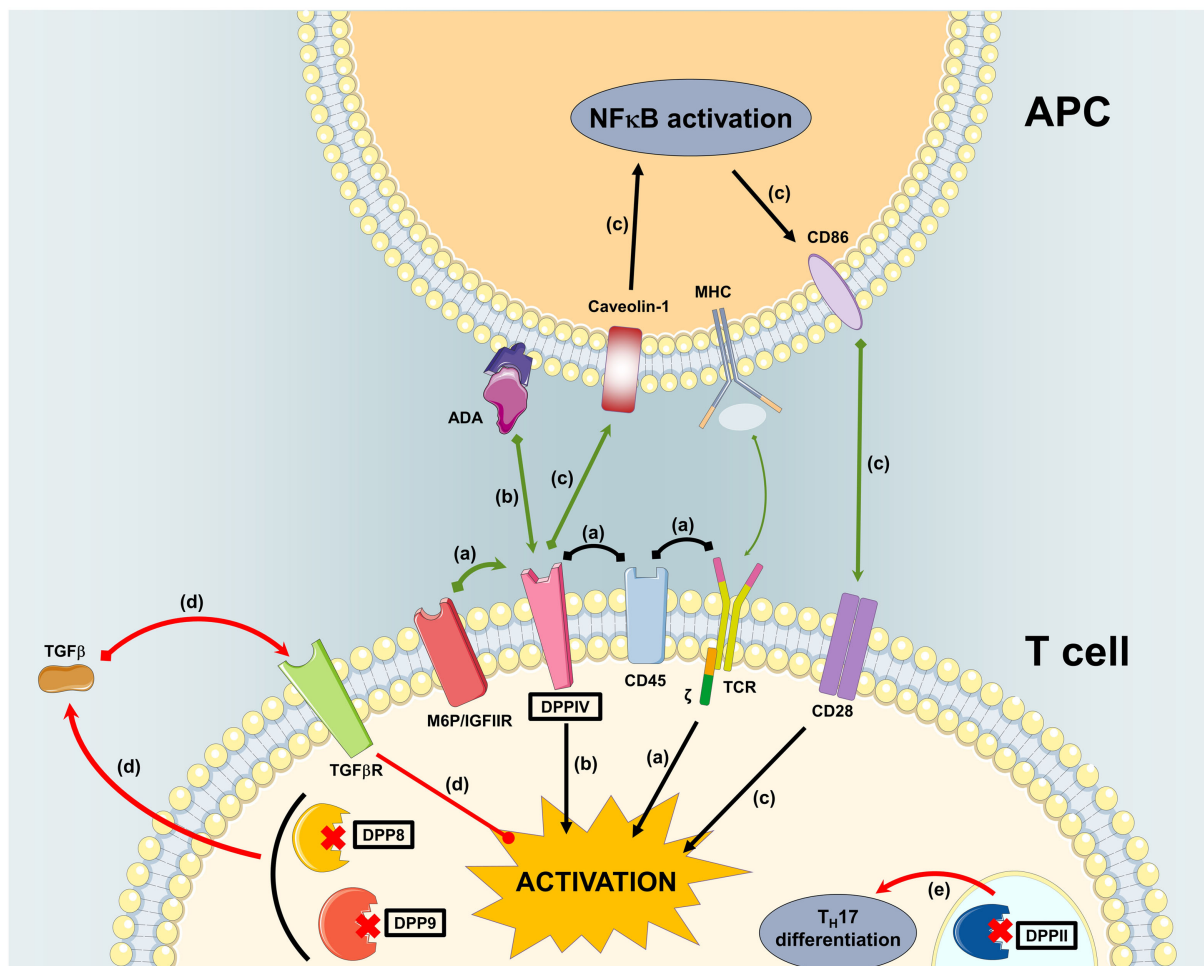


FIGURE 4 | Overview of *in vitro* data on DPP involvement in primary human T cell activation. (A) M6P/IGFIR associates with mannose-6-phosphorylated DPPIV causing it to associate with CD45 in lipid rafts. This facilitates co-localization with the TCR signaling molecules for T cell costimulation. **(B)** Interaction of ADA presented by ADA-anchoring proteins on dendritic cells with DPPIV on T cells

causes costimulation. **(C)** Interaction of DPPIV on T cells with caveolin-1 on monocytes induces the expression of CD86 on the latter. Interaction of CD86 with CD28 costimulates T cells. **(D)** Inhibition of DPP8/9 induces TGF β in PWM-stimulated T cells. TGF β attenuates T cell activation. **(E)** Inhibition or absence of DPPII steers T cells toward T_H17 differentiation.

arthritis. Whereas the density of CD26 on peripheral T cells is increased in patients, it is low on synovial fluid T cells (213–215). DPPIV activity in plasma, serum, or synovial fluid of patients has also been found to be decreased, similar to results in several rat models of arthritis (216–222). Interestingly, rats resistant to induction of arthritis show higher plasma DPPIV levels (222). By contrast, DPPII and PREP activity are increased in serum or synovial fluid of arthritis patients (219–221). Likewise, FAP immunoreactivity is much higher in fibroblast-like synoviocytes of rheumatoid arthritis patients compared to osteoarthritis controls (223). DPPIV's involvement in rheumatoid arthritis has been studied, but remains unclear. On the one hand, inhibition can suppress development of arthritis in rats (224). Note, however, that effects mediated through other DPPs are hard to exclude as these inhibitors were developed before DPP8 and DPP9 were discovered. On the other hand, induced arthritis is more severe in DPPIV-deficient mice (216). This may be due to increased levels of circulating CXCL12 (216), a DPPIV substrate shown to be involved in rheumatoid arthritis. Several case reports in patients seem to suggest a link between the development of rheumatoid arthritis and the use of DPPIV inhibitors (225–227). PRCP has also been associated with rheumatoid arthritis as its activity was shown in synovial fluid isolated from arthritic joints (149).

Inflammatory bowel disease shows a distinct expression pattern of the DPP family. DPPIV serum or plasma activity seems to be lower in patients, whereas there is an increase of circulating CD26⁺CD25⁺ cells with a higher CD26 surface expression (228, 229). FAP is heavily expressed by myofibroblasts in the submucosa strictures in Crohn's disease, and is upregulated after stimulation with TNF α or TGF β (230). In a mouse model, colonic DPPII and DPP8 mRNA and DPPII activity are increased, while colonic DPP8/9 activity only increases significantly in mice that are also DPPIV knockouts (231). In mouse models, inhibition or abrogation of DPPIV seems to at least partially ameliorate symptoms, possibly by increasing circulating GLP-2, impairing neutrophil recruitment, and maintaining T_{reg} populations (231–236). Some of those beneficial effects may be mediated in part by the other DPPs, as additive effects were found for DPPIV KO and the DPP inhibitors (231, 234, 237). A recent study suggests that the ameliorative effects of DPP inhibitors are most likely not mediated through GLP-2 protection (238).

The DPP family has also been studied in neuroinflammation. Ischemia-induced neuroinflammation in rats prompts a distinct expression and activity pattern of the DPPs. In the days following ischemia, the brain of these rats undergoes a complex reorganization of DPP expression with changes in mRNA, protein, and activity levels of DPPII, 4, 8, and 9 in cortical neurons, microglia, and macrophages (146). Similarly, PREP seems to be associated with astrocytes and microglia in lesioned inflamed brains of rats (239). DPPIV and PREP also may be involved in multiple sclerosis. CD26⁺ T cells were found to correlate with disease scores (240). Soluble DPPIV levels are elevated in cerebrospinal fluid of patients (241). Plasma PREP activity, on the other hand, is lower in patients with relapsing–remitting or primary progressive multiple sclerosis and in clinically isolated syndrome (118, 119). Interestingly, PREP inhibition seems to aggravate symptoms in a mouse model of multiple sclerosis (118).

In systemic lupus erythematosus, DPPs also seem to be dysregulated. In mouse models, DPPII and PREP activities are increased in plasma, spleen, kidney, and liver, whereas DPPIV activity is decreased (221, 242). Human patients also show elevated DPPII and reduced DPPIV activities in serum, along with reduced numbers of CD26⁺ T cells (221, 243). Interestingly, serum DPPIV levels are inversely correlated with disease score (243). FAP immunoreactivity is decreased in the synovium of lupus patients (244).

Finally, DPPIV has been studied in psoriasis, an immune-mediated chronic inflammatory disorder with primary involvement of skin and joints. Its mRNA, protein levels and activity are higher in psoriatic skin samples (245, 246). By contrast, serum DPPIV levels and activity seem to be lower in patients (247, 248), accompanied by a reduction of peripheral CD8⁺CD26⁺ T cells (249, 250). Two case reports suggest a link between the use of DPPIV inhibitor sitagliptin and psoriasis. While one woman developed a psoriaform eruption 6 days after starting sitagliptin treatment (251), another patient's psoriatic lesions gradually diminished and were effectively gone 3 months after the start of sitagliptin treatment (252).

The DPP Family in Atherosclerosis

Dipeptidyl peptidase IV has recently received much attention for its potential as a therapeutic target for the treatment of atherosclerosis (Box 3) (253). This is not surprising considering the current use of DPPIV inhibitors in the treatment of T2D and the fact that T2D is associated with a higher risk for atherosclerosis (28, 254). In the ApoE^{−/−} mouse model of atherosclerosis, DPPIV inhibition generally reduces plaque area and monocyte and macrophage plaque infiltration (255–257). A reduction in the number of plaque lesions or in smooth muscle cell content have also been observed (255, 256), as well as lower plaque MMP9 and higher plaque collagen levels, suggesting increased plaque stability (258). One study reported effects of DPPIV inhibition on atherosclerotic plaques of only diabetic ApoE^{−/−} mice (141), but more recently, Terasaki et al. found similar effects in non-diabetic and diabetic ApoE^{−/−} mice (259). Likely, such differences can be explained by the fact that different DPPIV inhibitors were employed. Effects of DPPIV on atherogenesis similar to those observed in ApoE^{−/−} mice have been reproduced in LDLR^{−/−} mice (142, 260). In human atherosclerotic plaques,

BOX 3 | Atherosclerosis.

Atherosclerosis is the most common underlying cause of cardiovascular diseases and should be regarded as an inflammatory disease. It starts with dysfunction of the endothelium leading to the expression of leukocyte adhesion molecules, such as selectins and integrins. Locally produced pro-inflammatory cytokines attract the immune cells into the inner layer of the endothelium. However, not only leukocytes are found in the plaque but also low-density lipoprotein particles (LDL) and their oxidized counterparts (oxLDL). In the plaque, monocytes differentiate into macrophages, phagocytose the oxLDL and turn into so-called pro-atherogenic foam cells. This process leads to a self-sustaining, local inflammation leading to plaque growth, and migration of smooth muscle cells into the core. A plaque is defined as stable as long as it is contained by a thick fibrous cap. However, the latter is slowly degraded by the proteolytic enzymes from the leukocytes. This eventually leads to rupture and the formation of arterial thrombi (264, 265).

DPPIV immunoreactivity could only be found on endothelium of neovessels (82). It was recently found that DPPIV activity may be a predictor for the onset of atherosclerosis in otherwise healthy Chinese individuals (261). Another prospective study investigated the influence of vildagliptin or sitagliptin treatment on intima-media

thickness, a surrogate marker for atherosclerosis. This study found that treatment with vildagliptin or sitagliptin reduced intima-media thickness, suggesting that DPPIV inhibition might be beneficial in atherosclerosis in humans as well (262). Moreover, treatment naïve T2D patients treated with alogliptin

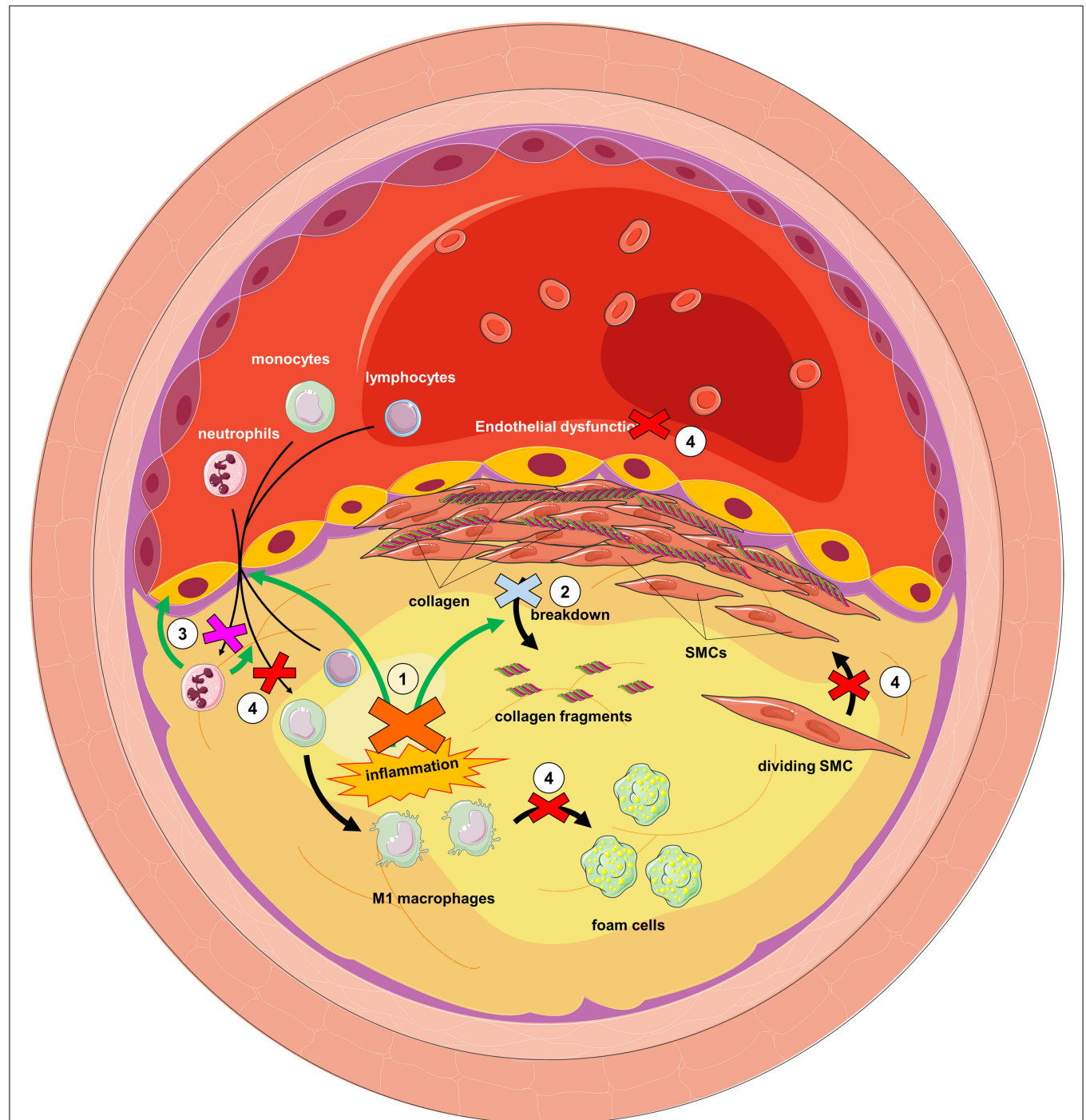


FIGURE 5 | Dipeptidyl peptidase inhibition as a putative strategy

for the treatment of atherosclerosis. (1) DPP9 inhibition would attenuate M1 macrophage activation, reducing local inflammation. Reduction in $\text{TNF}\alpha$ would reduce FAP on smooth muscle cells (SMCs). This and FAP inhibition (2) would reduce collagen degradation and

therefore plaque instability. PREP inhibition (3) would reduce neutrophil infiltration and consequently endothelial dysfunction and further monocyte infiltration. DPPIV inhibition (4) would prevent SMC proliferation, foam cell formation, endothelial dysfunction, and monocyte infiltration.

for 3 months saw a significant decrease in their circulating atherogenic lipids (263).

It has been suggested that DPPIV inhibitors' anti-atherogenic effects are mainly mediated through decreased monocyte infiltration, as DPPIV inhibitors suppress monocyte activation and chemotaxis *in vitro* (142, 258). DPPIV inhibition also reduces *in vitro* foam cell formation in exudate peritoneal macrophages from ApoE^{-/-} mice (255). Moreover, soluble DPPIV stimulates *in vitro* proliferation of smooth muscle cells and this can be reduced through the addition of a DPPIV inhibitor (256, 260). Finally, active circulating GLP-1 levels are augmented and this improves endothelial dysfunction (259, 266). Probably, DPPIV inhibition improves atherosclerosis through a combination of all these mechanisms. Indeed, incretin antagonists only partially attenuate the anti-atherogenic effects of DPPIV inhibition, suggesting that other mechanisms beyond incretin preservation are in play (259). Interestingly, monocyte-endothelial cell adhesion is abrogated by an anti-SDF-1 α antibody *in vitro* (267). LDL seems to induce SDF-1 α expression and leads to smooth muscle cell proliferation and inhibition of cell apoptosis (267, 268). SDF-1 α is a DPPIV substrate, which loses its biological activity after cleavage (216). As DPPIV inhibition seems to improve atherosclerosis, whereas intact SDF-1 α appears to be deleterious, it could be argued that SDF-1 α cleavage by DPPIV does not play a major role in atherosclerosis.

Dipeptidyl peptidase 8 and 9 have been found to be abundantly present in the macrophage-rich regions of human atherosclerotic plaques and considering DPP9's role in macrophage activation, it might potentially be involved in atherogenesis (82). FAP expression is enhanced in some, but not all types of human atheromata. It is found on smooth muscle cells, and its expression correlates with macrophage burden, probably due to the fact that TNF α upregulates FAP in smooth muscle cells *in vitro*. As it is mainly associated with collagen-poor regions and can digest type I collagen and gelatin *in vitro*, FAP probably contributes to plaque instability (269).

