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A functional heteromeric MIF receptor formed by CD74 and CXCR4

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Abstract

MIF is a chemokine-like inflammatory mediator that triggers leukocyte recruitment by binding to CXCR2 and CXCR4. MIF also interacts with CD74/invariant chain, a single-pass membrane-receptor. We identified complexes between CD74 and CXCR2 with a role in leukocyte recruitment. It is unknown whether CD74 also binds to CXCR4. We demonstrate that CD74/CXCR4 complexes formed when CD74 was expressed with CXCR4 in HEK293 cells. Expression of CD74-variants lacking an ER-retention signal showed CD74/CXCR4 complexes at the cell surface. Importantly, endogenous CD74/CXCR4 complexes were isolated by co-immunoprecipitation from monocytes. Finally, MIF-stimulated CD74-dependent AKT activation was blocked by anti-CXCR4 and anti-CD74 antibodies and AMD3100, whereas CXCL12-stimulated AKT activation was not reduced by anti-CD74. Thus, CD74 forms functional complexes with CXCR4 that mediate MIF-specific signaling.

Keywords

CD74; CXCR4; MIF; Chemokine receptor; GPCR; Invariant chain

1. Introduction

Macrophage migration inhibitory factor (MIF) is a structurally unique 12.5 kDa cytokine that is a critical mediator of acute and chronic inflammatory diseases such as septic shock, Crohn's disease, rheumatoid arthritis, and atherosclerosis, as well as cancer [1–4]. Contrary to its historic name, MIF has been recognized to exhibit chemokine-like properties [5,6] and was identified as a non-cognate ligand of the chemokine receptors CXCR2 and CXCR4 [5].

Chemokines are chemotactic cytokines that orchestrate the activation and recruitment of leukocytes during immune surveillance and inflammation. The structural classification of chemokines and their G protein–coupled receptors (GPCRs) has been well defined. Over 50

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chemokines bind to some 20 receptors, which constitutes a redundancy with respect to ligand action [7–10]. CXCR2 is the prototypic receptor for ELR+ chemokines such as CXCL8 (interleukin-8, IL-8) or CXCL1/GRO-α. Pathophysiological processes ranging from inflammatory leukocyte recruitment to cancer cell migration and angiogenesis are mediated by CXCL8/CXCR2 [7]. CXCR4 has been considered the sole receptor for the chemokine CXCL12 (stromal cell-derived factor-1α, SDF-1α), although CXCR7 has recently been suggested as an alternative receptor for CXCL12 [11]. The dogma of a monogamous relationship between CXCR4 and CXCL12 also has been challenged by the observations that HIV gp120 and MIF bind to CXCR4 [4,5,12]. The CXCL12/CXCR4 axis is involved in various homeostatic and inflammatory cell migration processes, including inflammatory and atherogenic T cell recruitment, stem cell homing, and cancer cell metastasis [7,13].

In spite of architectural similarities, MIF does not belong to the canonical CC, CXC, CX_3C, or C sub-families of chemokines and has been classified as chemokine-like function (CLF) chemokine [4,5,14]. Binding of MIF to CXCR2 is dependent on a pseudo(E)LR motif that is reminiscent of the ELR motif [15]. Through its dual interaction with CXCR2 and CXCR4, MIF uniquely triggers both monocyte and T cell arrest on atherogenic endothelium, promotes leukocyte transmigration, and increases the formation and progression of atherosclerotic plaques [4,5].

MIF also interacts with CD74, a single-pass type II membrane protein, also known as MHC class II invariant chain (Ii) [16]. Ii associates with MHC class II molecules during endoplasmic reticulum (ER)-endolysosomal traffic and functions as class II chaperone to control antigenic peptide loading. CD74 is the membrane form of Ii, which in smaller percentage traffics to the plasma membrane, presumably after modification by chondroitin sulfate. Surface CD74 is expressed on class II-positive cells but upon inflammatory stimulation, can be detected at the plasma membrane of class II-negative cells, including stromal and epithelial cell types [17,18]. There is ample evidence that surface interaction of MIF with CD74 leads to activation of ERK1/2-MAPK and AKT/PI3K signaling. CD74 also is important for the regulation of B cell survival by MIF [19]. MIF-triggered ERK1/2 signaling requires the recruitment of CD44 and a non-receptor tyrosine kinase [16,20]. We recently identified receptor complexes between CD74 and CXCR2. The precise molecular details of this interaction are unclear, but it has become evident that CD74/CXCR2 complexes play an important role in MIF-driven atherogenic leukocyte recruitment [5]. Binding of CD74 to CXCR2 exemplifies heteromeric receptor complex formation between a GPCR and a single-pass transmembrane protein and may be associated with cross-talk between receptor tyrosine kinase (RTK) complexes and GPCR [4,5].

We examined whether CD74 interacts with CXCR4, the second chemokine receptor utilized by MIF. Applying flow cytometry and fluorescence microscopy, we established that HEK293 cells transfected with tagged fusion proteins of CD74 and CXCR2 expressed both receptors. A CD74 variant lacking the ER retention signal that prevents surface trafficking of CD74 in the absence of class II was applied to verify interactions between CD74 and CXCR4 at the cell surface. We then investigated an interaction between these receptors by co-immunoprecipitation (CoIP)/pull-down analysis and confocal microscopy using overexpressed receptor fusion and endogenous proteins. A functional interplay between CD74 and CXCR4 was addressed in a MIF-triggered CD74-dependent signaling assay by applying a CXCR4-specific small molecule inhibitor and by comparing the effects of MIF and CXCL12 in the presence versus absence of blocking anti-CD74 and anti-CXCR4 antibodies.

2. Materials and methods

2.1. Plasmids, antibodies and reagents

Recombinant human MIF (rMIF) was prepared as described and contained negligible concentrations of endotoxin (<10 pg endotoxin/μg MIF) [21]. AMD3100 was purchased from Sigma.

