Role of CXC chemokine receptor type 4 as a lactoferrin receptor

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1 **Role of CXC chemokine receptor type 4 as a lactoferrin receptor**

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1 **Abstract**

2 Lactoferrin exerts its biological activities by interacting with receptors on target cells,
3 including LDL receptor-related protein-1 (LRP-1/CD91), intelectin-1 (Omentin-1), and
4 Toll-like receptor 4 (TLR4). However, the effects mediated by these receptors are not
5 sufficient to fully explain the many functions of lactoferrin. C-X-C-motif cytokine
6 receptor 4 (CXCR4) is a ubiquitously expressed G-protein coupled receptor for stromal
7 cell-derived factor-1 (SDF-1/CXCL12). Lactoferrin was found to be as capable as
8 SDF-1 in blocking infection by an HIV variant that uses CXCR4 as a co-receptor
9 (X4-tropic HIV), suggesting that lactoferrin interacts with CXCR4.

10 We addressed whether CXCR4 acts as a lactoferrin receptor using HaCaT human
11 keratinocytes and Caco-2 human intestinal cells. We found that bovine lactoferrin
12 interacted with CXCR4-containing lipoparticles, and that this interaction was not
13 antagonized by SDF-1. In addition, activation of Akt in response to lactoferrin was
14 abrogated by AMD3100, a small molecule inhibitor of CXCR4, or by a
15 CXCR4-neutralizing antibody, suggesting that CXCR4 functions as a lactoferrin
16 receptor able to mediate activation of the PI-3K/Akt signaling pathway. Lactoferrin
17 stimulation mimicked many aspects of SDF-1-induced CXCR4 activity, including
18 receptor dimerization, tyrosine phosphorylation, and ubiquitination. Cycloheximide
19 chase assays indicated that turnover of CXCR4 was accelerated in response to
20 lactoferrin. These results indicate that CXCR4 is a potent lactoferrin receptor that
21 mediates lactoferrin-induced activation of Akt signaling.

22

1 **Introduction**

2 Lactoferrin is an iron-binding glycoprotein present in external secretions and secondary
3 granules of neutrophils. Lactoferrin contributes to host defense through its anti-bacterial
4 and immune modulatory activities (Legrand 2012; Alexander et al. 2012). In addition,
5 lactoferrin can regulate the growth and differentiation of many types of mammalian
6 cells (Alexander et al. 2012). The sulfated chain of cell surface proteoglycans is
7 regarded as the primary lactoferrin-binding site (Legrand et al. 2006). Following initial
8 binding, lactoferrin interacts with specific cell surface receptors and activates
9 intracellular signaling pathways, such as the ERK1/2 and PI3K/Akt signaling pathways.
10 The effect of lactoferrin binding depends on the types and distribution of receptors on
11 the cell surface (Suzuki et al. 2005).

12 Low-density lipoprotein receptor-related protein-1 (LRP-1, also known as CD91) is a
13 ubiquitously expressed receptor that belongs to the low-density lipoprotein (LDL)
14 receptor family (May et al. 2005). LRP-1 recognizes more than 40 different ligands,
15 including lactoferrin. LRP-1 participates in hepatocyte uptake and clearance of
16 lactoferrin from circulation (Willnow et al. 1992). In addition, LRP-1 is a potent
17 lactoferrin signaling receptor that converts lactoferrin binding to activation of the
18 ERK1/2 signaling pathway in fibroblasts, osteoblasts, keratinocytes, and adipocytes
19 (Takayama et al. 2003; Grey et al. 2004; Tang et al. 2010; Ikoma-Seki et al. 2015).

20 Intelectin-1 (Omentin-1) is a galactofuranose-binding lectin abundantly expressed in the
21 small intestine. Cell surface intelectin-1 participates in both the binding and endocytosis
22 of lactoferrin in a calcium-dependent manner (Suzuki et al. 2001). Lactoferrin-induced
23 activation of ERK1/2 is dependent on intelectin-1 (Jiang and Lönnnerdal 2012). However,
24 studies using specific LRP-1 antagonists and RNA silencing of intelectin-1 suggest that
25 neither LRP-1 nor intelectin-1 are capable of mediating lactoferrin-induced Akt
26 activation (Grey et al. 2006; Jiang and Lönnnerdal 2012). Toll-like receptor 4 (TLR4) is
27 involved in lactoferrin-induced activation of the NF- κ B signaling pathway (Ando et al.
28 2010). However, a lactoferrin receptor that is exclusively involved in Akt activation has
29 yet to be identified.