Interestingly, many of the studies reviewed above show the potential of targeting DPP family members for the treatment of atherosclerosis (see Figure 5). FAP inhibition might reduce plaque instability by decreasing collagen breakdown; DPP9 inhibition is likely to attenuate M1 macrophage activation, reducing the local inflammatory cascade; DPPIV inhibition may decrease monocyte infiltration, foam cell formation, improve endothelial dysfunction, and reduce smooth muscle cell proliferation; and finally, PREP inhibition might reduce neutrophil infiltration, preventing endothelial dysfunction, and monocyte infiltration. All of this shows the possibilities of repositioning DPPIV inhibitors, currently being used to treat type 2 diabetes, as well as the potential of targeting other members of the DPP family.

Conclusion

Caution should be taken when interpreting results from literature data based on DPP inhibitors, especially from older studies. It is now known that, under the experimental conditions used, many of these inhibitors are not specific for one particular family member. The reported findings, however, remain interesting. This review has shown extensive involvement of members of the DPP family in the immune system. It is clear that these enzymes hold great potential as targets for the treatment of certain inflammatory disorders. Particularly, the possibility of targeting DPP family members for the prevention and treatment of atherosclerosis warrants further investigation.

Acknowledgments

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Dipeptidyl peptidase-4 regulation of SDF-1/CXCR4 axis: implications for cardiovascular disease

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Dipeptidyl peptidase-4 (DPP4) is a ubiquitously expressed protease that regulates diverse number of physiological functions. As a dipeptidase, it exerts its catalytic effects on proteins/peptides with proline, alanine, or serine in the penultimate (P1) amino acid residue from the amino terminus. The evidence to date supports an important effect of DPP4 in catalytic cleavage of incretin peptides and this perhaps represents the main mechanism by which DPP4 inhibition improves glycemic control. DPP4 also plays an important role in the degradation of multiple chemokines of which stromal cell-derived factor-1 (SDF-1, also known as CXCL12) is perhaps an increasingly recognized target, given its importance in processes, such as hematopoiesis, angiogenesis, and stem cell homing. In the current review, we will summarize the importance of DPP4-mediated enzymatic processing of cytokines/chemokines with an emphasis on SDF-1 and resultant implications for cardiovascular physiology and disease.

Keywords: SDF-1, CXCR4, dipeptidyl peptidase-4, cardiovascular, chemokine

Introduction

Dipeptidyl peptidase-4 (DPP4) is a type-II integral transmembrane glycoprotein that has recently gained attention owing to its role in the catalytic degradation of incretins and as a receptor for entry for the Middle Eastern respiratory syndrome (MERS) virus. DPP4 is best known for its catalytic function, whereby it proteolytically cleaves a number of peptide and protein substrates. It exhibits a strong preference for peptides with proline, serine, or alanine as the penultimate amino acid from the amino terminus. The N-terminus of glucagon insulotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) consist of Tyr-Ala and His-Ala, respectively, rendering them excellent substrates for DPP4. In addition to its role in modulation of incretin peptides, such as GLP-1 and GIP, DPP4 regulates immune responses via cleavage of many cytokines and chemokines, including stromal cell-derived factor-1 (SDF-1, also known as CXCL12), involved in immune function and physiological functions, such as angiogenesis. In this review, we will focus on the regulatory aspects of DPP4 on chemokines, such as SDF-1, and its potential implications in the pathogenesis and management of cardiovascular disease.

An Overview of DPP4 Biology

Dipeptidyl peptidase-4 also called cluster of differentiation-26 (CD26) or adenosine deaminase-binding protein (ADA-binding protein), is a member of S9b peptidases. The S9b family consists of structurally homologous proteolytic enzymes, including DPP2 (also called quiescent cell proline dipeptidase, QPP), DPP4, DPP8, DPP9, and fibroblast activation protein (FAP). DPP4 was first

identified as a new dipeptide naphthylamidase in 1966 (1) and subsequently found to be identical to T cell activation antigen CD26, ADA-binding protein, mouse thymocyte-activating molecule, and rat liver membrane glycoprotein gp110 (2). DPP4 consists of a short N-terminal intracellular domain (6 residues), a 22-residue-long transmembrane α -helix domain (23 amino acids), and a large C-terminal extracellular domain. The C-terminal extracellular domain is responsible for its catalytic activity and binding to a number of ligands, such as ADA and matrix proteins (3). The catalytic activity of DPP4 depends on its dimerization and glycosylation of specific residues (2). DPP4 can also assemble into tetramers on the cell surface, which may involve the linkage of dimers located on the surface of two different cells, enabling it to function as a cell–cell communication molecule. In addition to its membrane-bound form, DPP4 also circulates as a soluble form in the plasma, which lacks the cytoplasmic and transmembrane domain with preserved catalytic activity. Soluble DPP4 (sDPP4) is a homodimer with a molecular weight range of 210–290 kDa (4), but can form higher molecular weight assemblies migrating as 900-kDa complexes (5). Whether sDPP4 is cleaved from the membrane or is secreted is unclear. For instance, studies investigating viral liver infection suggested that sDPP4 is shed from membrane-bound DPP4 (6). sDPP4 has, however, also been detected in the lumen of secretory granules in pancreatic α cells and in the exocytic secretory lysosomes of natural killer cells (7, 8). sDPP4 is commonly elevated in many disorders, such as solid tumors, reactive airways disease, hepatitis C, type 2 diabetes, and obesity (6, 9, 10).

Tissue Distribution and Cell Specific Expression and Phenotype of *Dpp4*^{-/-} Mice

Dipeptidyl peptidase-4 is widely distributed throughout the body (Table 1), with particularly high expression on the apical surface of endothelial and differentiated epithelial cells. Bone marrow cells, brush border of the small intestine, proximal tubular cells, and glomerular cells in the kidney express high levels of DPP4 as well (11). DPP4 is present on endothelial cells and fibroblasts throughout the body. Among hematopoietic cells, DPP4 is expressed at the highest level on T cells with lower levels in monocytes and dendritic cells (12). DPP4 expression increases

as monocytes differentiate into antigen-presenting cells as well as during T cell activation (12, 13). DPP4 is expressed at high levels in kidney, spleen, lung, pancreas, and prostate (14). Mice lacking the gene encoding DPP4 are refractory to the development of obesity and hyperinsulinemia and demonstrate improved post-prandial glucose control (15, 16). Mice deleted for *Dpp4* are fertile and appear healthy. Only slight decrease of body weight in *Dpp4*^{-/-} mice was observed compared to wild types. They have normal fasting blood glucose level, but shows reduced glycemic excursion after an oral glucose challenge (16). Increased intact insulinotropic form of GLP-1 and circulating insulin were seen in *Dpp4*^{-/-} mice after oral glucose stimulation (16). Pair feeding and indirect calorimetry studies indicate that reduced food intake and increased energy expenditure accounted for the resistance to high fat diet-induced obesity in the *Dpp4*^{-/-} mice. Ablation/deletion of DPP4 is associated with improved metabolic control with improved insulin sensitivity, reduced pancreatic islet hypertrophy, and protection against streptozotocin-induced loss of β cell mass and hyperglycemia (15). Pharmacological inhibition of DPP4 enzymatic activity improves glucose tolerance in wild-type but not in *Dpp4*^{-/-} mice. Interestingly, DPP4 inhibitor's also improve glucose tolerance in *Glp1r*^{-/-} mice, indicating that DPP4 contributes to blood glucose regulation by controlling the activity of GLP-1 as well as additional substrates (16).

DPP4 in Cytokine Processing

Dipeptidyl peptidase-4 has been shown to be able to cleave a number of chemokines and cytokines, including SDF-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3), erythropoietin (Epo), regulated on activation normal T-cell expressed and presumably secreted (RANTES, also known as CCL5), macrophage-derived chemokine (MDC, also known as CCL22), eotaxin (also known as CCL11), monokine induced by IFN- γ (MIG, also known as CXCL9), IFN- γ -induced protein-10 (IP-10, also known as CXCL10), and interferon-inducible T-cell α chemoattractant (ITAC, also known as CXCL11) (Table 2). The regulation of cytokine levels through catalytic cleavage may influence their levels in tissue domains. The differential contribution of DPP4 in the regulation of each of these cytokines is obviously dependent on the levels of expression of DPP4, which may vary depending on the tissues, the cells predominantly expressing the cytokine of interest and the disease context. Additionally, circulating or cell free DPP4 may also contribute to catalytic activity. DPP4-mediated truncation of RANTES abolishes the chemotactic activity to monocytes but not to T cells (27). Eotaxin (CCL11) is an eosinophil chemotactic protein and has been shown to be involved in allergic responses. DPP4 truncation of eotaxin inactivates its chemotactic activity for eosinophils. DPP4-truncated eotaxin (3–74) shows impaired binding and signaling through CCR3. In addition, truncated eotaxin suppresses calcium signaling and chemotaxis of intact eotaxin (28). DPP4 inhibition by either genetic deletion or pharmacological inhibition, enhances eotaxin-induced mobilization of eosinophils into the blood and recruitment into the injury/injection site (29). MDC (CCL22) is a chemoattractant for monocytes, dendritic cells, NK cells, and chronically activated

TABLE 1 | Major distribution and potential function of DPP4.

Distribution	Potential function
Adipocyte	Serves as an adipokine mediating obesity-induced metabolic syndrome (9)
Adipose tissue macrophage and dendritic cells	Enhances T cell inflammation and obesity-induced insulin resistance (12)
T cells	Promotes T cell activation by providing co-stimulatory signaling (17, 18)
Endothelial cells	Regulates endothelial function and vascular tone (19, 20)
Epithelial cells	Expressed in the epithelial cells in the kidney, lung, and GI tract. Mediates MERS-CoV infection in the lung (21), kidney fibrosis (22), diabetic nephropathy (23), intestinal growth (24)
Hepatocytes	Involved in lipogenesis (25) and liver damage (26)

TABLE 2 | Cytokine substrates of DPP4.

Substrates	N terminal sequence	Consequence of cleavage
SDF-1 (34)	KPVSL ... ^a	Inactivation, truncated product antagonize intact protein
RANTES (27)	SPYSS ... ^a	Altered target cell specificity
Eotaxin (28)	GPASV ... ^a	Inactivation, truncated product antagonize intact protein
Erythropoietin (35)	APPRL ... ^a	Inactivation, truncated product antagonize intact protein
G-CSF (35)	TPLGP ... ^a	Inactivation, truncated product antagonize intact protein
GM-CSF (35)	APARS ... ^a	Inactivation, truncated product antagonize intact protein
IL-3 (35)	APMTQ ... ^a	Inactivation, truncated product antagonize intact protein
MDC (32)	GPYGA ... ^a	Altered target cell specificity
IP-10 (33)	VPLSR ... ^a	Inactivation, truncated product antagonize intact protein
MIG (33)	TPVVR ... ^a	Inactivation, truncated product antagonize intact protein
ITAC (33)	FPMFK ... ^a	Inactivation, truncated product antagonize intact protein

^aBold letters indicate dipeptides to be cleaved by DPP4.

T lymphocytes (30, 31). DPP4-truncated MDC displays preserved chemotactic activity toward monocytes, but less potency toward lymphocytes and dendritic cells (32). CXCR3 interacts with MIG (CXCL9), IP10 (CXCL10), and ITAC (CXCL11), all of which are targets of DPP4. Cleavage of those chemokines by DPP4 attenuates their chemotactic activity, with the cleaved products serving as endogenous antagonists for CXCR3 binding (33).

Dipeptidyl peptidase-4 is also involved in the inactivation of multiple colony-stimulating factors (CSFs) and thus regulates hematopoietic stem cells (HSCs) and hematopoietic progenitor cell (HPC) function. The proliferative action of GM-CSF, G-CSF, IL-3, and Erythropoietin (Epo) on HPCs is enhanced in DPP4 knockout mice or by pretreatment with a DPP4 inhibitor (35). Catalytic inhibition of DPP4 or DPP4 deficiency promotes the engraftment of HSCs and HPCs after bone marrow transplantation in mice (35). DPP4-truncated CSFs may suppress the activity of their respective full-length CSF via antagonism (35).

SDF-1 and CXCR4

Stromal cell-derived factor-1 is an 8-kDa peptide that is encoded by *Cxcl12* (36). It is a chemoattractant for T lymphocytes, bone marrow stem cells [such as HSC, endothelial progenitor cell (EPC), and mesenchymal stem cells (MSCs)], endogenous cardiac stem cells (CSCs), and adipose-derived regenerative cells (37–39). There are several isoforms of SDF-1 (SDF-1 α – ζ), resulting from alternative splicing of its mRNA (40). Among these isoforms, SDF-1 α is the best described. SDF-1 α is expressed in many tissues, including bone marrow, heart, liver, kidney, thymus, spleen, skeletal muscle, and brain (36, 40–43). In the cardiovascular system, SDF-1 α is expressed in stromal cells, endothelial cells, and cardiomyocytes (44, 45). SDF-1 is typically inactivated by

exopeptidases, such as DPP4, matrix metalloproteinase (MMP)-2, and -9 (34). Unlike cleavage of SDF-1 by DPP4 at position 2–3, MMPs cleave SDF-1 at position 4–5, leading to the loss of its binding activity to CXCR4 (46). The relative contribution of each of these peptidases in regulation of SDF-1 levels is unclear. CXCR4 is an alpha-chemokine receptor specific for SDF-1 and belongs to a family of G-protein-coupled receptors. CXCR4 is expressed on a range of progenitor cells (including hematopoietic, endothelial, and CSCs) and thus is important for cell migration and organ development during embryogenesis (39, 40, 47). Mice deficient for either CXCR4 or SDF-1 display abnormal B-lymphocyte, hepatic, and cardiac (ventricular septal defects) development, and die *in utero* (48–50). Loss-of-function CXCR4 mutations in humans also causes impaired neutrophil mobilization and B-cell lymphopenia (51). In addition to CXCR4, CXCR7 has also been suggested to be an important receptor for SDF-1 (52, 53). However, the relative contribution and interactions of CXCR4 and CXCR7 is not fully elucidated. The involvement of CXCR7 in cardiovascular disease, if any, is also not yet known (39). DPP4 may also play a more general role in regulating CSF activity and stem cell homing (35). It was previously believed that disruption of the interaction between CXCR4 receptor expressed by hematopoietic progenitors and SDF-1 expressed by bone marrow stromal cells is sufficient to detach anchored progenitors from their bone marrow niches, leading to their rapid mobilization to the peripheral blood. AMD3100 (also termed plerixafor) inhibits SDF-1-mediated migration *in vitro* by blocking the chemokine binding to its major receptor CXCR4 (54). AMD 3100 mobilizes immature progenitor cells from the bone marrow into the blood and has been approved for clinical mobilization in lymphoma and multiple myeloma patients undergoing autologous transplantation. When combined with G-CSF, AMD3100 synergistically augments mobilization of progenitor cells, with increased *in vitro* migration to SDF-1 gradients and facilitates repopulation of transplanted non-obese diabetic/severe combined immunodeficient mice (55). AMD 3100 has recently been shown to directly induce SDF-1 release from CXCR4⁺ human bone marrow osteoblasts and endothelial cells, with SDF-1 release from these cells into the circulation, representing a pivotal mechanism essential for steady-state egress and rapid mobilization of HPCs (56).

DPP4 and SDF-1/CXCR4 Axis in Cardiovascular Disease

SDF-1/CXCR4 and DPP4 Inhibition in Stem Cell Homing and Engraftment

The SDF-1/CXCR4 axis has been shown to be critical in tissue repair in multiple organ systems, including the eye, heart, kidney, liver, brain, and skin. Specific to the heart, the SDF-1/CXCR4 axis has been shown to be essential for cardiogenesis (57, 58). SDF-1 is now well known as a key regulator of stem cell migration to sites of tissue injury (44, 59). SDF-1 was first identified by Askari et al. as a key regulator of stem cell migration to ischemic cardiac tissue (44). CD34⁺ stem cells express the SDF-1 receptor CXCR4 at high levels (37, 60). During myocardial infarction, SDF-1 levels are elevated 1 h after infarction and return to baseline at day 7 and further reduced to a low level thereafter (44). Overexpression

of SDF-1 in ischemic cardiomyopathy by either engineered cell-based or plasmid-based approach improved cardiac function in rats via enhancing stem cell homing and promoting revascularization of the infarct area (61, 62). Therefore, the ability to express SDF-1 locally is believed to enhance the vasculogenic potential of adult cardiac progenitor cells (63). However, the enhancement of endogenous stem cell-based repair appears to be blunted due to the short half-life of SDF-1 at the time of acute myocardial infarction owing to its degradation by proteases (44). As a major enzyme mediating the degradation of SDF-1, DPP4 may represent a potential target for improving stem cell homing with stem cell-based therapy. Preservation of SDF-1 by DPP4 inhibition has been shown to promote stem cell repopulation and homing to ischemic tissues. DPP4 inhibitors diprotin A or Val-Pyr, enhance chemotaxis of HSCs and HPCs and greatly increase homing and engrafting capacity of HSCs (64, 65). Pretreatment of HSC with DPP4 inhibitor diprotin A, enhanced their repopulation ability in lethally irradiated mice (66). Enhancement of engraftment of human CD34⁺ cord blood cells with DPP4 inhibition has also been observed in xenogeneic mouse recipients (NOD/SCID or NOD/SCID/beta 2^{null}) (67, 68). Pretreating either donor cells *in vitro* or recipients *in vivo* is able to enhance the engraftment of stem cells (66, 69). In a lung transplantation model, systemic DPP4 inhibition by vildagliptin increases SDF-1 levels in plasma, spleen, and lung, accompanied by a significant increase of stem cells in the lung grafts. DPP4 inhibitor-treated mice also shows less alveolar edema compared with untreated recipients (70). Liebler showed that DPP4 inhibition enhances SDF-1/CXCR4 axis and increased the retention of human bone marrow-derived cells in the injured lungs of immune deficient mice by 30% (71). In addition to SDF-1, DPP4 inhibition also enhances bone marrow engraftment by preserving G-CSF and GM-CSF. Both G-CSF and GM-CSF are substrates for DPP4, with inhibition of DPP4 promotes bone marrow engraftment not only through SDF-1 but also CSF-dependent mechanisms (35). G-CSF and GM-CSF in turn may also increase the expression of DPP4 on CD34⁺ cells, which results in their decreased responsiveness to SDF-1 (72).