The plasmid for V5-tagged CD74, pcDNA3.1/V5-His-TOPO-CD74, has been described [16]. pEYFP-C1-CD74 was generated by cloning the cDNA encoding human CD74 into pEYFP-C1, wherein the cDNA for CD74 was amplified from pcDNA3.1/V5-His-TOPO-CD74 [16] using primers: 5′-TCCGCTCGAGCGATGCACAGGAGGAGA-3′ and 5′-AGCGAATTCCGTCACATGGGGACTGGGCC-3′. Untagged CD74 plasmid encoding for a p33 variant of human CD74 devoid of the ER retention signal [22] and that is abundantly targeted to the cell surface was termed pCD74minRTS and was a gift of Carolus Therapeutics (San Diego, CA, USA). pEYFP-C1-CD74minRTS was cloned by inserting CD74minRTS into pEYFP-C1 (see above). pECFP-N1-CXCR4 was generated by cloning human CXCR4 cDNA into pECFP-N1. The CXCR4 cDNA was amplified from pcDNA3.1-CXCR4$(HA)_3$ (Missouri S&T cDNA Resource Center) using primers: 5′-TCCGCTCGAGCCATGTACCCATACGAT-3′ and 5′-AGCGAATTCCGCTGGAGTGAAAACTTGAAG-3′. pBABEpuro was from B. Lüscher (RWTH Aachen University) and originally obtained from Addgene, USA.

Antibodies used were: mouse monoclonal antibody (mAb)-V5 (R960-25, Invitrogen, Karlsruhe, Germany), rat anti-HA mAb (3F10, Roche Diagnostics, Mannheim, Germany), mouse isotype control IgG1 (14-4714, eBioscience, San Diego, USA), human anti-CD74 mAb (M-B741, 555538, BD Pharmingen, San Diego), human anti-CD74 mAb (C-16, sc-5438, Santa Cruz Biotechnology), polyclonal rabbit anti-CXCR4 (c8352, Sigma, Taufkirchen, Germany), horseradish peroxidase (HRP)-conjugated anti-mouse antibody (1858413, Pierce Biotechnology, Rockford, USA), HRP-conjugated anti-rat antibody (NA9350, GE Healthcare/Amersham, Freiburg, Germany) and HRP-conjugated anti-rabbit IgG (1858415, Pierce) for CoIPs and pull-downs. Fluorescein-conjugated mouse anti-human CXCR4 (FAB170f, R&D Systems) and mouse anti-human CD74 (M-B741, 555540, BD Pharmingen) were applied for flow cytometry. Anti-phospho-AKT (Ser 473-specific, Cell Signaling, Danvers, MA, USA), anti-actin (Ab 691001, MP Biomedicals, Eschwege, Germany), HRP-conjugated sheep anti-mouse IgG (GE Healthcare) and HRP-conjugated donkey anti-rabbit IgG (GE) were used for the AKT signaling assay. For neutralization experiments, anti-human CXCR4 mAb (MAB171, R&D Systems, Wiesbaden, Germany), anti-human CD74 (Ab 555612, BD Pharmingen, Heidelberg, Germany), and an isotype IgG1 (eBio-sciences) were used. Other reagents were of the highest grade commercially available.

2.2. Cell culture and transfections

Cell culture reagents including media, supplements and antibiotics were from Invitrogen (Karlsruhe, Germany). Unless stated otherwise, cell culture experiments were performed at 37 °C in a humidified incubator with 5% CO_2.

Human embryonic kidney cells (HEK293) and MonoMac6 human monocytes were purchased from the German Society for Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with Glutamax™ containing 4.5 g/l D-glucose and sodium pyruvate, containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). MonoMac6 cells were cultured in RPMI 1640 medium containing 10% FCS, 1% P/S, 1% non-essential amino acids (NEAA), 9 μg/ml human insulin (Sanofi-Aventis) and 1 mM

sodium pyruvate. Jurkat T cells were purchased from ATCC (TIB152) and were cultured in RPMI 1640 medium containing 10% FCS and 1% P/S.

For transient transfections of HEK293, FuGENE HD transfection reagent from Roche Diagnostics (Grenzach-Wyhlen, Germany) was used.

2.3. Flow cytometry

HEK293 transfectants, MonoMac6 and Jurkat cells were subjected to flow cytometry. Jurkat T cells were treated in medium containing reduced concentrations of FCS (0.5%) for 24–48 h before flow cytometric analysis. For each measurement, 200 000 cells were washed once with buffer (1× phosphate-buffered saline (PBS), pH 7.2, containing 0.5% bovine serum albumin (BSA) and 0.1% azide). Cells were labeled with anti-CXCR4-FITC (1:3.5) or anti-CD74-FITC (1:20). After washing and resuspension, cells were analyzed on a FACS Canto™ (BD Bioscience, Heidelberg, Germany).

2.4. Immunofluorescence microscopy and colocalization analysis

One day after seeding, HEK293 cells were transiently transfected with pECFP-N1-CXCR4, pEYFP-C1-CD74minRTS, and pBABEpuro, respectively, and incubated for 24 h at 37 °C and 5% CO_2. Medium was changed and cells selected by addition of puromycin (1 μg/ml, Calbiochem, Darmstadt, Germany) for 24 h. Medium was aspirated, cells detached and centrifuged at 1250 rpm for 5 min. The supernatant was aspirated and the pellet resuspended in DMEM medium. To one well of a 6-well-plate containing a sterile poly-L-lysine-coated cover slip, 3 ml of the cell suspension were added. Seeded cells were incubated for 24 h at 37 °C and 5% CO_2, were washed under live-cell imaging conditions [5] and studied using an Axiovert 100 M confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.5. Coimmunoprecipitations and pull-down experiments