SDF-1/CXCR4 and Regulation by DPP4 in Angiogenesis

Angiogenesis and vasculogenesis are an immensely complex process that requires the coordinated action of a multitude of cells, transcription factors, and cytokines working in concert in a precisely choreographed manner. It is widely believed that these processes can be recapitulated in the adult through the participation of a progenitor cell population of which EPCs are perhaps the best described and widely believed to be important building blocks for the assembly of functional vasculature in adults. While the origins of EPCs are still controversial, what is clear is that these cells have the capacity to differentiate into mature endothelial cells (73). Implantation of *ex vivo*-expanded EPC has been shown to improve neovascularization of injured tissues in animal models (74–76). SDF-1 plays a pivotal role in the trafficking and homing of EPCs to ischemic tissues (77–79). SDF-1 levels increase in plasma and ischemic tissue shortly after ischemic injury, in response to hypoxia which upregulates HIF-1 α (79). HIF-1 α upregulates SDF-1, by binding to the promoter of SDF-1 and initiating its

transcription (80). *Ex vivo* priming with SDF-1, enhances the proangiogenic potential of EPC as evidenced by improved blood flow recovery when transplanted into a nude mouse model of hind-limb ischemia (81). Disease states, such as diabetes associated with upregulation of DPP4, may represent prototypical conditions associated with defective homing and integration of EPCs owing to rapid degradation of SDF-1 (82). Kanki et al. reported that SDF-1 could be cleaved by DPP-4 in both plasma and ischemic heart tissue (34). Shih demonstrated an improvement in EPC number and endothelial nitric oxide synthetase (eNOS) expression after DPP4 inhibition by MK-0626 (83).

Therapeutic Applications of SDF-1 and DPP4 Inhibition-Mediated Prolongation of SDF-1 Effects in Cardiovascular Disease

Transient engineered cell-based or plasmid-based overexpression of SDF-1 in ischemic cardiomyopathy has been shown to improve cardiac function in animal models (62). In a study that compared the effects of SDF-1 overexpressed on MSCs alone or mesenchymal stem cells engineered to overexpress SDF-1 (MSC-SDF) on cardiac function in Lewis rats after acute myocardial infarction, tail vein infusion of MSC and MSC-SDF-1, 1 day after acute myocardial infarction, led to improved cardiac function by echocardiography by 70.7 and 238.8%, respectively, compared with saline controls. The beneficial effects of MSC-SDF transplantation were suggested to be mediated through preservation rather than regeneration of cardiac myocytes within the infarct area (84). Cardiac progenitor cell and CXCR4 expression on cardiac myocytes are required for further local trophic effects of MSC (85). The mechanism of action of SDF-1 overexpression in myocardial infarction and heart failure are likely multifactorial, including both systemic and direct trophic effects. An important effect of SDF-1 is its effect on the recruitment of CSCs to the infarct and infarct border zone (59). Delivery of MSCs engineered to overexpress SDF-1 at the time of acute myocardial infarction has been shown to lead to improvement in cardiac function (61). The myocardial repair initiated by endogenous stem cell appears blunted because of the natural short-term expression of SDF-1 at the time of acute myocardial infarction. In light of these effects in regulation of SDF-1, DPP4 inhibition has been suggested to be of potential benefit in cardiovascular diseases, such as myocardial infarction and peripheral arterial disease. In combination with G-CSF, DPP4 inhibition augments myocardial regeneration and improves cardiac function after myocardial infarction in mice (86, 87). In combination with CXCR4 overexpression, diprotin A treatment has shown to improve myocardial function and repair of infarcted myocardium (88). A bioengineered protease-resistant form of SDF-1 has shown greater potency in promoting blood flow recovery after hind-limb ischemia (89) and improving cardiac function as well as capillary density in the infarcted heart (34). Dual injection of G-CSF and sitagliptin resulted in the mobilization of progenitor cells and relieved the symptom of end-stage heart failure in a 19-month-old boy (90). Protease-resistant forms of SDF-1 display an enhanced potency in improving blood flow in experimental peripheral artery disease and myocardial infarction (34, 89). It has been shown that parathyroid hormone treatment after myocardial infarction improves

survival and myocardial function with potential involvement of enhanced homing of bone marrow-derived stem cells. Huber et al. demonstrated that parathyroid hormone serves as a DPP4 inhibitor and increases cardiac SDF-1 level, which in turn enhances CXCR4⁺ bone marrow-derived stem cell homing to ischemic heart and attenuates ischemic cardiomyopathy after infarction (91). Haverslag showed SDF-1 preservation by DPP4 inhibitor increases monocyte extravasation and thus accelerating perfusion recovery without detrimental side effects on plaque stability in atherosclerosis-prone *ApoE*^{-/-} mice (92). **Figure 1** depicts modulation of SDF-1 levels in the myocardium by DPP4 inhibition and enhancement of myocardial angiogenesis by DPP4 levels. In a porcine model of HF, delivery of a plasmid SDF-1 with an endomyocardial injection catheter demonstrated safety at doses up to 100 mg while improving cardiac function and vasculogenesis up to 90 days post-injection at doses of 7.5 and 30 mg (59). In a Phase I dose escalation study with 12 months follow-up in ischemic cardiomyopathy, 17 subjects in New York Heart Association class III heart failure, with an ejection fraction $\leq 40\%$ on stable medical therapy, were enrolled to receive 5, 15, or 30 mg of plasmid SDF-1 via endomyocardial injection. The primary end points for safety and efficacy were at 1 and 4 months, respectively. The primary safety end point was a major adverse cardiac event while efficacy end points were changes in quality of life, New York Heart Association (NYHA) class, 6-min walk distance, single photon emission computed tomography, N-terminal pro-brain natriuretic peptide, and echocardiography at 4 and 12 months. The primary safety end point was met. At 4 months, all of the cohorts demonstrated improvements in 6-min walk distance, quality of life, and NYHA class (93). Stromal cell-derived factor-1 plasmid treatment for patients with heart failure (STOP-HF) was a Phase II, double-blind, randomized, placebo-controlled trial

to evaluate safety and efficacy of a single treatment of plasmid SDF-1 delivered via endomyocardial injection to patients with ischemic heart failure. The primary endpoint was a composite of change in 6 min walking distance and Minnesota Living from Heart Failure Questionnaire from baseline to 4 months follow-up. The primary endpoint was not met ($P = 0.89$). For the patients treated with pSDF-1, there was a trend toward an improvement in left ventricular ejection fraction at 12 months (placebo vs. 15 vs. 30 mg Δ LVEF: -2 vs. -0.5 vs. 1.5% , $P = 0.20$). Patients in the first tertile of EF ($<26\%$) that received 30 mg of pSDF-1 demonstrated a 7% increase in EF compared with a 4% decrease in placebo (Δ LVEF = 11% , $P = 0.01$) at 12 months (94). Although the reasons for the overall failure are currently unclear, the differential benefit in those with advanced left ventricle dysfunction raises the possibility of differential mechanisms that would be operational in more advanced patients. These include the possible overexpression of CXCR4 in cardiac myocytes in the infarct border leading to a negative inotropic state (95). The transient overexpression of SDF-1 in ischemic cardiomyopathy has been shown to lead to long-term down-regulation of cardiac myocyte CXCR4 expression, re-recruiting the contractile function of the border zone (61). Patients with greater left ventricle dysfunction are likely to have a greater volume of myocardial tissue under stress; therefore, a greater demonstrable response to SDF-1 overexpression. Another important reason could be that the upregulation of DPP4 in the border zone of the infarct or around ischemic areas may have resulted in rapid degradation of SDF-1 limiting the efficacy of such an approach. Since both the Phase I and Phase II studies were performed in the absence of DPP4 inhibition, it could be speculated that the results may have been different if the trials had been performed either in the presence of a DPP4 inhibitor.

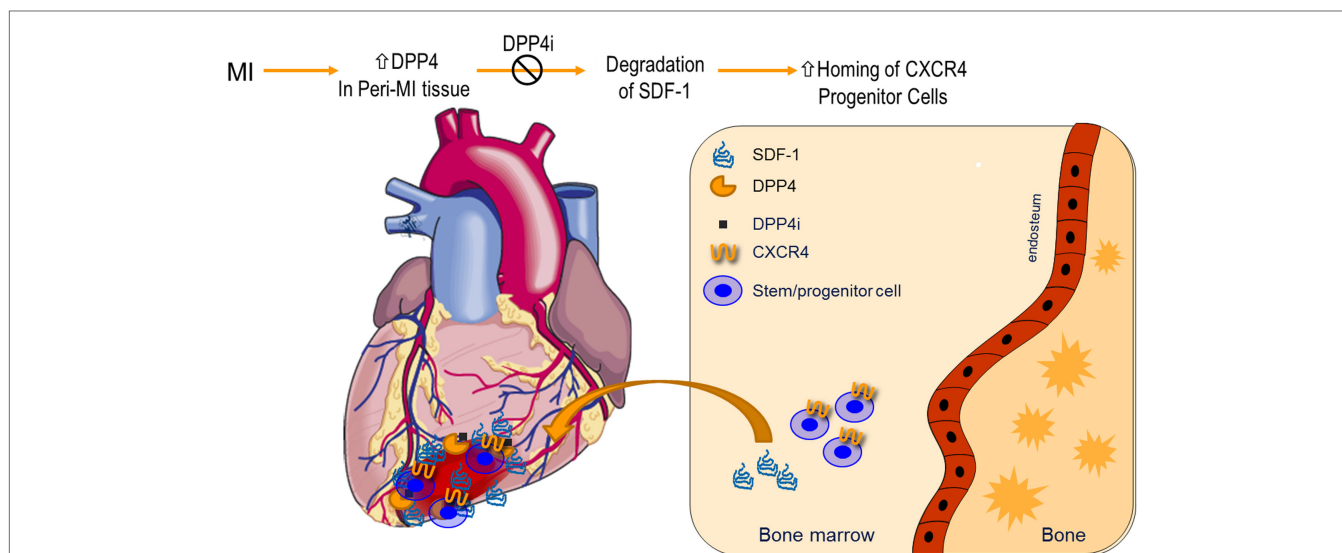


FIGURE 1 | Dipeptidyl peptidase-4 inhibition in modulation of SDF-1 and myocardial angiogenesis: expression of DPP4 increases in myocardial infarction. Suppression of DPP4 enzymatic activities by pharmacological inhibitors preserves SDF-1, which results in an enhanced homing of CXCR4⁺ progenitor cells from bone marrow to infarcted tissues. CXCR4, chemokine (C-X-C motif) receptor 4; DPP4i, DPP4 inhibitor; MI, myocardial infarction; SDF-1, stromal-derived factor-1.

Conclusion and Future Directions

Due to the importance of SDF-1/CXCR4 axis in the stem cell and progenitor cell survival and function, understanding this axis and molecules that modulate their production and action will be of utility for the treatment of cardiovascular disease. There are a number of clinically approved drugs, including DPP4 inhibitors and parathyroid hormone, which have the ability to enhance SDF-1/CXCR4 responsiveness and may improve the outcome of cardiovascular diseases. Several recent large scale clinical trials have indicated that unlike most other oral anti-diabetic drugs

that promote cardiovascular disease, DPP4 inhibitors are safe from cardiovascular standpoint despite lack of evidence showing beneficial effect (96–98). To what extent SDF-1/CXCR4 axis contributes to this effect requires further investigation.

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DPP4 in diabetes

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Dipeptidyl-peptidase 4 (DPP4) is a glycoprotein of 110 kDa, which is ubiquitously expressed on the surface of a variety of cells. This exopeptidase selectively cleaves N-terminal dipeptides from a variety of substrates, including cytokines, growth factors, neuropeptides, and the incretin hormones. Expression of DPP4 is substantially dysregulated in a variety of disease states including inflammation, cancer, obesity, and diabetes. Since the incretin hormones, glucagon-like peptide-1 and glucose-dependent insulintropic polypeptide (GIP), are major regulators of post-prandial insulin secretion, inhibition of DPP4 by the gliptin family of drugs has gained considerable interest for the therapy of type 2 diabetic patients. In this review, we summarize the current knowledge on the DPP4–incretin axis and evaluate most recent findings on DPP4 inhibitors. Furthermore, DPP4 as a type II transmembrane protein is also known to be cleaved from the cell membrane involving different metalloproteases in a cell-type-specific manner. Circulating, soluble DPP4 has been identified as a new adipokine, which exerts both para- and endocrine effects. Recently, a novel receptor for soluble DPP4 has been identified, and data are accumulating that the adipokine-related effects of DPP4 may play an important role in the pathogenesis of cardiovascular disease. Importantly, circulating DPP4 is augmented in obese and type 2 diabetic subjects, and it may represent a molecular link between obesity and vascular dysfunction. A critical evaluation of the impact of circulating DPP4 is presented, and the potential role of DPP4 inhibition at this level is also discussed.

Keywords: CD26/DPP4, soluble DPP4, type 2 diabetes mellitus, incretins, DPP4 inhibitors/gliptins, multifunctional enzyme

Introduction

Dipeptidyl-peptidase (DPP) 4, which is also known as CD26, is a ubiquitously expressed glycoprotein of 110 kDa, which was first characterized by Hopsu-Havu and Glenner (1). DPP4 is a type II transmembrane protein, which is also cleaved off the membrane and released into the circulation by a process called shedding (2, 3). The importance of DPP4 for the scientific and medical community raised substantially since the approval of DPP4 inhibitors for the treatment of type 2 diabetes mellitus (T2DM). These so-called gliptins increase the incretin levels and therefore prolong the post-prandial insulin action. Since soluble DPP4 is characterized as an adipokine (4) and also correlates with parameters of the metabolic syndrome (5), it might also be an important molecular biomarker. DPP4 is a multifunctional enzyme, which serves as a binding partner for numerous peptides, among which are adenosine deaminase (ADA) and extracellular matrix proteins (2, 6, 7). Moreover, as a serine protease, DPP4 cleaves numerous substrates, which further amplifies its complexity of action. Thus, DPP4 is involved in signaling processes, immune cell activation, and its dysregulated expression and release is associated with numerous diseases.

In the present review, we wanted to emphasize the complex function of DPP4 with special focus on its association to T2DM. Furthermore, we wanted to offer a different perspective of the current view of DPP4 beyond the inhibition of its protease activity (8–10). The first part of the present review is dealing with general information about DPP4 and its numerous biological functions in regard to T2DM and its treatment. The last section collects the current knowledge about how DPP4 with its pleiotropic functions, as described before, affects several organs, thereby playing a pivotal role in the development of T2DM and its comorbidities.

General Information on DPP4

Biology of DPP4

The following part will deal with the domain architecture and respective relevance of these domains for the functionality of DPP4.

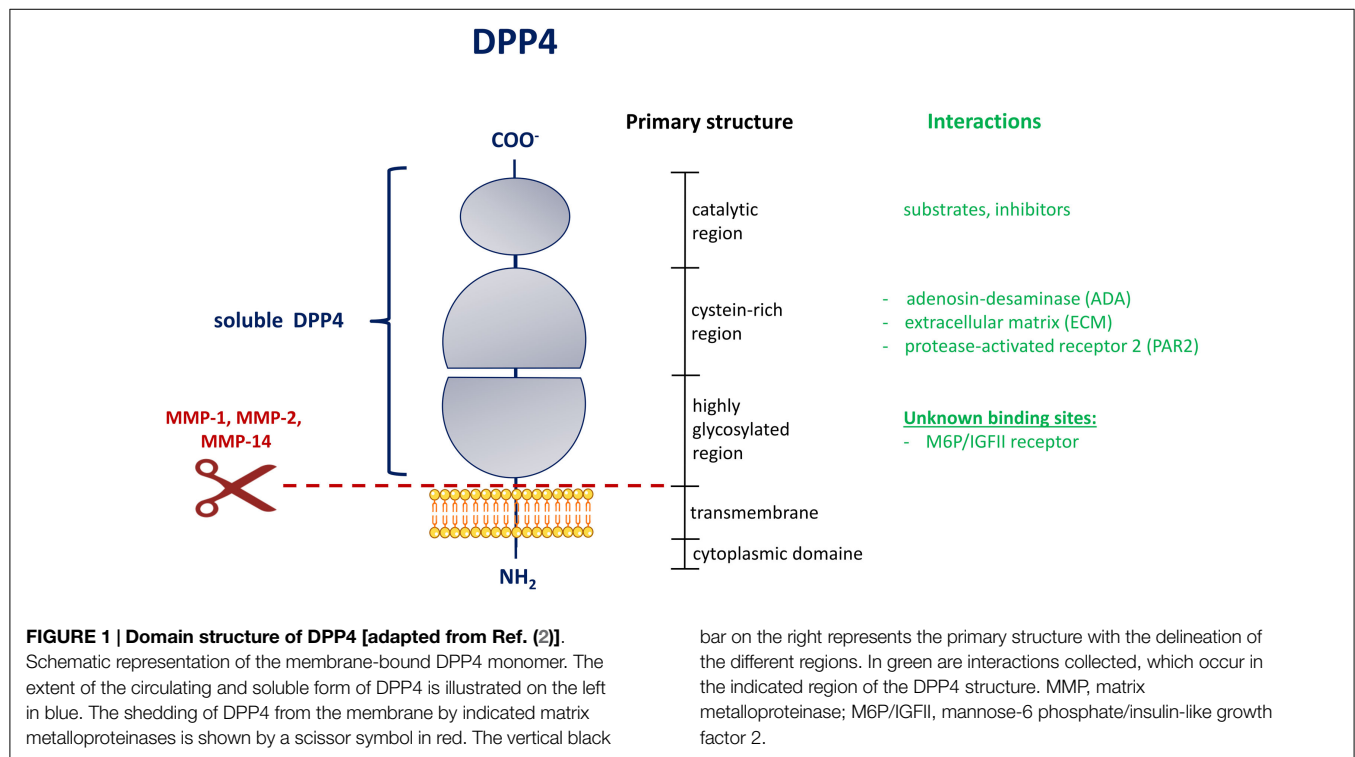
Dipeptidyl-peptidase 4 (EC3.4.14.5) is a type II transmembrane protein, which groups together with fibroblast-activation protein α (FAP), the resident cytoplasmic proteins, DPP8 and DPP9, and the non-enzymatic members, DPP6 and DPP10, to the serine peptidase subfamily S9B. All of these proteins share a typical α/β hydrolase fold (2, 6). The DPP4 protein consists mainly of 4 domains: a short cytoplasmic domain (1–6), a transmembrane domain (TMD) (7–28), a flexible stalk segment (29–39), and the extracellular domain (40–766), which can be further separated by a highly glycosylated region, the cysteine-rich region, and the catalytic region (Figure 1).

As a member of the type II transmembrane proteins, DPP4 contains a typical signal peptide, which is necessary for the targeting to the endoplasmic reticulum and the initiation of the translocation across the cell membrane. In contrast to the

classically secreted proteins, the signal peptide is not cleaved off, but serves as a membrane anchor. We were able to show that the circulating form of DPP4 (sDPP4), which lacks the cytoplasmic domain and the transmembrane region, is cleaved off the membrane of human adipocytes and smooth muscle cells in a process called shedding by the involvement of matrix metalloproteases (MMPs) (3).

Within the TMD, it could be shown that proline residues play an important role for the translocation of membrane-anchored proteins, such as DPP4. Chung and colleagues (11) studied single proline substitution throughout the TMD of DPP4. They were able to show that translocation and integration into the membrane are determined by the hydrophobicity, conformation, and also the location of proline within the TMD. Furthermore, the position of proline relative to other prolines and the location of highly hydrophobic residues within the TMD are important for correct translocation and membrane integration of DPP4.

In addition to the TMD, the glycosylation of DPP4 is also important for the correct trafficking of DPP4. Carbohydrates account for approximately 20% of the total molecular mass of DPP4 and cause heterogeneity of this protein depending on the location on different cell types. Two highly conserved glutamate residues (205 and 206) within the glycosylated region are essential for the activity of DPP4 (12). Interestingly, six of the nine N-glycosylation sites are located within the glycosylated region. These glycosylation sites are mostly conserved among species. They are necessary for folding, stability, and intracellular trafficking (13). Other modifications like sialylation and/or O-glycosylation have an impact on targeting DPP4 to the cell membrane. Sialylation of DPP4 increases significantly with age, and hypersialylation occurs in patients with HIV infection (14).



Not only glycosylation and residues within the TMD are important for the cellular function of DPP4 but also dimerization. DPP4 can be found as monomer, homodimer, or even as homotrimer on the cell surface of cells. DPP4 needs dimerization for enzymatic activity, and this is the predominant form of DPP4 (15). Dimerization occurs upon interaction with DPP4 itself or with other binding partners, e.g., FAP (16, 17), and occurs via interaction with the cysteine-rich region. Through its interaction with several proteins, DPP4 can act also in an enzymatic activity-independent way. Through this interaction, DPP4 is linked to various mechanisms like immune response and tumor invasion. The heterodimerization and interaction with different binding partners will be discussed in a later section.

The serine in the active site of DPP4 is located in the sequence Gly–Trp–Ser–Tyr–Gly and is part of the catalytic triad (Ser 630, Asp 708, His 740) within the catalytic region of DPP4. DPP4 is an exopeptidase, which cleaves dipeptides from the penultimate N-terminal position of its substrates and thereby either inactivates these peptides and/or generates new bioactive compounds (7). There are numerous different DPP4 substrates known to date and they will be addressed in a separate section within this review.

DPP4 Expression and Its Regulation

Dipeptidyl-peptidase 4 is ubiquitously expressed on numerous different cell types among which are epithelial cells, fibroblasts, and leukocyte subsets. Mechanisms that regulate DPP4 gene transcription and enzymatic activity are not fully understood so far and may be dependent on the studied cell type.

The human DPP4 gene is located on chromosome 2, spans 70 kb, and consists of 26 exons (2). The DPP4 promoter region contains consensus sites for different transcription factors like NFκB, SP-1, EGFR, and AP-1 factor NF-1 (18). At least in chronic B lymphocytic leukemia cells, it could be shown that there is a consensus interferon γ-activated sequence (GAS), which is a binding motif for STAT1. The interferons, α, β, and γ, stimulate STAT1α binding to this region and thus lead to an increased DPP4 expression and activity (19). Interleukin (IL) 12, which is a key factor in differentiation of naïve T-cells into the Th1 subtype, is also able to upregulate DPP4 expression. Therefore, DPP4 is important in immune cell activation (20, 21). Our group was able to show that release of soluble DPP4 is increased upon TNFα stimulation and insulin *in vitro* (4). However, IL-12 and TNFα also seem to play a regulatory role in translation and translocation of DPP4. In activated lymphocytes, IL-12 upregulates DPP4 translation whereas TNFα decreases cell surface expression, which might be due to elevated sDPP4 release (22). Also transcription factors, such as HIF-1α and HNFs, target DPP4 expression (23), which fits to the observation of our group that hypoxia induces DPP4 release in human smooth muscle cells, which might be mediated by MMPs (3).

Non-Enzymatic Interactions of DPP4

Through its cysteine-rich region, which is separated from the catalytic region, DPP4 is able to interact with different proteins, and further broadens its spectrum of activity and highlights its multifunctional role in different processes.

Binding Partners of Membrane-Bound DPP4

The best-studied interaction in this regard is certainly the binding of DPP4 and ADA. It was already identified in 1993 by Morrison and colleagues (24). Importantly, the interaction of DPP4 and ADA preserves the enzymatic function of both binding partners. It has been shown that residues 340–343 of DPP4 are essential for the interaction with ADA. Regulation of the DPP4/ADA interaction occurs, e.g., via tetramerization of DPP4 or glycosylation at Asn281, which interferes with ADA binding (25). Also, the HIV envelope glycoprotein, gp120, which interacts with DPP4 on lymphocytes via its C3 region, is able to inhibit the association with ADA (2). Upon ADA binding, activation of plasminogen-2 occurs, which raises plasmin levels. This leads to a degradation of matrix proteins and an activation of MMP, thereby indicating that the interaction of DPP4 and ADA might be involved in tissue remodeling (26).

Furthermore, ADA catalyzes the irreversible deamination of adenosine and 2'-deoxyadenosine and is therefore a crucial player in the cellular and humoral immunity. Via interaction with CD45, the complex of ADA and DPP4 enhances T-cell activation. Interestingly, DPP4 is also able to promote T-cell proliferation independent from ADA binding or even its enzymatic activity (27). Zhong et al. were able to show that the interaction of DPP4 and ADA on dendritic cells might potentiate inflammation in obesity upon activation and proliferation of T-cells, which could be competitively inhibited by exogenous sDPP4, but not by inhibiting DPP4 enzymatic function (28). Furthermore, ADA activity is elevated in T2DM patients and may serve as a marker of inflammation and obesity (29).

Beside its role in inflammation, adenosine is also an important player in glucose homeostasis. Already in 1988 it was shown that, by lowering endogenous adenosine levels, ADA contributes to a reduced insulin sensitivity of glucose transport stimulation (30). Additionally, adenosine seems to facilitate insulin action in adipocytes (31). Another study could show a correlation of increased ADA activity in T2DM with fasting plasma glucose, HbA1c, aspartate, and alanine aminotransferase (ALT). DPP4 inhibitors exert no additional effects on ADA activity despite glycemic control or HbA1c-dependent effects (32). All these studies emphasize that the effects of ADA/DPP4-interaction are independent of DPP4 enzymatic activity.

Another known interaction partner of DPP4 is Caveolin-1, which is present on antigen-presenting cells (APCs) and binds to residues 630 and 201–211 of DPP4 expressed on T-cells. Thereby, an upregulation of CD68 occurs and initiates a signaling cascade, which might be implicated in the pathogenesis of arthritis, and may be relevant for other inflammatory diseases as well (33). Intracellular signaling is also initiated by DPP4 via interaction with Caspase recruitment domain containing protein 11 (CARMA-1) (6).

Another well-known interaction of DPP4 is with extracellular matrix proteins like collagen and fibronectin (34, 35). The interaction of DPP4 with fibronectin was revealed via nitrocellulose binding assays in rat hepatocytes and seems to play a role in the interaction of these cells with the ECM and with matrix assembly (36). Interaction of DPP4 with FAPα leads to a local degradation of ECM and thus migration and invasion of endothelial cells (37).

Potential Receptors for sDPP4

Since DPP4 is shed from the membrane of cells with intact enzymatic and cysteine-rich region, it can also exert biological functions in a paracrine or endocrine manner. These functions might also involve intracellular signaling events in the targeted cells. Therefore, it would be of great importance to know receptors of sDPP4 to better understand the multiple role of sDPP4 on different cells and in different disease conditions where serum levels are elevated. However, there is not much known about DPP4 receptors so far.

Ikushima et al. were able to show that DPP4 needs to associate with mannose-6 phosphate/IGF-IIR to exhibit its function as T-cell activator. This is due to the fact that for this activation, internalization of DPP4 is necessary, but DPP4 lacks a signal for exocytosis. The binding with M6P/IGF-IIR occurs via M6P residues in the carbohydrate moiety of DPP4 and the complex is then internalized and able to exert its biological function (38).

Our group showed that at least in human vascular smooth muscle cells, protease-activated receptor 2 (PAR2) might be activated by sDPP4. We were able to show that sDPP4-mediated ERK activation and proliferation, as well as upregulation of inflammatory cytokines could be prevented by silencing of PAR2. The same was shown by use of a specific PAR2 antagonist. We propose that sDPP4 acts as an activator of PAR2, since a sequence within the cysteine-rich region of DPP4 is highly homologous to the auto-activating tethered ligand of PAR2 (39).

Genetic Alterations of DPP4 and Predisposition to T2DM-Associated Diseases

There are only few studies aiming to identify modifications in the DPP4 gene and their association with T2DM. Some of these are reviewed in the following section.

In 2009, Boucharde et al. analyzed single nucleotide polymorphisms (SNPs) in the DPP4 gene and searched for association with blood pressure, lipids, and diabetes-related phenotypes in obese individuals, to verify whether DPP4 gene polymorphisms could explain the individual risks of obese patients to develop metabolic complications. Three of the analyzed SNPs showed significant association with plasma total-cholesterol levels or plasma triglyceride level or total cholesterol level. But none of the polymorphisms or cardiovascular disease risk factors showed a significant correlation with DPP4 mRNA levels in omental adipose tissue. Therefore, the authors concluded that, at least in their studied group, DPP4 gene polymorphisms seem to be unrelated to the inter-individual risk of developing obesity-related metabolic complications (40).

In another study, visceral adipose tissue DNA of 92 severely obese, non-diabetic female patients was analyzed for methylation rate in the DPP4-promoter CpG island and compared between different DPP4 polymorphisms. These cytosine- and guanine-rich regions are prone to epigenetic modification like methylation, and thus inactivate or activate transcription of certain genes. Different methylation levels of the DPP4 gene were identified in three DPP4 SNPs. Interestingly, the methylation level was negatively associated with DPP4 mRNA abundance and positively with plasma total/HDL-cholesterol ratio. These observations suggest that plasma lipid profile is improved by a higher methylation

status of the investigated CpGs (41). Two years later, the same group analyzed DPP4 gene methylation levels between obese subjects with and without the metabolic syndrome in visceral adipose tissue. They observed no significant difference in the percentage of methylation levels of the CpGs within or near the second exon of the DPP4 gene between non-diabetic severely obese subjects with or without metabolic syndrome. However, they were able to show a correlation between plasma cholesterol levels and the percentage of methylation when the subjects were classified into quartiles (42). This further underpins a link between epigenetic modification of the DPP4 gene and plasma lipid metabolism.

Aghili et al. analyzed 875 patients with angiographically documented coronary artery disease (CAD), and divided them in two subgroups dependent of their myocardial infarction (MI) status. By a genome-wide association study, loci, which predispose to MI, were assessed and associated with SNPs in the DPP4 gene. They found that polymorphisms in the DPP4 gene increase the risk of MI and progression of atherosclerosis in terms of plaque stability in patients with already existing CAD. Especially, one SNP was identified in both dominant and additive inheritance modes, which associates with low plasma DPP4 levels and which may increase the risk of MI in CAD patients (43).

Dyslipidemia, which is characterized by excessive lipids in the blood, is a common feature of T2DM. The status of this risk factor is quantifiable by the measurement of apolipoprotein B (ApoB) in the blood. In a very recent study by Baileys and colleagues, they aimed to identify novel SNPs associated with ApoB level. Especially in South Asians, who tend to develop risk factors for T2DM and MI at younger ages and lower BMI, they found an association of a DPP4 SNP with ApoB level (44).

The DPP4 Deficiency in Animal Models

To date, there are several studies dealing with the question, which role DPP4 plays *in vivo*. Animal models are useful tools to study the involvement of DPP4 in different organs. Upon triggering different diseases like insulin resistance (IR) or MI, it is possible to understand the role of DPP4 in these comorbidities of T2DM.

DPP4 Deficiency in Rats

A major part of the literature is dealing rather with DPP4-KO in rats than in mice. Most research groups work with the F344/DuCrj (DPP4-deficient) strain. Rats developing IR due to high-fat diet (HFD) feeding showed improved HOMA-IR values and blood glucose levels in oral glucose tolerance test (oGTT) and more active glucagon-like peptide-1 (GLP-1) and insulin in plasma (45). The same improved glucose tolerance with increased GLP-1 and leptin levels was found in DPP4-depleted Dark Agouti rats with diet-induced obesity (46). Another research group also found improvement in serum lipid profile despite increased visceral fat. They also performed insulin tolerance tests (ITT) in addition to GTT and saw an increased phosphorylation of Akt and reduced expression of gluconeogenic genes, concluding that DPP4-KO improved insulin sensitivity. Furthermore, the KO rats showed increased adipocyte maturation by increased expression of genes involved in triglyceride uptake and in PPAR γ expression and increased adiponectin and leptin levels. In addition, adipose tissue is less inflamed illustrated by lower TNF α , IL-6, PAI1, and

CCL7 levels (47). The observed effects were attributed to elevated glucose-dependent insulinotropic polypeptide (GIP) levels in the KO rats. Furthermore, the same group could also show attenuated liver damage under HFD challenge in the KO rats due to improved bile secretory function. They postulate that the enhanced export of bile acids out of hepatocytes and a reduction of bile acid synthesis via inhibition of CYP7A1, which converts cholesterol to bile acids, were mediated by increased GLP-1 in DPP4-KO rats (48). Interestingly, at least Yasuda and colleagues also saw a significant reduced food intake in the KO rats irrespective of the diet (45), which might be due to changed receptor specificity of neuropeptide Y (NPY), which was shown to be more potent in KO rats to influence food intake and feeding motivation (49). Although several independent working groups saw increased NPY levels in KO rats (49–51), the effect on food intake is controversial (45, 50). When diabetes is induced via streptozotocin (STZ) treatment in F344/DuCrj-DPP4-deficient rats, onset of hyperglycemia was delayed, but KO rats showed impaired creatinine clearance and more severe dyslipidemia, which might be caused by a dysregulated expression of factors involved in steroid and lipid metabolism (52, 53). The authors concluded that DPP4 might be responsible for preservation of renal function. Another effect of the whole-body KO of DPP4 in rats is induction of behavioral changes like a blunted stress phenotype (46, 51) and also effects on the immune-regulatory system like blunted NK cell and T-cell function and differential leukocyte subset composition or altered cytokine levels (46, 47).

DPP4 Deficiency in Mice

Most of the observations already described in deficient rats are also true in whole-body DPP4-KO mice. Marguet et al. showed enhanced glucose tolerance, lower plasma glucose, and higher plasma insulin and GLP-1 after a 15 min oral glucose load without further characterizing the diet, age, or sex of the used C57BL/6 DPP4-KO mice (54). Conarello and colleagues found less weight-gain independent of the diet, and marked hypertrophy in the HFD-fed KO mice in epididymal white (eWAT) and brown adipose tissue (BAT). Importantly, they admitted that the reduction in caloric intake accounted for ~70% of the observed changes in bodyweight. Although they still observed differences in the bodyweight between KO and WT when they used pair-feeding, they carried out their further analysis in *ad libitum* fed mice and it is therefore difficult to judge the influence of DPP4 irrespective of bodyweight. However, they found improved insulin sensitivity and islet morphology, and improved liver biology in respect to lipid content and marker gene expression (55). The observation that DPP4 might be involved in the immune-regulatory system was also investigated in DPP4-KO mice, which were treated with pokeweed mitogen that stimulates growth and proliferation of B-cells. DPP4 seems to be involved in maturation and migration of immune cells, cytokine secretion, and percentages of spleen lymphocytes (56).