Fourty-eight hours after transfection with combinations of plasmids pcDNA3.1/V5-His-TOPO-CD74 and pcDNA3.1-CXCR4$(HA)_3$, or pCD74minRTS and pcDNA3.1-CXCR4$(HA)_3$, HEK293 transfectants were disrupted with CoIP buffer (50 mM Tris–HCl, pH 7.4, containing 100 mM NaCl, 15 mM EGTA, 1% Triton X-100, 1% CHAPSO, 0.02% azide, and protease inhibitor cocktail (Calbiochem, Darmstadt, 1:100)). After clearance of lysates, samples were incubated for 2 h at 4 °C with either anti-V5 or -HA antibodies or isotype mouse IgG_1 (negative control). Protein-G beads (NEB, Frankfurt) were blocked with PBS, containing 1% BSA, and resuspended in 200 μl CoIP buffer. Lysates were incubated with pre-treated protein-G beads overnight at 4 °C. Beads with bound immune complexes were washed once with 500 μl CoIP buffer and 4× with a 1:5 dilution CoIP buffer. Beads were resuspended in sample buffer and samples subjected to SDS-PAGE/Western blotting. Blots were revealed with rat anti-HA (1:2000), mouse anti-V5 (1:5000), rabbit anti-CXCR4 (1:1000), or anti-CD74 (1:500) antibodies. HRP-conjugated anti-mouse (1:1000) or anti-rat (1:1000) antibodies were used as secondary antibodies. Chemiluminescence was detected by ECL (Pierce). For corresponding pull-down controls, blots were inversely developed with anti-V5, anti-HA, or anti-CXCR4, respectively.

For immunoprecipitation of endogenous receptor complexes, MonoMac6 cells were cultured in the presence of human interferon-γ (Peprotech, 1:100) for 72 h, before the above protocol was applied. Pull-downs of receptor complexes from the lysates of these cells were performed with an anti-CD74 antibody versus IgG_1 as control. For Western blot, anti-CXCR4 (1:1000) antibody was used as primary antibody and HRP-conjugated anti-rabbit IgG (1:1000) as secondary antibody. CD74 pull-down controls were revealed by Western blotting with anti-CD74 (1:500).

2.6. AKT signal transduction assay

For AKT activation analysis in Jurkat cells, cells were grown inRPMI 1640 medium containing 10% FCS and medium replaced by RPMI 1640/0.5% FCS for 24 h. Untreated Jurkat cells and those pre-incubated with the CXCR4-specific inhibitor AMD3100 (1 μg/ml, 30 min) were incubated with rMIF (0-1250 ng/ml, 10 min). For blocking CD74 or CXCR4, cells were pre-treated with 10 μg/ml neutralizing anti-CD74 mAb or 10 μg/ml anti-CXCR4 mAb for 30 min. For control, pre-treatment with isotype IgG was performed. Phosphorylation ratios (phospho-Ser473-AKT/actin) were calculated by band densitometry using Aida Image Analyser Software (Raytest Isotopen, Berlin, Germany).

2.7. Statistical analysis

Statistical significance between two groups of data was evaluated by Student's *t*-test. P values below 0.05 were considered statistically significant.

3. Results

3.1. Colocalization and protein complex formation of CD74 and CXCR4 receptor fusion proteins

To study the interaction between CD74 and CXCR4, we applied the following fusion proteins: (i) a V5-His-tagged p35 form of CD74, (ii) an HA-tagged CXCR4, and (iii) CXCR4-CFP with the CFP-tag fused C-terminally to CXCR4. A variant of CD74 lacking the ER retention signal mediating ER retention of CD74 (CD74minRTS) in cells that do not express MHC class II was used as untagged protein (iv) and YFP-tagged fusion protein with YFP linked to the N-terminus of CD74 (v).

Using Western blotting we first established that CD74 and CXCR4 fusion proteins were efficiently overexpressed in HEK293 cells, which do not endogenously express CD74 and only low levels of CXCR4 (data not shown). Flow cytometry histograms confirmed expression of HA-tagged CXCR4 and demonstrated surface localization of this receptor fusion protein (Fig. 1a). However, V5-tagged CD74 could not be detected on the surface of HEK293 cells (Fig. 1b). It is known that only a minor portion of Ii/CD74 is targeted to the surface, whereas most of it remains localized in the ER/endolysosomal compartment. We thus ectopically expressed the CD74minRTS variant, which proved to exhibit abundant surface expression (Fig. 1c). To further confirm surface expression of CXCR4 and CD74, we applied CXCR4-CFP and YFP-CD74minRTS in confocal fluorescence microscopy studies. These experiments verified marked surface expression of both receptor constructs (Fig. 1d) and provided evidence that YFP-CD74 and CXCR4-CFP co-localized in sections of the plasma membrane of the double-transfectants. In line with the notion that CXCR4 also is rapidly re-internalized following surface targeting [23], intracellular co-localization between the fluorescent fusion receptors was also observed, especially at subcellular spots of excessive YFP-CD74 expression (Fig. 1d).

We next studied receptor complex formation of CD74 and CXCR4 by biochemical methods. V5-tagged CD74 was pulled-down from HEK293 lysates by anti-V5 antibody and co-precipitated CXCR4 revealed by Western blotting using anti-HA or anti-CXCR4 antibody. A strong CXCR4 band was detected under these conditions whereas blots from pull-downs applying protein G beads alone were devoid of CXCR4, indicating that overexpressed V5-CD74 strongly bound to HA-CXCR4 in HEK293 cells (Fig. 2a, left). Pull-down efficiency was confirmed by anti-V5 Western blot (Fig. 2a, right). For confirmation, the pull-down/Western blot experiment was performed in reverse setting, i.e. pull-down of CXCR4 with anti-HA and blot development with anti-V5 antibody. This experiment yielded a specific band, again suggesting CD74/CXCR4 complex formation, whereas the control pull-down

only showed a weak non-specific band (Fig. 2b, left). Pull-down efficiency was confirmed by anti-HA or anti-CXCR4 Western blot (Fig. 2b, right). To ask whether increased plasma membrane expression of CD74 would lead to an improved pull-down rate of CXCR4/CD74 complexes, HEK293 cells were transfected with pCD74minRTS together with pHA-CXCR4 and complexes pulled-down by anti-HA antibody. In fact, the band for co-precipitated CD74 devoid of the ER retention signal was more pronounced than that for V5-full length CD74 (compare Fig. 2c, left with Fig. 2b, left), indicating that CXCR4/CD74 complex formation is favoured at the plasma membrane. Again, pull-down efficiency was confirmed by anti-HA Western blot (Fig. 2c, right).