All these studies have in common that they use whole-body KO animals. The disadvantage here is that one cannot distinguish between direct effects of the KO and side effects caused, for example, by different immune cell status or decreased caloric intake.

To really decipher the role of DPP4 in different tissues and their crosstalk with other target tissues, it is of great importance to study tissue-specific KO models.

Because of this and because we were the first to describe DPP4 as a novel adipokine linked to parameters of the metabolic syndrome (4), we decided to develop an adipose tissue-specific KO mouse model. The AT-specific DPP4-KO mouse was generated using a Cre-lox strategy under control of the aP2 promoter on the C57BL/6J background. Interestingly, we found out that KO mice gained significantly more weight, fat, and lean mass under HFD with no effect on energy expenditure or food intake. However, KO mice showed improved HOMA-IR and lower fasting insulin. The observations that within AT, KO mice display a shift toward significantly more smaller adipocytes, and an increased expression of M2 macrophage marker genes points toward a beneficial role of DPP4 deletion in adipose tissue remodeling during HFD (57, 58).

Enzymatic Function of DPP4

Dipeptidyl-peptidase 4 exerts its enzymatic action by clipping dipeptides from the penultimate position of its substrates. The active center, which is housed in an internal cavity, is surrounded by the β -propeller domain and the catalytic domain. Inhibitors and substrates enter/leave the active center by a so-called “side opening” (59, 60). The following section deals with known substrates of DPP4 in respect of T2DM, and with DPP4 as a drug target for T2DM treatment, which will include current knowledge on DPP4 inhibitors and the impact of DPP4 on organs involved in complications of T2DM.

DPP4 Substrates

In theory, numerous peptides are potential DPP4 substrates since they contain the cleavable amino acid sequence at their penultimate position, but not for all of them it could be shown that DPP4 is able to cleave them *in vivo*. There seems to be a size limitation at least for cytokines, where DPP4 is more prone to cleave substrates of around 24 amino acids (aa) length. Furthermore, the substrate recognition is also dependent on the aa sequence around the penultimate position (61, 62). It turned out to be difficult to find physiological targets of DPP4 in the literature, reasons of that are excellently summarized in a recent review by Mulvihill and Drucker (6). We decided to focus here on the (potential) substrates of DPP4, which might play a role in T2DM or its complications. The list of DPP4 substrates mentioned here is not fully complete and aims to highlight the importance of DPP4 in T2DM also beyond its well-known incretin effect.

Incretin Hormones

The incretin hormones account for approximately 50% of the insulin secretion after meal intake and are secreted from the gut within minutes after the meal intake. Through binding to distinct receptors on beta cells in the pancreas, they stimulate insulin secretion and suppress glucagon release depending on the blood glucose level. Most potent in their glucose-lowering action are glucagon-like peptide 1 (GLP-1) and GIP. Both peptides belong

to the same glucagon peptide superfamily and share significant aa character.

Glucagon-like peptide-1 is secreted from L-cells of the gut into the bloodstream. Upon binding to G-protein-coupled receptors on the beta cells, intracellular cAMP level is elevated and the protein kinases, Epac1 and 2, are activated, which leads to an increase of insulin secretion. Furthermore, GLP-1 enhances beta-cell mass by mediating proliferation and differentiation and inhibiting apoptosis (8). By inhibiting gastric emptying, GLP-1 also improves blood sugar excursion, delays food absorption, and is therefore a regulator of satiety and appetite also through the hypothalamus (63).

Glucose-dependent insulinotropic polypeptide is a 42 aa peptide, which mainly originates from enteroendocrine K cells (64). Subjects with diabetes or impaired glucose tolerance show significantly reduced levels of meal-stimulated circulating GIP and the levels are negatively correlated with the severity of IR in the patients (65, 66). GIP has, in contrast to GLP-1, no effect on glucagon secretion, but also regulates fat metabolism in adipocytes (67).

Since inhibition of DPP4 due to genetic deletion or use of DPP4 inhibitors was shown to elevate GLP-1/GIP levels in numerous studies, this effect is the main focus of developing therapeutic targets for treatment of T2DM. There are numerous reviews, which focus on DPP4- and GLP-1-mediated effects, and this topic will not be further discussed here.

Stromal Cell-Derived Factor-1 α /CXCL12

Stromal cell-derived factor-1 (SDF-1) is a chemokine that promotes angiogenesis and attracts endothelial progenitor cells (EPC) by binding to its receptor C-X-C motif chemokine receptor type 4 (CXCR4). SDF-1 is thus discussed in the literature as important mediator of cardioprotective effects addressed to the use of DPP4 inhibitors (further discussed in “DPP4 Substrates: SDF-1- and BNP-Dependent Effects of DPP4 Inhibitors”). It is a well-known physiological target of DPP4 (68, 69). SDF-1 α also plays a role in diabetes itself by protecting stem-cell-derived insulin-producing cells from glucotoxicity under high glucose conditions (70) or promoting pancreatic beta-cell survival in mice via Akt activation (71). Furthermore, it was shown that some genetic variants of SDF-1 α are associated with late stage complications in T2DM patients (72, 73).

NPY and PYY

Neuropeptide Y and peptide YY are members of the polypeptide family. They are highly expressed in the hypothalamus but are also present in peripheral tissues like islets. NPY regulates energy balance, memory, and learning, while PYY reduces appetite, inhibits gastric motility, and increases water and electrolyte absorption in the colon (74). Both NPY and PYY play a role in beta-cell survival and in glucose homeostasis (74). NPY is able to suppress insulin secretion acutely (75). Both polypeptides have in common that DPP4 truncation shifts their receptor specificity and thus alters their biological role in different cellular processes. *In vitro* experiments in adipocytes could show that DPP4 inhibition has an impact on lipid metabolism mediated by NPY (76, 77).

Substance P

Substance P is a physiological target of DPP4, which is sequentially converted to SP (3–11) and SP (5–11) *in vivo* in F344-DPP4-positive rats (78). SP is a neurotransmitter and modulator, which is involved in neurogenic inflammation. Serum levels in diabetes are controversially discussed with one study showing a decrease in diabetic patients (79), and another one showing an increase in fasting blood samples with correlation to diabetic risk factors like BMI and blood pressure (80). This discrepancy in serum levels could be addressed to the fact that it is not always stated which form of SP (full length versus truncated) is measured. However, SP was shown to promote IR *in vitro* in human preadipocytes by interacting with proteins that are involved in the inhibitory phosphorylation of IRS-1. Furthermore, SP can directly inhibit insulin-dependent glucose metabolism in rat adipocytes (81). SP also promotes diabetic corneal wound healing, as shown by Yang and colleagues (82).

Brain Natriuretic Peptide

Brain natriuretic peptide is responsible for vasodilation, natriuresis, and suppresses renin secretion. It is so far only a predicted DPP4 substrate, which was cleaved *in vitro* by DPP4 to BNP (3–32). This truncation was inhibited by a DPP4 inhibitor in a dose-dependent manner (83). Truncated forms of BNP with lower enzymatic activity are discussed as an indicator of heart failure severity. In 2013, dos Santos et al. could show an improved cardiac performance in sitagliptin-treated rats, which they attributed to increased levels of active BNP (84).

Pituitary Adenylate Cyclase-Activating Polypeptide

Pituitary adenylate cyclase-activating polypeptide (PACAP) is very rapidly degraded by DPP4 to the fragments (3–27), (5–27), and (6–27). These fragments lack PACAPs insulinotropic ability, but are no feasible treatment options for T2DM because of their actions on glucose homeostasis and glucagon secretion (85). Several studies have shown that PACAP is a powerful stimulator of insulin secretion, which enhances glucose uptake in adipocytes and augments antilipolytic action of insulin (86, 87). After DPP4-inhibitor treatment in mice, PACAP-induced insulin secretion was enhanced (88). However, a proof that PACAP also plays a role in humans is lacking so far.

Regulated on Activation, Normal T-Cell Expressed and Secreted/CCL5

Regulated on activation, normal T-cell expressed and secreted (RANTES) recruits leukocytes into inflammatory sites and is cleaved by DPP4 to RANTES (3–68). Due to this truncation, RANTES (3–68) is not able anymore to increase cytosolic calcium concentrations and to induce chemotaxis of human monocytes *in vitro*. This is explained by a shift in receptor subtype-specificity toward enhanced activation of CC-motif-chemokine-receptor 5 (CCR5) (89). Elevated serum levels of RANTES in T2DM are associated with post-prandial hyperglycemia (90). Interestingly, RANTES and its receptor CCR5 are important mediators of obesity-induced inflammation, which was shown in CCR5-KO mice (91). Levels of RANTES and CCR5 were reduced in adipose tissue of obese patients upon exercise (92). RANTES reduces

glucose-stimulated GLP-1 secretion *in vitro* and *in vivo* in mice, by acting most probably through the intestinal glucose transporter SGLT1 (93).

Eotaxin/CCL11

Eotaxin mediates mobilization of eosinophils into the bloodstream, which was shown to be increased in DPP4-deficient F344 rats (94). DPP4 cleaves eotaxin to eotaxin (3–74). However, there was no significant correlation of eotaxin seen in patients with T2DM or impaired glucose tolerance in the KORA cohort (95).

DPP4 as a Drug Target for the Treatment of T2DM

Deactivation of DPP4 Enzymatic Activity

DPP4 inhibitors

Major DPP4 substrates are the so-called incretin hormones, which are key regulators of post-prandial insulin release. DPP4 inhibition leads to greater bioavailability of these proteins and therefore prolongs the half-life of insulin action. The majority of effects seen upon DPP4-inhibitor treatment are ascribed to an increase in GLP-1 levels. Because of this, DPP4 became a major target for the treatment of T2DM. This section deals with the most recent knowledge around DPP4 inhibitors, their mode of action – if known – and the newest developments in the inhibition of DPP4 enzymatic activity. There are numerous modifications and potential optimizations of the five so far approved gliptins reported. However, most of them are not in clinical trials yet and not much is known about their advantage in a head to head comparison to established gliptins. Therefore, we decided to focus on the most recent data on approved gliptins in this review. The data are also summarized in **Table 1**.

Dipeptidyl-peptidase 4 inhibitors lower DPP4 activity by 70–90%. They do not pass the blood–brain barrier and have no direct effect on satiety or on altering gastric emptying (8). The benefit for diabetes therapy clearly is their indifference on bodyweight gain and the low risk of hypoglycemia. There are five gliptins approved so far for clinical use, namely sitagliptin, vildagliptin, saxagliptin, linagliptin, and alogliptin. Three more gliptins, teneligliptin, anagliptin, and trelagliptin are only approved in the Japanese and Korean market. Despite the same mode of action, the different gliptins diverge in their pharmacodynamic and pharmacokinetic properties, which might be clinically relevant for some patients (9, 96). The peptide mimetic compounds, vilda-, saxa-, and teneligliptin, were identified by

replacement experiments of peptide-based substrates, whereas the non-peptide mimetic compounds, sita-, alo-, and linagliptin, were derived from initially found inhibitors of random screenings. The diverse chemical structures also explain the unique binding modes of the inhibitors to DPP4 (10).

The six inhibitors have been classified into three classes depending on their different binding modes in the DPP4 active center (10). Class 1 contains vilda- and saxagliptin, which only bind to the S1 and S2 subsites and form a covalent bond with the nitrile group of their cyanopyrrolidine moiety and Ser630 of DPP4. Saxagliptin has a fivefold higher activity in blocking DPP4 than vildagliptin. Group 2 contains alo- and linagliptin, which also interact with the S1' subsite or even in case of linagliptin with the S2' subsite. The uracil rings of both gliptins induce a conformational change in the Tyr547 of the S1' subsite. Because of the additional interaction of linagliptin with S2' subsite, it has an eightfold higher activity than alogliptin. The third class has the highest inhibitory function toward DPP4, because both sita- and teneligliptin interact with the S2-extensive subsite of the DPP4 active center, and an increasing number of interactions seems to increase the potency of the gliptin (10). Teneligliptin, which is only approved for T2DM treatment in the Japanese and Korean market so far, also has a unique structure characterized by a J-shape and an anchor-lock domain, which explains the strong inhibitory function and the low IC₅₀ value of this drug [for review, see Ref. (97)]. The binding of the DPP4 S2-extensive subsite of some inhibitors also guarantees a high specificity toward DPP4 since other close-related peptidases like DPP8, DPP9, and FAP lack this subsite. All DPP4 inhibitors have in common that they build salt bridges with Glu-residues in the S2 subsite (10). At least for sitagliptin, it is also known that it lowers the level of free fatty acids (FFA) and thereby also comprises insulin-sensitizing properties (98). Furthermore, sitagliptin was shown to have potent anti-inflammatory properties by suppressing expression of pro-inflammatory genes in mouse and humans (98, 99). In patients with renal impairment, which is a very common complication of T2DM, sitagliptin is more suitable than sulfonylureas (100). Anagliptin, which is only approved since 2012 in the Japanese market, seems to have serum lipid-lowering and anti-atherogenic actions as well, which makes it unique among the gliptins approved so far (101, 102). Anagliptin has an IC₅₀ of 3.3 nM and its main excretion route is renal elimination (101, 103). However, since this gliptin is only on the market since 2012,

TABLE 1 | Summarized properties of gliptins.

Inhibitor	Approved since	Binding mode	Kind of inhibition	Route of excretion	IC ₅₀ value	Reference
Sitagliptin	2006 FDA	S1, S2, and S2 extensive subsites	Competitive inhibition	Mostly renal route	19 nM	(10, 98–100)
Vildagliptin	2007 European medicines agency	Only S1 and S2 subsite	Substrate–enzyme blocker	Mostly renal route	62 nM	(10, 209)
Saxagliptin	2009 FDA	Only S1 and S2 subsite	Substrate–enzyme blocker	Mostly renal route	50 nM	(10, 209)
Linagliptin	2011 FDA	S1, S2, and S1' subsites	–	Through biliary route	1 nM	(10)
Alogliptin	2013 FDA	S1, S2, and S1' subsites	Competitive inhibition	Mostly renal route	24 nM	(8, 10)
Teneligliptin	2012 Japan 2014 Korea	S1, S2, and S2 extensive subsites	Very potent because of unique anchor-lock domain and J-shape of molecule	Mostly renal route	0.37 nM	(97)

comparative head-to-head trials and data on the long-term use are missing at the moment. Shinjo and colleagues demonstrated that anagliptin exerts anti-inflammatory effects on macrophages and adipocytes *in vitro* and on inflamed mouse livers *in vivo*. In this very recent study, anagliptin was more potent in its anti-inflammatory actions than sitagliptin (104). To improve the quality of life of T2DM patients, development of novel agents now is more and more focused on long-acting agents. There are two more gliptins, which only have to be applied once weekly, namely SYR-472 (trelagliptin) and MK-3102 (omarigliptin) (105, 106). Trelagliptin is approved in Japan since 2015.

Although some authors claim that DPP4 inhibitors are only beneficial in early stages of diabetes, this could be rebutted by the work of Kumar and Gupta (107). They could show beneficial effects of three gliptins (sitagliptin, saxagliptin, and vildagliptin) in lowering HbA1c also in patients with longstanding T2DM for more than 10 years. Thus, DPP4 inhibition also plays an important role irrespective of the duration of diabetes.

What has to be mentioned in respect of the beneficial roles of DPP4 inhibitors is that more and more studies about their beneficial pleiotropic effects are upcoming, which are also discussed in the following section of this review dealing with different organs. There are reports that gliptins themselves have effects on lipid profile and blood pressure as well as on inflammatory processes (108). In addition to the incretins, there are some DPP4 substrates, like SDF-1 α , which might explain potential cardioprotective effects, which are discussed for gliptins. However, cardiovascular outcomes are still widely debated and controversially evidenced. Ongoing long-term studies will further shed light on the respective role of DPP4i beyond glucose homeostasis. Furthermore, one has to keep in mind that also DPP4 has direct effects independent of its enzymatic activity, like activation of downstream signaling events upon receptor binding, which are not well understood so far. Which role DPP4 inhibition plays on T2D relevant organs/comorbidities will be the topic of the following sections.

Alternative modes of DPP4 inhibition

Very recently, Pang and colleagues published a different strategy to inhibit DPP4 activity. They used DPP4-targeted immune therapy by vaccines in a C57BL/6J mouse model and were able to show comparable effects like in treatments with gliptins regarding GLP-1 plasma levels and post-prandial glucose excursion and insulin sensitivity in HFD-fed mice. Furthermore, they observed no side effects on immune cell activation by the DPP4 vaccine. An advantage of this method is the long-lasting effect of the vaccine in the mouse model, which could, if transferable to human patients, be a convenient alternative to the daily intake of gliptins (109).

Further research in developing alternatives toward Gliptins especially for long-acting medications would be an interesting new approach to improve lifestyle of patients.

Incretin-Based Therapies: Comparing DPP4i and GLP-1 Analogs

It is well accepted that incretin-based therapies are able to lower blood glucose levels and are therefore a treatment option for T2DM. There are mainly two approaches to target the incretin

system (1) via inhibiting the enzymatic action of DPP4 and thereby upregulating GLP-1 levels physiologically and (2) via increasing GLP-1 levels pharmacologically. While GLP-1 receptor agonists (GLP-1-RA) directly target GLP-1, GLP-1-independent effects are also possible with the use of DPP4 inhibitors (DPP4i). These drugs might also affect the level of other DPP4 substrates and might therefore have a more complex mode of action.

However, there have been a lot of attempts to compare the effects of GLP-1-RA versus DPP4i in clinical studies. The results of these head-to-head comparisons are summarized in many current reviews (110–114). Most of these comparative studies agree that GLP-1 analogs are more effective in respect of glycemic control. Both incretin-based therapies are equally potent in lowering blood pressure and total cholesterol (110). Furthermore, both have the advantage of low incidence of hypoglycemia (110, 111). The results for body-weight lowering effects of DPP4i are heterogeneous throughout the studies (113), whereas beneficial effects on body-weight are well accepted for GLP-1-RA (110–112, 114). Therefore, some authors tend to prefer GLP-1-RA over the use of DPP4i (112). However, one should be aware of the fact, that GLP-1-RA have a higher incidence of gastrointestinal adverse events like nausea (110, 112, 114), which might be disadvantageous for elderly people who may be more prone to these side effects (114).

Furthermore, there are reports that DPP4i might also have cardioprotective effects (113), which will also shortly be discussed in section “Effect of DPP4 Inhibition on the Cardiovascular System” of this review. Despite the clear beneficial effects of incretin-based therapies, there are also concerns reported in respect of the risk for long-term complications like pancreatitis (115). These potential risks might, however, outweigh the benefits. These controversial discussions are well summarized by the reviews of Nauck and Butler (115, 116).

To really assess which medication is of more importance always depends on the special patient characteristic.

Impact of DPP4 on T2DM-Relevant Organs and Associated Comorbidities

Dipeptidyl-peptidase 4 inhibitors exert glucose regulatory actions by prolonging the effects of GLP-1 and GIP, ultimately increasing glucose-mediated insulin secretion and suppressing glucagon secretion (117). Beside the glucose-lowering properties of DPP4 inhibitors, emerging evidence suggests that incretin-based therapies may also have a positive impact on inflammation, cardiovascular and hepatic health, sleep, and the central nervous system (118). However, the underlying mechanisms of these effects cannot be fully explained by lower blood glucose levels or increased GLP-1 bioavailability or signaling, and has to be further elucidated. Thus, the next section is focused on the role of DPP4 action in T2DM-relevant organs and associated comorbidities.

Adipose Tissue

Adipose tissue is the primary storage organ for excess energy. While the role of adipose tissue as a central source of energy has been recognized for centuries, in the past decade, it has become increasingly clear that adipose tissue also displays characteristics of an endocrine organ releasing a number of adipose

tissue-specific factors, known as adipokines. During the progression of obesity, the ability of adipocytes to function as endocrine cells and to secrete multiple biologically active proteins is affected (119). Thus, adipose tissue has been shown to be a central driver of T2DM progression, establishing and maintaining a chronic state of low-level inflammation (120).

DPP4 Expression and Release Within Adipose Tissue

Recently, we showed that DPP4 is highly expressed in human primary adipocytes (4). Furthermore, DPP4 expression in adipose tissue is increased in obese compared to lean individuals in both subcutaneous and visceral adipose tissue (4, 5). Interestingly, visceral fat of obese patients exhibits the highest DPP4 level. According to the increased expression, we could identify sDPP4 as a novel adipokine released from primary human adipocytes. *In vitro*, the DPP4 release increased substantially during fat cell differentiation, and comparison with preadipocytes and adipose tissue, macrophages showed that adipocytes most likely represent the major source of DPP4 released from the intact organ to the circulation. Furthermore, the release of sDPP4 was elevated in adipose tissue explants of obese patients compared to lean controls and correlates with various classical markers of the metabolic syndrome, namely BMI, waist circumference, plasma triglycerides, and HOMA as an index of IR, as well as with fat cell volume and the adipokine leptin (4, 5).

How DPP4 expression affects adipocyte homeostasis can only be speculated. DPP4 might be involved in adipose tissue lipolysis. DPP4 recruits ADA, a monomeric enzyme catalyzing deamination of adenosine to inosine and ammonia (121, 122). It has been shown that DPP4-bound ADA has a 1000-fold greater activity than free ADA (123), which in turn may modulate the well-established antilipolytic effects of adenosine. Moreover, DPP4 is a strong inhibitor of the antilipolytic activity of NPY (76), which is one of the best peptide substrates of the enzyme (89). In this regard, Rosmaninho-Salgado and colleagues demonstrated that DPP4 stimulates lipid accumulation and PPAR- γ expression through cleavage of NPY suggesting that sDPP4 might stimulate adipocyte differentiation (77). However, it is noteworthy that the authors of this study were using tremendously high and non-physiological concentrations of sDPP4. On the contrary, a recent published study showed that DPP4 expression was strongly upregulated during adipocyte dedifferentiation *in vitro*. Hence, the authors concluded that DPP4 might be a major component in adipose tissue remodeling and cell plasticity (124). Nevertheless, enhanced abundance of DPP4 within adipose tissue of obese subjects may be involved in adipose tissue remodeling and substantially augments the lipolytic activity of enlarged adipocytes (57, 58).

Moreover, dendritic cells and macrophages resident in visceral adipose depots exhibit an increased DPP4 expression in response to inflammation or in the obese state (28). Since it is known that DPP4 exerts immunomodulating properties, Zhong et al. showed that membrane-bound DPP4 is co-localized with membrane-bound ADA on human dendritic cells resulting in an increased T-cell proliferation (28). Thus, it can be speculated that DPP4 might also play an important role in the chronic low-grade inflammation taking place in obesity and T2DM.

Adipose Tissue as Relevant Source of Circulating DPP4

Serum levels of sDPP4 are altered in many pathophysiological conditions, including different types of cancer, allergic asthma, or hepatitis C (7). Our group was the first analyzing circulating sDPP4 in the context of obesity and the metabolic syndrome. DPP4 serum levels of morbidly obese men are elevated compared with lean controls and significantly correlated with BMI, the size of adipocytes in subcutaneous and visceral fat, and the adipocyte hormones adiponectin (negatively) and leptin. These data suggest that sDPP4 is related not only to increased body weight but also to other important parameters of adipose tissue physiology. In addition, sDPP4 release and serum concentration can be reversed to normal levels by surgery-induced weight loss (4). Thus, in obesity, both circulating levels of sDPP4 and sDPP4 release by adipose tissue are increased and correlate strongly with the metabolic syndrome but can be reduced to control levels by substantial weight loss. Thus, indicating that enlargement of visceral adipocytes in obesity may substantially contribute to the augmented level of circulating sDPP4 in obese patients.

Endocrine Effects of Soluble DPP4

Although there is clear evidence that increased circulating levels of sDPP4 are associated with hallmarks of obesity and type diabetes, such as whole-body IR, elevated BMI, and adipocyte hypertrophy, there are only few studies investigating the endocrine effects of sDPP4. We were the first showing that DPP4 consistently impairs insulin signaling at the level of Akt in primary human adipocytes (4). Enzymatic activity of sDPP4 appears to be involved in this process; however, since this work was done *in vitro*, it is most unlikely that the sDPP4-induced impairment of insulin action is due to an increased bioavailability of any DPP4 substrate. It might rather be that DPP4 inhibitors may also affect the binding properties of sDPP4 to its receptors, namely M6P/IGFII receptor (38) or PAR2 (39). For the latter, it is not only known that PAR2 signaling induces IR in adipocytes (125), but PAR2 might also be a substantial contributor to inflammatory and metabolic dysfunction (126). Although there is a hint that circulating sDPP4 itself might affect adipose tissue function, the exact mechanism has to be further investigated.

Impact of DPP4 Inhibition on Adipose Tissue

To further investigate the role of DPP4 in adipose tissue, several studies with DPP4 inhibitors were conducted. Interestingly, the administration of the DPP4-inhibitor des-fluoro-sitagliptin ameliorates linoleic acid-induced adipose tissue hypertrophy in β -cell-specific glucokinase haploinsufficient mice, a model of non-obese T2DM (127). Moreover, des-fluoro-sitagliptin protects against linoleic acid-induced adipose tissue inflammation illustrated by CD8⁺ T-cell infiltration. Due to the loss of GLP-1 receptors in adipose tissue, the authors exclude the involvement of GLP-1 and claim that the observed effects are due to the huge variety of DPP4 substrates. Thus, DPP4 inhibition might have pleiotropic effects in adipose tissue. A similar outcome has been observed in C57BL/6 mice fed a HFD. After linagliptin treatment, a significantly lower expression of the macrophage marker F4/80 was

found compared with vehicle treatment. In line with these data, the authors demonstrated an increased insulin sensitivity after linagliptin treatment suggesting that DPP4 and adipose tissue inflammation play a pivotal role in the induction of IR. In 3T3-L1 cells, a murine preadipocyte cell line, Rosmaninho-Salgado et al. demonstrated that the DPP4-inhibitor vildagliptin reduces lipid accumulation by inhibiting adipogenesis, without affecting lipolysis through NPY cleavage and subsequent NPY Y2 receptor activation (77).

With the recognition that adult humans also have BAT, an organ with substantial capacity to dissipate energy, BAT gained considerable interest as a novel target to treat or prevent obesity and its associated diseases. In 2013, the group around Shimasaki was the first reporting that des-fluoro-sitagliptin attenuated body adiposity, without affecting food intake, in C57BL/6 mice with diet-induced obesity (128). The increase in energy expenditure could be explained by enhanced levels of PPAR- α , PGC-1, and uncoupling protein-1 (UCP-1) in BAT as well as elevated levels of proopiomelanocortin in the hypothalamus. The beneficial effects of des-fluoro-sitagliptin on energy expenditure could only partly be ascribed to increased GLP-1 levels and have to be further validated. Shortly afterward, Fukuda-Tsuru et al. could confirm these data in the same animal model by administration of teneligliptin (129). Moreover, in this study, teneligliptin also reduces fat mass and suppresses HFD-induced adipocyte hypertrophy.

Collectively, there is clear evidence that DPP4 expression and release by adipose tissue play a key role in obesity and T2DM-associated processes, such as inflammation, adipocyte hypertrophy, and IR. However, the underlying mechanism of these beneficial effects is not fully understood and remains unclear in most of the publications.

Pancreatic Islets

β -cells play a central role in the etiology of T2DM. Due to failure of β -cell sensitivity to glucose and loss of β -cell mass, insulin secretion of these cells is not sufficient to counterbalance IR, finally leading to T2DM. Although DPP4 inhibitors are now widely used for glycemic control, many debates are ongoing about their exact mode of action and their beneficial effects on pancreatic β cells.

Regulation of DPP4 Expression Within Pancreatic Islets

Interestingly, within the pancreatic islets, DPP4 localization differs between species. Islets of rodents showed a near-exclusive expression of DPP4 in β cells, with little expression in α -cells. In contrast, human and pig islets express DPP4 almost exclusively in α cells (130, 131). The species difference in the localization of DPP4 expression, and the possible physiological consequence of that difference, is unclear. Moreover, in a recent published study, it has been demonstrated that DPP4 activity was detectable in the conditioned medium of human islets suggesting that DPP4 is released from human islets as well (132). Under pathological conditions, islets of obese mice chronically fed a HFD that exhibit an increased DPP4 activity. The contrary was found in human islets from type 2 diabetic donors, showing a decreased DPP4 activity (131).

Impact of DPP4 Inhibition on Pancreatic Islets

Accumulating *in vitro* and pre-clinical data show that DPP4 inhibition has beneficial effects on T2DM induced β -cell dysfunction and apoptosis. Omar and colleagues demonstrated that DPP4 is not only present and active in mouse and human islets, but inhibition of islet DPP4 activity also has a direct stimulatory effect on insulin secretion, which is GLP-1 dependent (131). The same effect could be observed with a 2-week des-fluoro-sitagliptin treatment leading to increased insulin exocytosis by β cells from *db/db* diabetic mice (133). Furthermore, it could be shown that DPP4 inhibition is clearly associated with significantly increased β -cell mass and function in several models of T2DM (134–136). These beneficial effects were associated with the transcriptional activation of anti-apoptotic and pro-survival genes, as well as the suppression of pro-apoptotic genes in β cells (137). Additionally, Shah and collaborators showed that the DPP4-inhibitor linagliptin protects isolated human islets from gluco-, lipo-, and cytokine-toxicity (132). Accordingly, Akarte et al. reported anti-oxidative properties of vildagliptin shown by a dose-dependent decrease in nitric oxide concentrations in both serum and pancreatic homogenates of vildagliptin-treated diabetic rats (138).

Beside these pre-clinical and *in vitro* studies, only few are known about the beneficial effect of DPP4 inhibitors on β cells in human. In the short-term, 12-weeks vildagliptin treatment leads to a small increase in the capacity for insulin secretion (139). Treatment with vildagliptin over a longer period of time could also confirm an increased β -cell function in humans as a result of improved sensitivity of β cells to glucose (140, 141). However, this effect was not maintained after washout period, indicating that this increased capacity was not a disease modifying effect on beta cell mass and/or function. In the SAVOR-TIMI 53 trial, which was originally performed to assess the cardiovascular safety of saxagliptin, Leibowitz and colleagues recently reported that DPP4 inhibition may attenuate the progression of diabetes (142). This was evidenced by a decreased requirement for intensification of treatment associated with better preservation of glycemic control, as well as better sustained β -cell function as reflected in the fasting HOMA-2 β during the 2-year follow-up period.

The exact mechanism how DPP4 inhibitors augment insulin secretion and increase β -cell mass *in vitro* and *in vivo* is still not fully understood, since not all these effects could be explained by elevated GLP-1 level or improved glycemic control associated with less glucotoxicity.

Liver

Non-alcoholic fatty liver disease (NAFLD) describes a disorder with excessive deposition of fat within the liver with increasing prevalence in parallel to obesity and diabetes, which are major risk factors for NAFLD (143). Indeed, NAFLD is now the most common cause of chronic liver disease (144) and is present in one-quarter to one-half of diabetes patients (145). In the obese state, elevated triglyceride degradation in adipose tissue causes an increased hepatic uptake of fatty acids leading to fat accumulation within the tissue. Furthermore, reactive oxygen species (ROS), produced during lipid oxidation, are assumed to induce hepatocyte death and inflammatory reactions. Liver cirrhosis can be defined as the end stage of chronic liver diseases and is caused

by progressive fibrosis. This process is characterized by excessive accumulation of ECM and activated hepatic stellate cells (146, 147) that ultimately results in nodular regeneration with loss of function (148).

Regulation of DPP4 Expression in the Liver

Although DPP4 exhibits a widespread organ distribution, the liver is one of the organs that highly expresses DPP4 (149). In the healthy human liver, intense staining for DPP4 was found in hepatic acinar zones 2 and 3, but not in zone 1. This heterogeneous lobular distribution suggests that DPP4 might be involved in the regulation of hepatic metabolism (150). Furthermore, mRNA expression levels of DPP4 were significantly increased in NAFLD livers compared to that in control livers (151). In accordance to that, DPP4 expression levels of NAFLD patients were negatively correlated with HOMA-IR and BMI, and positively correlated with total cholesterol levels, but not with ALT, lactate dehydrogenase (LDH), or triglyceride levels. Moreover, under conditions of high glucose, DPP4 expression was increased in HepG2 cells. However, other nutritional conditions, such as high insulin or the presence of fatty acids and cholesterol, did not affect DPP4 expression in these cells. Thus, the authors claim that enhanced DPP4 expression in NAFLD liver may rather be associated with IR than triglyceride accumulation and may promote the progression of liver disease via subsequent deteriorations in glucose metabolism. How increased DPP4 expression might affect liver function is still unknown. There are only a few hints that DPP4 might play a role in fibronectin-mediated interaction of hepatocytes with extracellular matrix (2, 36, 152). Beside DPP4 expression, there is only indirect evidence that hepatocytes also release DPP4 to the circulation, which will be further discussed in the next section.

Serum Level of DPP4 in Liver Disease

As previously discussed, hepatic DPP4 mRNA expression level in the livers is significantly higher in patients with NAFLD compared to healthy subjects (151). This upregulation of hepatic DPP4 expression is thought to be responsible for elevated DPP4 serum level in patients with liver disease (153–155). In line with this observation, serum DPP4 activity can be correlated with hepatic steatosis and NAFLD grading (156). Similarly, in patients with NAFLD, DPP4 activity in serum correlates with markers of liver damage, such as serum gamma-glutamyltranspeptidase and ALT levels, but do not correlate with fasting blood glucose levels and HbA1c values (156, 157). Thus, hepatic DPP4 expression in NAFLD may be directly associated with increased DPP4 serum level and may be involved in hepatic lipogenesis and liver injury.

Impact of DPP4 Inhibition on Liver Function

Since DPP4 inhibitors are widely used in clinical practice, this drug was also investigated as a potential new therapeutic strategy against the development of liver fibrosis and steatosis. Kaji and collaborators demonstrated that sitagliptin markedly inhibits liver fibrosis development in rats via suppression of hepatic stellate cell proliferation and collagen synthesis (158). These suppressive effects were associated with dephosphorylation of ERK1/2, p38, and Smad2/3 in the hepatic stellate cells. Additionally, hepatic steatosis could be prevented in several different animal models

by DPP4 inhibition (127, 159, 160). Shirakawa and colleagues studied the effects of sitagliptin in glucokinase \pm diabetic mice with diet-induced hepatic steatosis (127). Here, sitagliptin prevented fatty liver in both wild-type and glucokinase \pm mice paralleled by decreased expression of sterol regulatory element-binding protein-1c, stearoyl-CoA desaturase-1, and fatty acid synthase, and increased expression of peroxisome proliferator-activated receptor- α in the liver. Furthermore, in a mouse model of non-alcoholic steatohepatitis, further studies indicated that linagliptin improves insulin sensitivity and hepatic steatosis in mice with diet-induced obesity (161) and ameliorates liver inflammation (162). The underlying mechanism of these beneficial effects has been further investigated by Ohyama et al. in *ob/ob* mice (163). The novel DPP4-inhibitor MK-0626 attenuates hepatic steatosis by enhancing AMPK activity, inhibiting hepatic lipogenic gene expression, increasing triglyceride secretion from liver, and elevating serum adiponectin levels.