3.2. Protein complex formation of endogenously expressed CD74 and CXCR4 receptors

To verify that CD74 and CXCR4 bind to each other, we studied receptor complex formation of the endogenously expressed proteins in human MonoMac6 monocytes. These cells endogenously expressed substantial levels of both receptors on their surface (Fig. 3a and b). Importantly, when lysates of MonoMac6 cells were immunoprecipitated with anti-CD74, endogenous CXCR4 was found to be coprecipitated as revealed by anti-CXCR4 Western blot showing a strong band at ~40 kDa which was not present in the control CoIP (Fig. 3c). A second, apparently specific, band was seen at 45–47 kDa and may represent a differentially glycosylated CXCR4 species. Pull-down efficiency was confirmed by anti-CD74 blot (Fig. 3d). Thus, portions of endogenously expressed CD74 can bind to fractions of endogenously expressed CXCR4 in monocytes.

3.3. MIF-stimulated AKT activation in T lymphocytes is dependent on both CD74 and CXCR4

We previously demonstrated CD74-dependent AKT activation by MIF in mouse embryonic fibroblasts [24]. Here we asked whether complex formation between CD74 and CXCR4 was accompanied by a functional interplay between these receptors during MIF signaling. AKT activation in Jurkat T cells, which endogenously express appreciable levels of both CXCR4 and CD74 on the plasma membrane (Fig. 4a and b), was triggered by rMIF. To ask whether MIF-stimulated AKT activation was mediated by CXCR4/CD74 complexes, CD74 was blocked with a neutralizing CD74 antibody and CXCR4 was inhibited by AMD3100 or CXCR4 antibody. Results were compared to MIF-stimulated cells in the absence of inhibitor. Phospho-AKT levels were measured 10 min post-stimulation. Fig. 4c and d demonstrates that AMD3100 blocked this MIF signaling process. Densitometric scanning of the phospho-AKT to actin ratio showed a significant decrease in MIF-dependent AKT phosphorylation due to AMD3100. Similarly, both anti-CXCR4 and anti-CD74 inhibited MIF-stimulated AKT signaling. In contrast, only anti-CXCR4 markedly blocked AKT phosphorylation induced by SDF-1α (Fig. 4e and f). Together, this confirmed that MIF- but not SDF-1α-induced AKT signaling is mediated by CD74/CXCR4 complexes.

4. Discussion

MIF is an inflammatory cytokine [1] with chemokine-like properties [5,6,25]. The receptor mechanism(s) by which MIF activates target cells have long been unclear. Today it is known that, dependent on the target cell and inflammatory context, MIF can engage three receptors: (i) CD74, the membrane form of MHC class II invariant chain [16], (ii) CXCR2, the cognate receptor for ELR+ CXC chemokines such as CXCL8 [5], and (iii) CXCR4, the cognate receptor for SDF-1α/CXCL12 [5]. Through interaction with CD74, MIF stimulates ERK1/2 and AKT signaling, regulating cell proliferation and survival [16,20,24,26]. The MIF/CXCR2 and MIF/CXCR4 ligand/receptor axes are critical for atherogenic monocyte/neutrophil and T cell recruitment, respectively, and involve activation of G_i protein-, PI3K-, calcium- and integrin-mediated signaling [4,5,25,27]. Antibody blockade experiments in

atherogenic arteries revealed that atherogenic leukocyte recruitment by MIF is dependent on CD74 and an overlap/cross-talk between the MIF/CD74 and MIF/CXCR2 or MIF/CXCR4 signaling pathways has been suggested [5]. Receptor binding studies showed that MIF can individually bind to CD74 or CXCR2/CXCR4 with high affinity independent of whether the other receptor type was coexpressed; yet coexpression of CD74 with CXCR2 or CXCR4 as it occurs on monocytes amplifies MIF-triggered responses [5]. Thus, it has been proposed that functional heteromeric CD74/CXCR receptor complexes can form. In fact, following overexpression of CD74 and CXCR2 or under semi-endogenous expression conditions, heteromeric CXCR2/CD74 receptor complexes have been demonstrated [5].

Here we asked whether CXCR4/CD74 receptor complexes exist and are responsive to MIF. We provide evidence that portions of CXCR4 and CD74 can form a complex under overexpression conditions, and, importantly under endogenous conditions in monocytes and T cells. Moreover, our study suggests that CXCR4/CD74 complexes are responsive to MIF, as MIF-triggered CD74-dependent AKT activation in T cells was blocked by AMD3100, a small molecule inhibitor of CXCR4, as well as by CD74 and CXCR4 antibodies.

Both CD74 and CXCR4 form homomeric oligomers. The oligomeric species of CD74/Ii thought to interact with MHC class II during endoplasmic reticulum/Golgi/endolysosomal passage is a CD74/Ii trimer. It is unknown whether CD74 trimers occur on the cell surface, although plasma membrane appearance of nonameric MHC II/Ii complexes in combination with high surface turnover has been suggested to contribute to endosomal targeting of MHC II/Ii complexes [18,28–31]. In situations of relative Ii abundance over class II molecules in the Golgi/endolysosomal compartment, excess Ii molecules travel to the plasma membrane. Also, CD74 can be transported to the plasma membrane in the absence of class II [18]. CXCR4, like CXCR2, has been found to form dimeric complexes, and higher order oligomers have been observed under certain local or inflammatory conditions [32]. Here we demonstrate that portions of CXCR4 and CD74 can form heteromeric receptor complexes.