Clinical data are very limited; however, several non-randomized trials conducted in small groups of diabetic patients demonstrated that DPP4 inhibitors improved the levels of liver transaminases and liver fat (164–166). Accordingly, Iwasaki et al. found a decrease in ballooning and non-alcoholic steatohepatitis scores in post-treatment liver biopsies (165, 166). Recently, in a comprehensive retrospective review of 459 type 2 diabetic patients, treated with DPP4-inhibitors, it was shown that DPP4 inhibitors improved the abnormality of the liver transaminases AST and ALT independent of HbA1c and body weight (167). Again in the majority of publications, the authors postulate that these beneficial actions were mediated through potentiation of direct GLP-1 actions on hepatocytes; however, it seems unlikely that hepatocytes express the canonical GLP-1 receptor (168).

In conclusion, accumulating studies indicate that DPP4 inhibitors are clinically useful for patients with T2DM accompanied by liver dysfunction based on fatty liver, and that DPP4 inhibition affects liver function regardless of diabetic status and obesity.

Cardiovascular System

Cardiovascular complications (CVD) are common in patients with T2DM and a major cause of mortality (169). Atherosclerosis is the dominant cause of CVD and usually develops many years before any clinical symptoms are manifested. The underlying pathogenesis of atherosclerosis involves an imbalanced lipid metabolism and a maladaptive immune response entailing a chronic low-grade inflammation of the arterial wall. Endothelial cells and intimal smooth muscle cells represent the major cell types of the artery wall preserving vessel wall homeostasis. Together with leukocytes, they are the major players in the development of this disease. Beside atherosclerosis, T2DM also exacerbates heart failure associated with diastolic heart failure and coronary microangiopathy (170–172).

Regulation of DPP4 Expression and Release in Vascular Cells

Dipeptidyl-peptidase 4 is expressed in both microvascular endothelial cells of different human tissues, such as liver, spleen,

lung, brain, heart (170, 172), and in human vascular smooth muscle cells (3). Under conditions of high glucose, DPP4 expression and activity were increased in human glomerular endothelial cells (173). Additionally, in STZ-induced diabetic rats, activity of membrane-bound DPP4 was increased, thereby reducing cardiac SDF-1 concentrations and causing impaired angiogenesis (174). Also hypoxia has been shown to regulate DPP4 expression in vascular cells. Regarding endothelial cells, there are conflicting data on the influence of hypoxia on DPP4 expression. In human microvascular endothelial cells as well as human umbilical vein endothelial cells, Eltzschig and colleagues showed that hypoxia increased DPP4 mRNA and protein level (175), whereas another study by Shigeta et al. observed a decreased protein level of DPP4 under hypoxic conditions in the same cells (174). However, in human vascular smooth muscle cells, we observed an increased DPP4 expression in response to hypoxia (3). In this particular study, we could also show that DPP4 is released from human vascular smooth muscle cells. However, only very little is known about the physiological role of the membrane-bound DPP4 within the vasculature. There is only one study showing that DPP4 forms a complex with ADA capable of degrading extracellular adenosine to inosine in endothelial cells. Increased inosine levels in turn are known to induce vasoconstriction due to mast cell degranulation (176).

Effect of DPP4 Inhibition on the Cardiovascular System

In several *in vitro* and pre-clinical studies, DPP4 inhibitors have been shown to exert important protective effects on the cardiovascular system. In this regard, it has been shown that DPP4 inhibitors decrease myocardial infarct size, stabilize the cardiac electrophysiological state during myocardial ischemia, reduce ischemia/reperfusion injury, and prevent left ventricular remodeling following MI (177, 178). Additionally, DPP4 inhibitors also exert vascular protective properties, including anti-inflammatory and anti-atherosclerotic effects and the ability to induce vascular relaxation (179, 180). To confirm cardiovascular safety or even protection of DPP4 inhibitors in humans, several cardiovascular outcome studies were conducted. However, several clinical trials, namely SAVOR-TIMI 53, EXAMINE, or VIVID in patients with established cardiovascular disease failed to confirm a cardio-protective effect (181–183). Even an increased cardiovascular risk for DPP4 inhibitors was discussed, since in the SAVOR-TIMI 53 trial, a significant increased hospitalization due to heart failure in the saxagliptin-treated group was observed. However, in the most recent published outcome study TECOS, the authors could show that among patients with T2DM and established cardiovascular disease, sitagliptin did not appear to increase the risk of major adverse cardiovascular events, hospitalization for heart failure, or other adverse events (184). As sDPP4 is an adipokine upregulated in obesity and T2DM that triggers IR and metabolic complications (4, 5), it might be speculated that the beneficial effects of DPP4 inhibitors would be higher in those early phases of the metabolic disorders previous to the development of established cardiovascular disease.

However, whether these beneficial effects observed in pre-clinical settings are due to increased levels of different DPP4

substrates or inhibition of direct effects of DPP4 remains unclear and will be assessed in more detail in the following section.

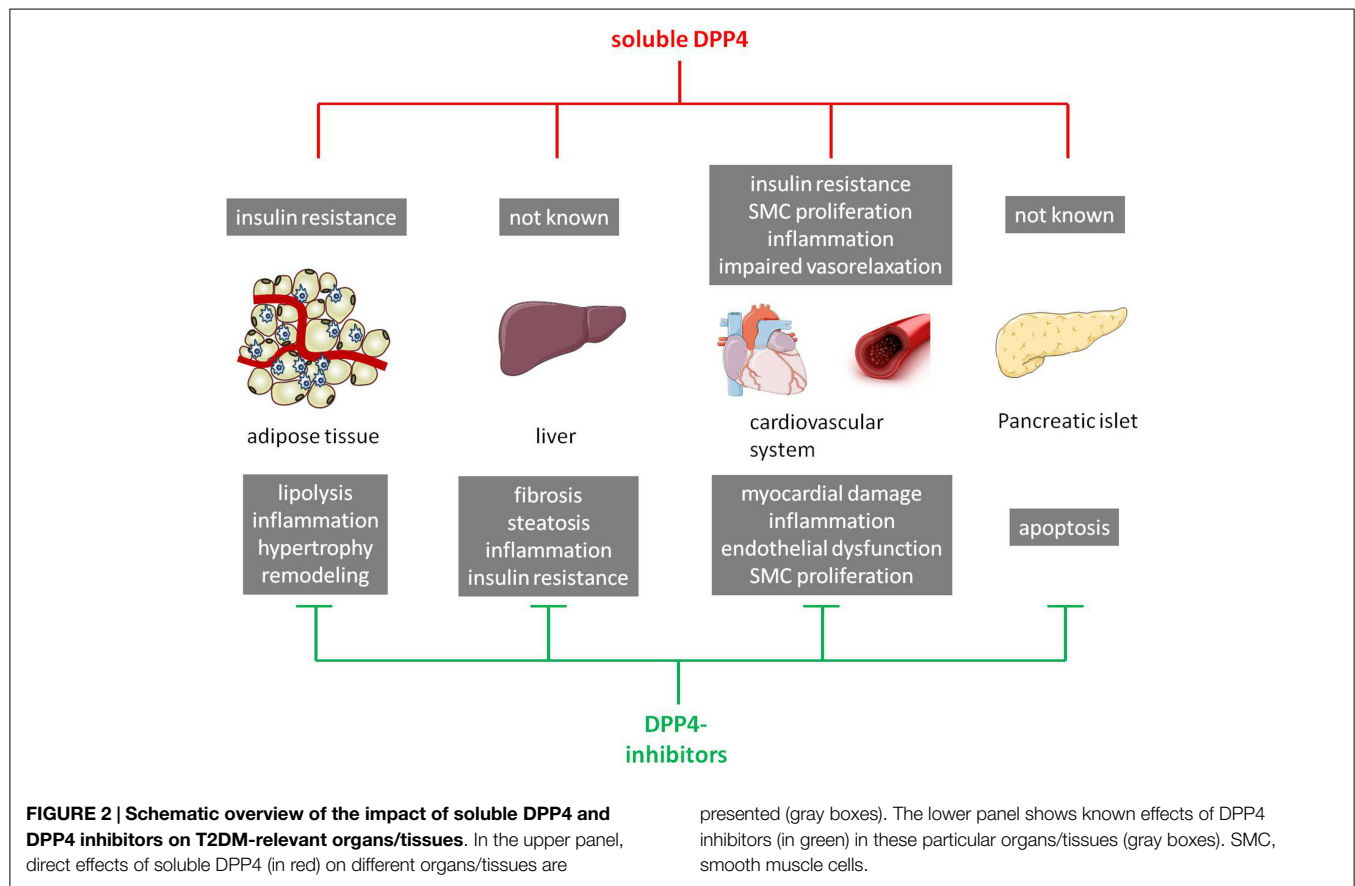
DPP4 Substrates: GLP-1 Dependent Effects of DPP4 Inhibitors

Since several studies have identified a role for GLP-1 receptor (GLP-1R) signaling in DPP4-dependent cardioprotection, it is suggested that GLP-1 itself has favorable cardiovascular effects. Indeed, mRNA transcripts of the GLP-1R have been detected in the heart of rodents (185, 186) and humans (187). Furthermore, GLP-1R has also been localized to mouse aortic smooth muscle and endothelial cells, as well as monocytes and macrophages (188).

Regarding MI and heart failure, pre-clinical studies have demonstrated that DPP4-deficient rats subjected to 45 min of ischemia with 2 h or reperfusion exhibited cardioprotection illustrated by reduced infarct size, improved cardiac performance, and reduced levels of BNP compared to control rats (189). These beneficial effects could be partially reversed by co-administration of the GLP-1R antagonist exendin (9–39). Accordingly, administration of exendin (9–39) reversed the sitagliptin-induced improvement in ventricular function in Sprague Dawley rats with transient cardiac ischemia (190). Additionally, in a rat model of chronic heart failure, GLP-1 analogs were able to improve cardiac function and morphology, with a concomitant amelioration of hyperglycemia and hyperinsulinemia (191).

Regarding the vascular system, continuous infusion of the GLP-1 analog exendin-4 reduced monocyte adhesion to aortic endothelial cells, associated with a reduction in atherosclerotic lesion size in non-diabetic C57BL/6 and *ApoE*^{−/−} mice. Furthermore, treatment for 1 h with exendin-4 reduced the expression of the pro-inflammatory cytokines, TNF α and MCP-1, in response to lipopolysaccharide (LPS) (188). In addition, exendin-4 stimulates proliferation of human coronary artery endothelial cells through endothelial nitric oxide synthase (eNOS)-, protein kinase A (PKA)-, and PI3K/Akt-dependent pathways (192, 193). Accordingly, in humans, preliminary data confirm the ability of GLP-1 to protect from high glucose-induced endothelial dysfunction in the post-meal phase (194). In a model of vascular injury, it has been shown that continuous infusion of exendin-4 reduces neointimal formation at 4 weeks after injury without altering body weight or various metabolic parameters (195). From *in vitro* studies, Goto et al. suggest that this effect was mediated by the ability of GLP-1 to suppress platelet-derived growth factor (PDGF)-induced proliferation of vascular smooth muscle cells. In contrast, in a pre-clinical study, combining HFD and STZ treatment in *ApoE*^{−/−} failed to detect evidence for GLP-1R-dependent reduction of lesion size in the thoracic or abdominal aorta (168). The authors discuss that the duration of treatment, the dose of the GLP-1 agonist, or the age of mice might be responsible for the lack of anti-atherogenic activity in this study.

However, in patients with heart failure, pilot studies also suggest cardioprotection by GLP-1 infusion (196, 197). Accordingly, a large retrospective analysis indicates that patients treated with the GLP-1 analog exenatide had a significant 20% reduction of CVD events compared with patients on other glucose-lowering agents (198). Nevertheless, studies showing cardiovascular protective



effects of GLP-1 were carried out using either native GLP-1 or recombinant GLP-1 analogs at high concentrations or in a way that induced supraphysiological GLP-1 signaling. Considering that DPP4 inhibition restores GLP-1 signaling within the physiological range, beneficial effects of DPP4 inhibitors might be different to those of GLP-1 analogs.

DPP4 Substrates: SDF-1- and BNP-Dependent Effects of DPP4 Inhibitors

But beside GLP-1, there are further substrates of DPP4, which might play a role in the favorable cardiovascular effects of DPP4 inhibitors. Two of the most promising candidates are SDF-1 α and brain natriuretic peptide (BNP). As already mentioned in section “Stromal Cell-Derived Factor-1 α /CXCL12,” SDF-1 is a chemokine promoting stem-cell homing of EPCs by binding to its receptor C-X-C motif chemokine receptor type 4 (CXCR4). EPCs are derived from the bone marrow and are known to promote vascular repair and neovascularization. When vascular damage occurs, local growth factors and cytokines signal the bone marrow to release EPC targeted to the injured sites. EPC then differentiate into mature endothelial cells and assist in the reconstruction of the vasculature (199). In mice, genetic deletion or pharmacologic inhibition of DPP4 is able to increase the homing of CXCR4+ EPC at sites of myocardial damage, resulting in a reduced cardiac remodeling and improved heart function and survival (200). In a human study, Fadini et al. demonstrated that

type 2 diabetic patients receiving a 4-week course of therapy with the DPP4-inhibitor sitagliptin show increased SDF-1 α plasma concentrations and circulating EPC levels (199). Additionally, SDF-1 engineered to be resistant to DPP4 cleavage, and delivered by nanofibers, improves blood flow in a model of peripheral artery disease (201). Collectively, these studies implicate a rationale to use DPP4 inhibitors for vascular repair through stimulation of EPC and neovascularization.

Brain natriuretic peptide, another substrate of DPP4, plays an important role in regulating body fluid homeostasis and vascular tone through binding and subsequent activation of the cGMP-coupled natriuretic peptide receptor type A (NPR-A) (202). BNP is secreted predominantly by ventricular cardiomyocytes in response to increased wall stress. Thus, elevated BNP is a sensitive marker of heart failure and appears to play a role in cardiac remodeling and healing after acute MI (203–205). DPP4 cleavage of the physiologically active BNP (1–32) to BNP (3–32) effectively lowers plasma cGMP levels, reduces diuresis and natriuresis, and inhibits vasodilatation (83, 202).

Endocrine Effects of Soluble DPP4 on Cardiovascular Homeostasis

Although it is well established that serum levels of sDPP4 are altered in several pathological conditions and that sDPP4 is released from vascular cells, only a minor part of research has focused on potential endocrine effects of this proteolytic enzyme.

Considering that DPP4 is discussed in immunomodulation, it might be speculated that the inhibition of DPP4 modulates responses occurring within early or late atherosclerotic lesions. In low-density lipoprotein receptor-deficient (LDLR^{-/-}) mice, Shah et al. could demonstrate that exogenously injected DPP4 increases monocyte migration *in vivo* (180). Although these pro-migratory properties of DPP4 could be completely inhibited by sitagliptin, the underlying mechanism of these effects remains unclear. Moreover, the combined treatment of sDPP4 and LPS leads to increased expression and secretion of the pro-inflammatory cytokines, TNF α and IL-6. This upregulation was achieved by elevated levels of ERK, c-Fos, NF- κ B p65, NF- κ B p50, and CUX1, all factors known to bind to the promotor of TNF α and IL-6 (180). In accordance to that, Ikushima and collaborators observed that sDPP4 binds to the M6P/IGF-IIR resulting in enhanced transendothelial T cell migration (206). In a further study, sDPP4 binding to M6P/IGF-IIR leads to elevated ROS levels in HUVECs. In both studies, binding of DPP4 to this particular receptor was completely prevented by a DPP4 inhibitor (207).

In human vascular smooth muscle cells, we could show that sDPP4 activates the MAPK and NF- κ B signaling cascade resulting in pro-atherogenic changes in human vascular smooth muscle cells illustrated by an increased proliferation, the induction of iNOS and elevated expression, and secretion of pro-inflammatory cytokines (39). Additionally, we observed that all these detrimental effects of sDPP4 were PAR2 mediated, since both a PAR2 antagonist and PAR2 silencing completely prevented the sDPP4-induced effects. In collaboration with the group of Sánchez-Ferrer, we further showed that sDPP4 exhibits direct effects on vascular function illustrated by vascular reactivity of murine mesenteric arteries (208). sDPP4 impaired the endothelium-dependent relaxation to acetylcholine in a concentration-dependent manner by up to 75%, without modifying endothelium-independent relaxation to sodium nitroprusside. Again, enzymatic activity of DPP4 appears to be involved in this process. Similarly, the cyclooxygenase inhibitor indomethacin and the thromboxane A2 receptor antagonist SQ29548 abrogated the impairing action of DPP4. These data suggest that DPP4 directly impairs endothelium-dependent relaxation through a mechanism that involves cyclooxygenase activation, and likely the release of a vasoconstrictor prostanoid. Since sDPP4 has been reported not only to contribute to monocyte migration and macrophage-mediated inflammatory reactions but also stimulates the proliferation of

human coronary artery smooth muscle cells as well as impairs endothelium-dependent vasorelaxation, it might be speculated that sDPP4 itself acts as a risk factor for atherosclerosis.

Collectively, this section emphasizes that both membrane-bound and sDPP4 and its inhibition are not only playing an important role in glucose homeostasis but also in several other processes and organs involved in the pathogenesis of T2DM (Figure 2). This supports the notion that DPP4 exhibits pleiotropic properties that are not fully understood so far and have to be further elucidated in the future.

Conclusion

Dipeptidyl-peptidase 4, originally identified as an enzyme nearly 50 years ago, has now been recognized to exert pleiotropic functions with substantial impact for a variety of diseases. The complexity of DPP4 action stems from (i) a long list of substrates cleaved by the enzyme including hormones, growth factors, and cytokines, (ii) an additional function of this protein being a binding partner at the surface of different cells, specifically immune cells, and (iii) the recent discovery that DPP4 is an adipokine with different endocrine functions. Thus, an integrated view on this molecule is required to more precisely understand its impact for metabolic diseases like type 2 diabetes. For this disease, DPP4 inhibition has gained substantial interest, mostly related to the DPP4 substrate, GLP-1. As shown here, other substrates like SDF-1 and BNP should also be taken into account and may help to better understand the therapeutic potential of DPP4 inhibitors. In this context, the direct effects of DPP4 inhibitors require to be assessed in more detail, and several aspects like the cardio-protective function of DPP4 inhibition remains controversial. Finally, soluble DPP4 is emerging as a new research line, putting this molecule to the list of adipo-cytokines with pro-inflammatory and proliferative function. Combining the accumulated knowledge on DPP4 will lead to an improved understanding of its impact for health and disease.

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The role of dipeptidyl peptidase – 4 inhibitors in diabetic kidney disease

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Despite major advances in the understanding of the molecular mechanisms that underpin the development of diabetic kidney disease, current best practice still leaves a significant proportion of patients with end-stage kidney disease requiring renal replacement therapy. This is on a background of an increasing diabetes epidemic worldwide. Although kidney failure is a major cause of morbidity the main cause of death remains cardiovascular in nature. Hence, diabetic therapies which are both “cardio-renal” protective seem the logical way forward. In this review, we discuss the dipeptidyl peptidase 4 (DPP4) inhibitors (DPP4inh), which are glucose-lowering agents used clinically and their role in diabetic kidney disease with specific focus on renoprotection and surrogate markers of cardiovascular disease. We highlight the novel pleiotropic effects of DPP4 that make it an attractive additional target to combat the fibrotic and inflammatory pathways in diabetic kidney disease and also discuss the current literature on the cardiovascular safety profile of DPP4inh. Clearly, these observed renoprotective effects will need to be confirmed by clinical trials to determine whether they translate into beneficial effects to patients with diabetes.

Keywords: dipeptidyl peptidase 4, diabetic kidney disease, fibrosis, inflammation, renoprotection

The Incretin System

Under physiological conditions, blood glucose is tightly regulated by a number of mechanisms including the incretin system of hormones that are secreted in response to a meal. Glucagon like peptide 1 (GLP-1) and gastrointestinal peptide (GIP) are released from the gut and regulate postprandial glucose excursions. Proglucagon is secreted by the intestinal mucosa and is subsequently cleaved to GLP-1, which acts on the GLP-1 receptor in the pancreas in a glucose-dependent manner and functions to stimulate pancreatic insulin release and suppress glucagon secretion with the net effect of regulating postprandial glucose (1). The half-life of GLP-1 is very short (minutes) as it is rapidly degraded by dipeptidyl-peptidase 4 (DPP4). DPP4 cleaves two amino acids at the amino terminal end of peptides that have a proline (or less commonly an alanine) at the penultimate position (2). Hence GLP-1 (7–36) amide is cleaved to GLP-1 (9–36) amide, which is the major circulating form. GLP-1 (9–36) does not have insulinotropic effects.

Dipeptidyl peptidase 4 inhibitors (DPP4inh) are novel oral diabetic agents used to lower blood glucose in patients with type 2 diabetes mellitus. They inhibit DPP4, which degrades GLP-1 resulting in raised endogenous GLP-1. They promote insulin release and inhibit glucagon secretion, causing lower blood glucose. Favorable aspects of DPP4inh in comparison to other diabetic agents include its oral formulation, reduced risk of hypoglycemia and lack of weight gain. In addition, DPP4inh have benefits beyond glucose lowering as DPP4 cleaves a host of peptides/substrates in addition to GLP-1, resulting in a broad range of altered biological functions.

In this review, we focus on the pleiotropic properties of DPP4 beyond glucose lowering with specific reference to renoprotection and cardiovascular aspects. This is summarized in **Figure 1**. The clinical relevance of this is the fact that up to a third of patients with diabetes suffers renal complications with the main cause of mortality being cardiovascular in origin. Hence, continued inquiry into mechanisms of renal failure in patients with diabetes that will lead to examination of novel strategies to limit cardiovascular and renal disease is the logical step forward.

DPP4 – A Multifunctional Protein

DPP4 is a 110-kDa type 11 integral membrane glycoprotein and is expressed ubiquitously in most organs and cell types. DPP4 exists in both a soluble and membrane bound form, both of which are capable of proteolytic activity. The soluble form in the circulation is thought to arise from shedding of the membrane bound DPP4 and is the target for DPP4inh as hypoglycemic agents in clinical use (1). Identified substrates include GLP-1, regulated on activation, normal T cell expressed and secreted (RANTES), which is relevant in renal disease (3, 4), brain natriuretic peptide 1–32 (5, 6), neuropeptide Y (7), high mobility group protein 1 (HMGB1) (8), and Substance P (9). More recently, aggregates of amyloid beta peptides (pathological hallmarks of Alzheimers disease) have also been identified as substrates of DPP4 (10). Many substrates have been identified as being pharmacological substrates (cleaved *in vitro*) but are not cleaved *in vivo*. Hence, this proteolytic effect can either lead to a gain in function, a loss of function or have no functional significance. An example of the physiological relevance of these non-GLP-1 substrates is highlighted by DPP4 truncation of certain chemokines, colony-stimulating factors, and interleukins, which have recently been linked to regulation of hematopoietic stem/progenitor cells where DPP4inh has been shown to enhance engraftment of cord blood in patients with hematological malignancies (11, 12).

The membrane bound form of DPP4, expressed on the surface of many cell types including kidney tubular cells, endothelial cells and T cells, is of major interest with respect to the pleiotropic actions of DPP4. Membrane-bound DPP4 also exerts non-enzymatic actions by virtue of co-localizing with other membrane proteins and modulating their intrinsic actions (2, 13). DPP4 is known to interact with adenosine deaminase (14), caveolin 1 (15, 16), cation independent mannose 6 phosphate

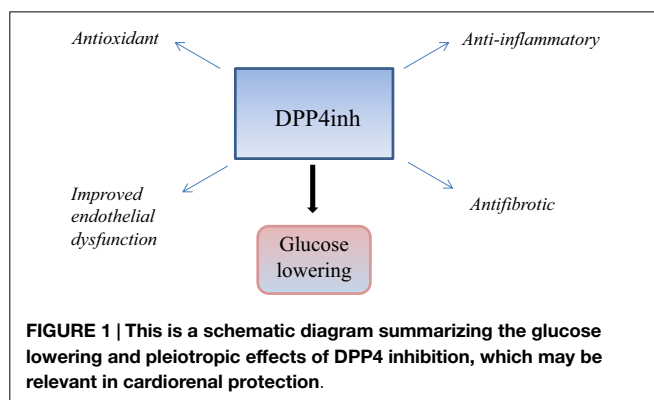
receptor (CIM6PR) (also known as insulin growth factor receptor 2) (17, 18), and glypican 3 (19). Interestingly, it has been recognized that DPP4 interacts with human coronavirus-EMC spike protein and acts as a receptor for this virus, which provides new knowledge to facilitate the direction of interventional strategies in related respiratory illness (20).

DPP4 and the Kidney

The proteolytic functions of DPP4 in the kidney have been described using liquid chromatography mass spectrometry-based peptidomics, where kidney tissue from DPP4^{+/+} and DPP4^{-/-} mice were compared. This revealed 10 peptides regulated by DPP4 *in vivo* (21, 22). Further studies with brush border membranes showed that aminopeptidase activity is required to generate DPP4 substrates. This suggests that DPP4 is involved in the extracellular catabolism of proteins in the kidney, specifically the degradation/catabolism of proline-containing peptides (23). As DPP4 is present on the brush border (apical side) of kidney proximal tubular cells, DPP4 inhibition is likely to alter the degradation/regulation of peptides in the lumen and thus influence the tubular cell structure or function in diabetes. Identifying the functionally relevant renal substrates of DPP4 will help us understand and anticipate long-term effects of DPP4 inhibition on the kidney in patients with diabetes. The tools required to validate identified substrates are limited given that specific antibodies must be able to distinguish between cleaved and non-cleaved peptides, which differ by only two amino acids at the N-terminal end.

Transforming growth factor beta 1 (TGFβ1) is a major driver of fibrosis in diabetic kidney disease. We have reported that linagliptin, a DPP4inh, reduces high glucose-induced active TGFβ1 in human kidney proximal tubular cells (24) with a downstream reduction in phosphorylated smad2 and fibronectin transcription and expression. As high-glucose-induced total secreted TGFβ1 was unchanged by linagliptin, we postulated that the mechanism was related to interference with the conversion from latent to active TGFβ1. TGFβ1 is secreted in a latent form and requires a complex interplay of soluble signaling molecules in the activation process, which releases it from the latency associated peptide (LAP) (25). Once released from the LAP, the unbound TGFβ1 can then bind to its receptor to initiate cell signaling via the Smad pathway. In pursuing this further, we showed that the interaction between CIM6PR and DPP4 is “switched on” by high glucose, and hence is maximally modulated by linagliptin in this environment. In the presence of excess M6P, the CIM6PR binding sites become saturated, resulting in the reduction in CIM6PR/DPP4 interaction, which would imply that the interaction is occurring through a M6P residue on the DPP4 molecule. The fact that linagliptin also reduced this interaction, suggests a M6P-mediated mechanism, which is independent of GLP-1 as our *in vitro* system is lacking in GLP-1 (unpublished data). This novel finding adds to the body of knowledge that DPP4 inhibition can exert antifibrotic effects through its non-proteolytic properties. This is discussed further below with respect to endothelial cell dysfunction.

In interpreting preclinical studies, it is important to appreciate the confounding aspects of concomitant glucose lowering by DPP4inh. There are two studies, which have looked at the



effect of DPP4inh (using vildagliptin and sitagliptin) on the diabetic animal kidney and both show renoprotection. However, the HbA1c (hemoglobin A1C) in the DPP4inh-treated diabetic animals was lower than in the diabetic-control animals (26, 27). So in both these *in vivo* studies, it is difficult to conclude that the renal effects of DPP4inh lie above and beyond glucose lowering.

Kanasaki and colleagues investigate the antifibrotic properties of linagliptin in a Type 1 model of diabetic nephropathy and showed that after 4 weeks, linagliptin ameliorated diabetic kidney fibrosis, an observation that occurred in association with the inhibition of endothelial-mesenchymal transition (EndMT) and the restoration of microRNA29s (28). An insulin-deficient model of diabetes enables evaluation of the effects of the DPP4inh independent of glycemic control as DPP4inh will not have a significant hypoglycemic effect due to the lack of a significant insulin response mediated by GLP-1. As a result, the new findings provide information regarding renal benefit of linagliptin independent of glycemic control.

The antifibrotic properties of DPP4inh have also been shown in other models of kidney fibrosis such as the unilateral ureteral obstruction (UUO) model (29). In this TGF β -driven model, LC15-0444 (a DPP4inh) reduced inflammatory and fibrotic markers such as levels of phosphorylated Smad2/3, TGF β 1, toll-like receptor 4, HMGB1, NADPH oxidase 4, and nuclear factor kappa B. However, this study lacked specific mechanistic data to explain the findings.

There is also experimental evidence to suggest that DPP4 is involved in the advanced glycation end product – receptor axis, which is highly relevant in diabetic kidney disease. Both DPP4 deficiency and linagliptin reduced renal damage in animal models of Type 1 diabetic nephropathy (30, 31). The mechanism proposed was related to reducing endothelial cell oxidative stress.

Likewise, DPP4inh has been explored in animal models of obesity, insulin resistance, and renal disease. The DPP4inh MK0626 was shown to reduce obesity-induced renal injury (MK0626) and attenuated filtration barrier injury and oxidant stress in the Zucker obese rat (linagliptin) (32, 33).

Endothelial dysfunction is an inherent aspect of diabetic kidney disease. Shi et al. demonstrated that in endothelial cells, DPP4 and integrin β 1 physically interacted. DPP4-associated endothelial to mesenchymal transition was inhibited by integrin β 1 deletion. In addition, DPP4 or integrin β 1 deficiency resulted in the inhibition of TGF β 2-stimulated heterodimer formation of TGF β receptors. Along with this, the interaction between DPP4 and integrin β 1 induced vascular endothelial growth factor receptor (VEGF-R) 1 expression with the concomitant suppression of VEGF-R2 levels (34). Importantly, linagliptin was able to reduce the TGF β 2 physical interaction between integrin and DPP4. These results indicate that the non-proteolytic interaction between DPP4 and integrin β 1 is relevant in diabetic kidney fibrosis.

Mechanistic studies designed to delineate whether effects on glucose-induced endothelial impairment are related or independent of GLP-1/GLP-1R showed that in addition to GLP-1 receptor effects, DPP4inh with linagliptin exerted direct effects on rat mesenteric vessels independent of any glucose lowering effect. The mechanism was shown to be related to its antioxidant effect through direct radical scavenging (35).

DPP4 inhibitors also exert anti-inflammatory effects. This has been demonstrated in a non-diabetic animal model of glomerular injury where alogliptin reduced macrophage infiltration (36) and likewise in a type 1 diabetic nephropathy model where PKF275-055 reduced macrophage infiltration and nuclear factor kappa B activity (37).

It is increasingly evident that although DPP4inh have similar capacity to improve glucose control they have very different pharmacokinetic and pharmacodynamic profiles as they bind to different sites on DPP4 (38). All DPP4 inhibitors can block the enzymatic (i.e., cleaving) property of DPP4 but may differ in their ability to influence the co-stimulatory/receptor properties of DPP4 (2). The mode of clearance of DPP4 inhibitors may also be relevant (39). DPP4 is highly expressed on the luminal aspect of the proximal tubular brush border which implies that drugs which are preferentially filtered by the kidney have better access to tubular DPP4.

In summary, preclinical studies suggest that DPP4inh offer renoprotection above and beyond glucose lowering through its proteolytic, protein–protein interaction, and antioxidant properties. Given the increased cardiovascular risk in this population, it would be highly desirable to have a glucose-lowering drug, which also offers cardiovascular benefit in addition to renoprotection. Regulatory agencies now mandate that new diabetic agents undergo cardiovascular assessment prior to marketing. The agreed-on primary endpoint for cardiovascular safety analyses is Major Adverse Cardiovascular Events Plus (MACE-plus), defined as a composite endpoint consisting of the following adjudicated events: cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, and hospitalization for unstable angina (40). The next section summarizes major clinical trials in this area with cardiovascular and renal endpoints.

Cardiovascular Aspects

Clinical trials evaluating both cardiovascular and renal endpoints have commenced with the CARMELINA study, which will include more than 8,000 adults with Type 2 Diabetes and linagliptin (ClinicalTrials.gov Identifier: NCT01897532). The primary endpoint will be time to the first occurrence of either cardiovascular death (including fatal stroke and fatal myocardial infarction); non-fatal myocardial infarction; non-fatal stroke; or hospitalization for unstable angina pectoris. The renal outcome will be measured as a composite of renal death, sustained end-stage renal disease and sustained decrease of $\geq 50\%$ eGFR.

Although DPP4inh have offered some promise with pre-clinical studies and *post hoc* analyses of clinical trials suggesting a positive cardiovascular risk profile, a recent randomized placebo controlled trial (SAVOR-TIMI 53) published in the New England Journal of Medicine by Scirica et al. showed no change in ischemic events but higher hospitalizations for heart failure with saxagliptin. This study enrolled 16,492 patients with type 2 diabetes with a history of, or were at risk for, cardiovascular events and followed up for a median of 2.1 years (41). The EXAMINE study evaluated alogliptin use in patients with type 2 diabetes after acute coronary syndromes showed that there was no increase in the rates of MACE-plus (42). This randomized

placebo-controlled trial involved 5,380 patients and had a median follow up of 18 months. The US Food and Drug Administration (FDA) independent advisory committee recently voted that the cardiovascular safety profile of these two drugs could be managed by updating the drugs' labels (43). Recently, a large multicentre clinical trial TECOS (Sitagliptin on Cardiovascular Outcome Study) where 14,671 patients with established cardiovascular disease and Type 2 diabetes were randomized to receive either saxagliptin or placebo (in addition to their usual therapy) showed no increase in the risk of MACE-plus, hospitalization for heart failure, or other adverse events (44). Another large multicentre trial with linagliptin CAROLINA (Cardiovascular Outcome Study of Linagliptin Versus Glimepiride in Patients with Type 2 Diabetes) is currently in progress (44). As DPP4inh raise GLP-1, it is also important to take into account the cardiovascular profile of GLP-1 analogs. In a large retrospective analysis by Best et al., patients treated with the GLP1 analog exenatide were shown to

have a 20% reduction in cardiovascular events in comparison with other glucose-lowering agents (45). However, the studies that have demonstrated cardiovascular benefit using GLP1 or an analog do so using supra physiological doses. DPP4inh promote GLP1 action only within the physiological range and DPP4inh are likely to have pleiotropic effects independent of their effects on GLP1 as outlined above. Further details about these trials can be found in www.clinicaltrials.gov. There are several DPP4 inhibitors in clinical use e.g., sitagliptin, saxagliptin, vildagliptin, alogliptin and linagliptin. They differ structurally and in their pharmacokinetic profiles and hence the outcome of ongoing trials is important as a class effect cannot be presumed.

In conclusion, DPP4 inhibitors hold promise as an antifibrotic and anti-inflammatory agent in kidney disease with a large clinical trial currently underway to evaluate hard renal endpoints. The jury is still out on whether the risk of heart failure is a class effect.

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