Although unusual, interactions between the chemokine/GPCR system and single-pass receptors are not unprecedented. In addition to the CD74/CXCR2 interaction, numerous reports have suggested signaling cross-talk between GPCRs and receptor tyrosine kinases (RTK) [33,34]. Cross-talk between CXCR4 and an RTK also has been observed [35]. Of note, it was demonstrated that CD74 directly interacts with angiotensin II type I receptor (AGTR1), a typical GPCR [36]. Interestingly, the binding site for CD74 on AGTR1 in localized in the carboxy-terminal tail, a site identified as important for the exit of AGTR1 from the ER and conserved in many GPCRs [36].

Overall, there has been scarce knowledge about the potential different functions of oligomeric CXCR chemokine receptor species. The same is true for CCR chemokine receptors for which several oligomeric species have been detected [32]. Thus, our study adds to an understanding of the functionality of homo- and heteromeric chemokine receptor oligomerization.

Identification of the CD74/CXCR4 interaction adds new protein entities to the limited number of proteins known to bind to CD74 or CXCR4. So far, HLA-DR, CD44, AGTR1, and MIF have been shown to bind to CD74 [16,29,36,37]. The current list of protein interaction partners for CXCR4 is also limited. In addition to CXCL12 [38,39] and MIF [5,12], only HIV gp120 was found to bind to CXCR4 [38].

CD74 interacts with CD44 and CD44 participates in mediating MIF signaling [20,37]. In fact, CD44 has been considered a signal-transducing co-receptor for CD74 [20]. Based on the interaction between CXCR4 and CD74, it will be important to study if CXCR4/CD74 complexes comprise CD44 or whether CD44 can be recruited to such complexes [20,24].

Along this notion, it is intriguing to speculate about heterotrimeric CXCR2/CD74/CXCR4 or heterotetrameric CXCR2/CD74/CXCR4/CD44 complexes. It remains unknown whether CXCR4/CD74 complexes exist preformed or whether they inducibly form upon ligand activation. Another unresolved question is whether CXCL12/SDF-1a interacts with CXCR4/CD74 complexes. Our AKT experiments, in which CXCL12-stimulated AKT phosphorylation was inhibited by anti-CXCR4 but hardly by anti-CD74 argue against this possibility. Hints towards a functional interaction between CD74 and CXCR4 have been obtained before. For example, Marsh and coworkers observed coexpression of CD74 and CXCR4 in type II alveolar epithelial cells, but did not test for direct functional interactions [40].

The functional implications of the herein identified CD74/CXCR4 interaction might be important. However, given that only a portion of CD74 is expressed on the cell surface of most Ii/class II-positive cells and that chemokine receptor expression such as that of CXCR4 is intracellular to an appreciable percentage, it should be emphasized that functional surface-expressed CD74/CXCR4 complexes may only represent a smaller portion of the CD74 and CXCR4 pools expressed in a given cell. Yet, such distribution might change under inflammatory situations in favour of the surface-expressed pool.

CD74 mediates several critical activities of the inflammatory cytokine MIF. Similarly, the CXCL12/CXCR4 axis controls key processes such as T cell trafficking in inflammation and immunity [7,9,41], tumor cell invasion, metastasis [42], and stem cell homing [43]. MIF shares with CXCL12 some of its activities [5]. On the other hand, MIF and CXCL12 exhibit distinct activity profiles, at least in atherogenic T cell recruitment and atheroma progression [5]. Thus, in addition to the biochemical challenge of unraveling the molecular details of CD74/CXCR4 complex formation and stoichiometry, CD74/CXCR4 complexes may turn out to be important for the specification of MIF versus CXCL12 actions.

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Abbreviations

CoIP	coimmunoprecipitation
CXCR	CXC-type chemokine receptor
CXCL	CXC-type chemokine ligand
GPCR	G protein-coupled receptor
Ii	invariant chain
MIF	macrophage migration inhibitory factor
SDF-1α	stromal cell-derived factor-1α

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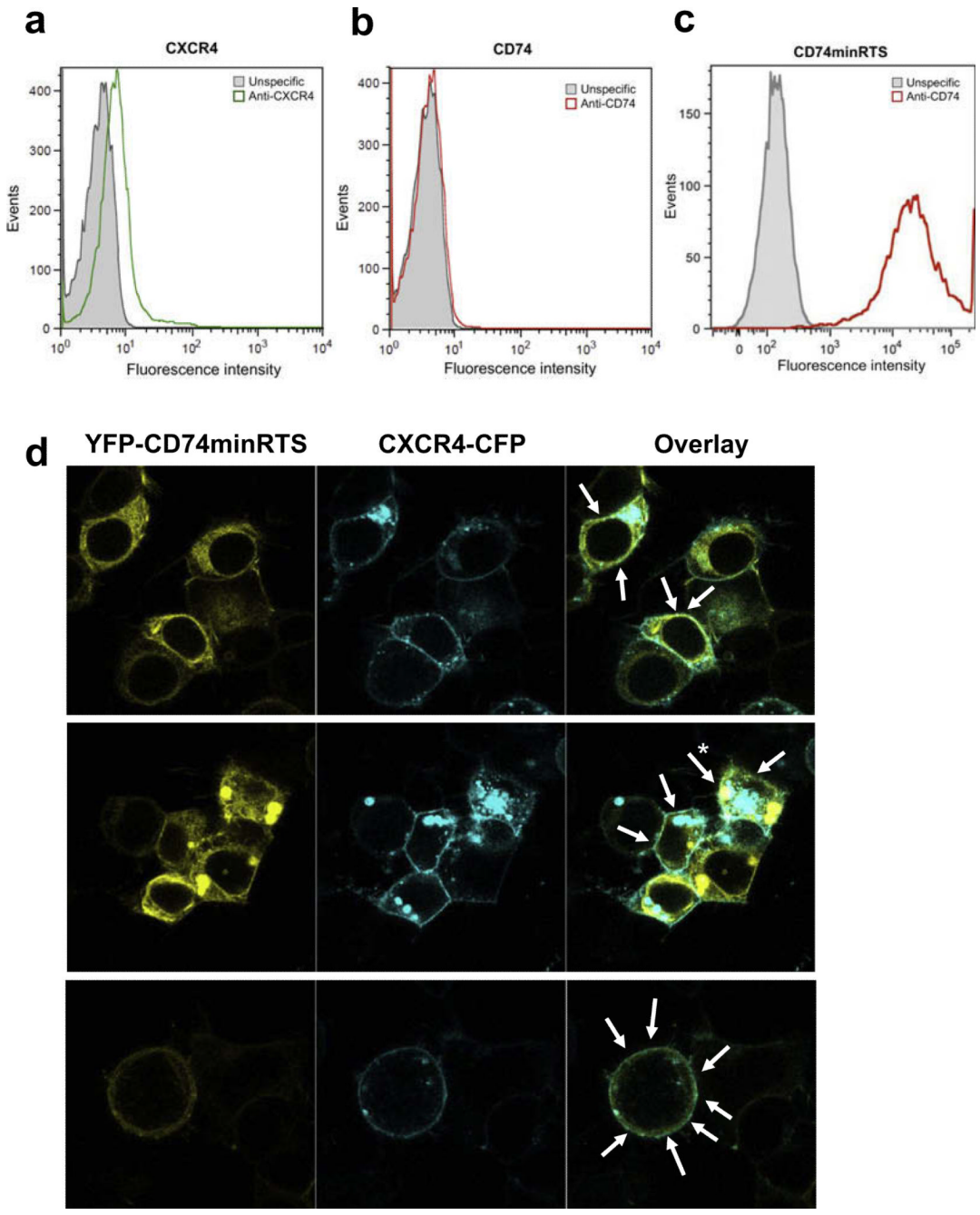

Fig. 1.

Cell surface expression of the MIF receptor fusion proteins following overexpression. Fusion proteins HA-CXCR4, V5-CD74, CD74minRTS as well as YFP-CD74minRTS and CXCR4-CFP were ectopically expressed in HEK293 cells. (a) Flow cytometry histogram of HEK293 cells expressing HA-tagged CXCR4. Fluorescence intensity of anti-CXCR4-stained cells (green) is compared with that of cells stained with FITC-IgG (grey). (b) Flow cytometry histogram of HEK293 cells expressing V5-tagged CD74. Comparison between anti-CD74-stained cells (red) and cells stained with FITC-IgG (grey). (c) Flow cytometry histogram of HEK293 cells expressing CD74minRTS. Fluorescence intensity of anti-CD74-stained cells (red) is compared with that of cells stained with FITC-IgG (grey). Note:

deletion of the ER retention signal in CD74 potentiated cell surface expression of CD74 (compare c with b). (d) Colocalisation of CXCR4-CFP and YFP-CD74minRTS in HEK293 cells. Fluorescence microscopy analysis with an Axiovert 100M/Zeiss LSM510 confocal microscope. Arrows indicate spots of strong colocalisation between CXCR4-CFP and YFP-CD74minRTS on the cell surface. Arrow + asterisk indicates a spot of marked intracellular colocalisation (possibly endolysosomal). The cell in the bottom shows colocalisation essentially all over the plasma membrane.

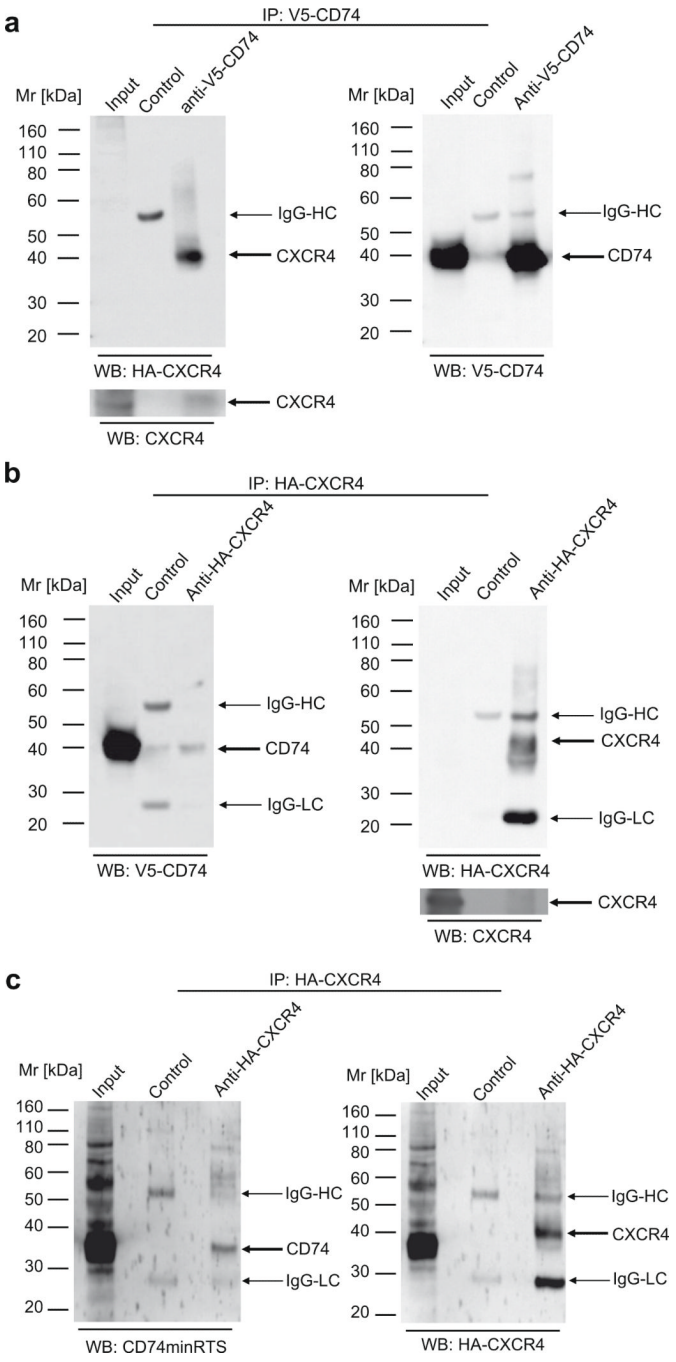

Fig. 2.

Protein complex formation of overexpressed CXCR4 and CD74 as revealed by coimmunoprecipitation. HA-CXCR4 and V5-CD74 fusion proteins or HA-CXCR4 together with the CD74minRTS protein were ectopically expressed in HEK293 cells. (a, left) V5-CD74 was immunoprecipitated with an anti-V5 antibody and coprecipitated CXCR4 detected by anti-HA or anti-CXCR4 antibody. (a, right) Control blot developed for CD74. (b, left) Inverse setting as in (a) with immunoprecipitation performed with anti-HA antibody and Western blot with anti-V5 antibody. (b, right) Control blot developed for CXCR4 by anti-HA or anti-CXCR4 antibody. (c, left) Setting as in (b) except that pCD74minRTS was transfected and blots developed with an anti-CD74 antibody. (c, right) Control blot

developed for CXCR4. Control immunoprecipitations were performed with beads alone (control); input lanes indicate Western blot analysis performed on 5% of the corresponding HEK293 lysates. Molecular weight standards were electrophoresed in the same gels (Mr). IP, immunoprecipitation; WB, Western blot. Non-specific staining of immunoglobulin heavy (IgG-HC) and light (IgG-LC) chains by the secondary WB antibody is indicated. Please note that the anti-HA antibody is a poor WB antibody; thus input controls for CXCR4 showed a weak or no band, but were detectable with anti-CXCR4 (lower panel in a, left and b, right).

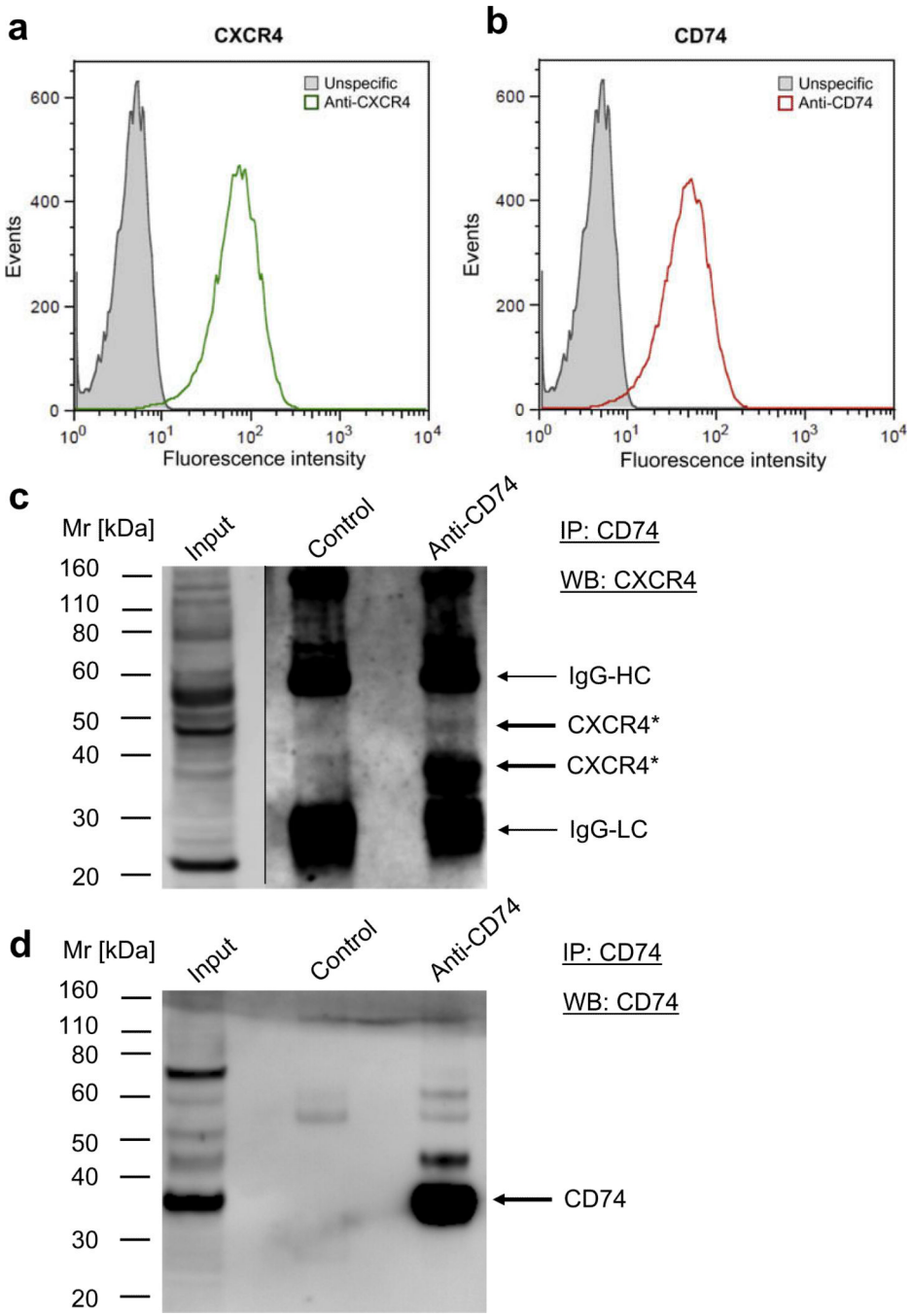

Fig. 3.

Formation of endogenous CXCR4/CD74 receptor complexes in monocytes. Co-immunoprecipitation of endogenous CD74/CXCR4 complexes from lysates of MonoMac6 cells. (a) Flow cytometry histogram showing abundant surface expression of CXCR4 in MonoMac6. Fluorescence intensity of anti-CXCR4-stained cells (green) is compared with that of cells stained with FITC-IgG (grey). (b) Flow cytometry analysis as in a but staining with anti-CD74 (red). (c) Formation of endogenous CXCR4/CD74 complexes as revealed by coimmunoprecipitation. MonoMac6 lysates were subjected to immunoprecipitation with anti-CD74 and complexed CXCR4 detected by Western blotting using anti-CXCR4. (d) Control blot developed for CD74. Control immunoprecipitations were performed with beads

alone (control); input lanes indicate Western blot analysis performed on 5% of MonoMac6 lysate. Molecular weight standards were electrophoresed in the same gel (Mr). IP, immunoprecipitation; WB, Western blot. Non-specific staining of immunoglobulin heavy (IgG-HC) and light (IgG-LC) chains by the secondary WB antibody is indicated. * indicates that in addition to the main CXCR4 band at 40 kDa a second specific band was detected which is likely to represent a differently glycosylated CXCR4 variant (47 kDa).

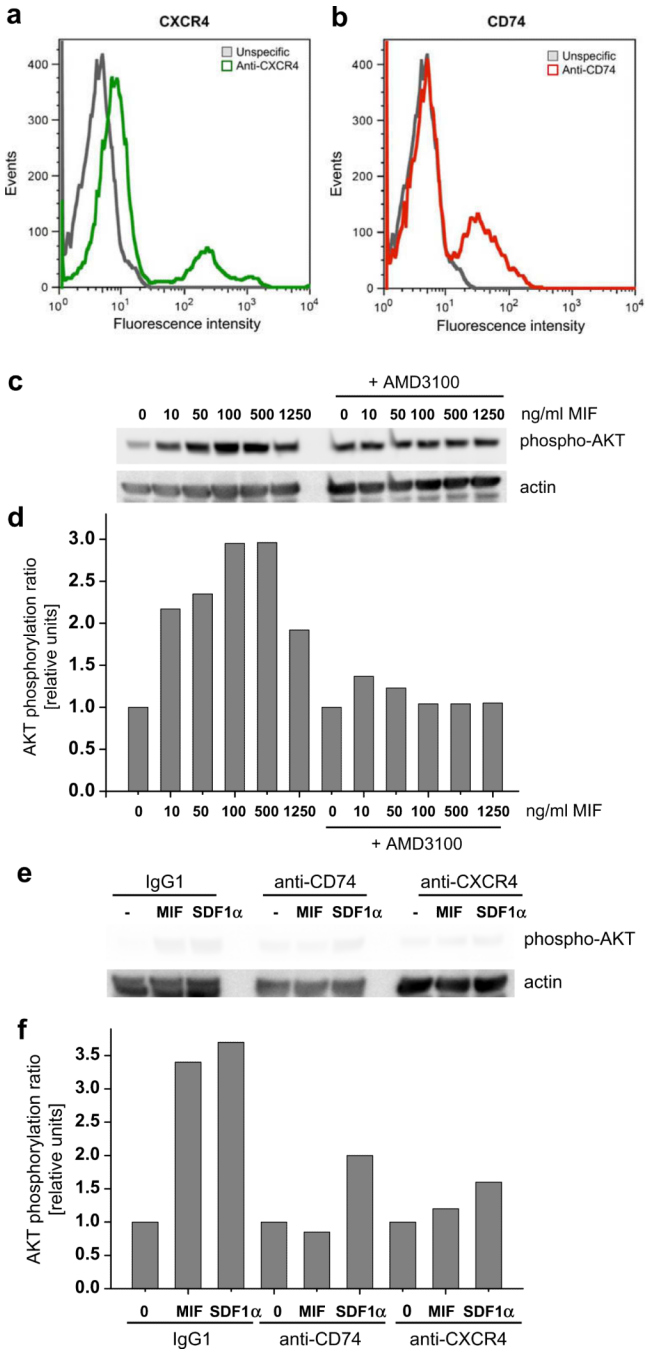

Fig. 4.

CXCR4/CD74 receptor complex formation correlates with a functional interplay between CD74 and CXCR4. The CXCR4 inhibitor AMD3100 as well as anti-CXCR4 and anti-CD74 antibodies block MIF-mediated AKT activation in Jurkat T cells, whereas SDF-1α-mediated AKT activation is only reduced by anti-CXCR4 but not anti-CD74. (a) Flow cytometry histogram showing surface expression of CXCR4 in Jurkat T cells. Fluorescence intensity of anti-CXCR4-stained cells (green) is compared with that of cells stained with a non-specific FITC-IgG (grey). (b) Flow cytometry histogram showing surface expression of CD74 in Jurkat T cells. Fluorescence intensity of anti-CD74-stained cells (red) is compared with that of cells stained with a non-specific FITC-IgG (grey). (c) Jurkat cells pre-treated with

AMD3100 (1 μg/ml, 30 min; +AMD3100) or with control buffer were incubated with rMIF at indicated concentrations for 10 min and AKT activation measured by Western blot using a phospho-Ser473-AKT antibody. Actin staining was performed for standardization of the blot. (d) Quantification of the blot according to (c) (phosphorylation ratio: AKT/actin; mean of $n = 2$). (e) As in c except that Jurkat cells were pre-treated with anti-CD74, anti-CXCR4 antibody or control IgG1 (10 μg/ml each, 30 min). MIF was added at a concentration of 100 ng/ml and for comparison cells were stimulated with 100 ng/ml SDF-1α. (f) Quantification of the blot according to (e) (phosphorylation ratio: AKT/actin; mean of $n = 2$).