30 C-X-C-motif cytokine receptor 4 (CXCR4) is a C-X-C-type seven transmembrane
31 G-protein coupled receptor (GPCR). CXCR4 is expressed in a wide range of tissues,
32 including brain, heart, kidney, liver, lung, and spleen (Rossi and Zlotnik 2000; Wu and
33 Yoder 2009). The predominant ligand for CXCR4 is stromal cell-derived factor-1

(SDF-1, also known as CXCL12). The SDF-1/CXCR4 axis is involved in a wide range of physiological processes, such as embryonic development and stem cell motility (Nagasawa 2014). CXCR4 activation in response to SDF-1 binding triggers the activation of multiple signaling pathways, including PI-3K/Akt and ERK1/2 signaling (Busillo and Benovic 2007; Wu and Yoder 2009).

In addition to its physiological function as a chemokine receptor for SDF-1, CXCR4 acts as a co-factor for HIV-1 and -2 viral entry into T-cells (Wu and Yoder 2009; Arnolds and Spencer 2014). Infection of host cells by HIV-1 is triggered by binding of the viral envelope glycoprotein gp120 to both the CD4 cell surface receptor and either the CXCR4 or CCR5 chemokine receptor. In addition, CD4-independent association of gp120 with CXCR4 has been reported (Hesselgesser et al. 1997). Interestingly, lactoferrin was capable of blocking infection by an HIV variant that uses CXCR4 as a co-receptor (X4-tropic HIV) (Berkhout et al. 2002; Berkhout et al. 2004; Saidi et al. 2006), and bovine lactoferrin interacts with the V3 loop of gp120 (Berkhout et al. 2002). Bovine lactoferrin is protective against a broad spectrum of HIV strains that use CXCR4 for infection, suggesting that bovine lactoferrin binds CXCR4 as effectively as gp120 (Moriuchi and Moriuchi 2001).

In this study, we addressed whether CXCR4 acts as a lactoferrin receptor and found that CXCR4-containing lipoparticles interacted with bovine lactoferrin. We tested the involvement of CXCR4 in lactoferrin-induced Akt activation, and found that lactoferrin-induced Akt activation was inhibited by AMD3100, a small molecule CXCR4 antagonist, and by a CXCR4-neutralizing antibody. Lactoferrin stimulation mimicked many aspects of CXCR4 activity induced by SDF-1 binding, including dimerization, tyrosine phosphorylation, mono-ubiquitination, and ligand-induced down-regulation. This study identifies CXCR4 as a potent lactoferrin receptor that mediates lactoferrin-induced activation of Akt signaling.

Materials and methods

Materials

Bovine lactoferrin was obtained from Fonterra (Auckland, New Zealand). The iron saturation of bovine lactoferrin was less than 30%, <20 endotoxin units (EU)/mg, and >95% purity. HaCaT human immortalized keratinocytes were provided by Cell Lines Service (Eppelheim, Germany). Caco-2 human intestinal epithelial cells were obtained

1 from RIKEN Bioresource Center (Ibaraki, Japan). High-glucose (4.5 g/ml) Dulbecco's
2 Modified Eagle's Medium (DMEM), Eagle's Minimum Essential Medium (MEM),
3 non-essential amino acid solution (NEAA), protease inhibitor cocktail, and
4 cycloheximide were purchased from Sigma (St. Louis, MO, USA). Biotinylated
5 CXCR4 lipoparticles and control (null) lipoparticles were obtained from Integral
6 Molecular (Philadelphia, PA, USA). The lactoferrin ELISA quantitation kit was
7 purchased from Bethyl Laboratories (Montgomery, TX, USA).
8 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic substrate, streptavidin conjugated
9 to horseradish peroxidase (HRP), and ELISA diluent solution were purchased from
10 eBioscience (San Diego, CA, USA). AMD3100 was obtained from Merck (Merck
11 Millipore, Darmstadt, Germany). LY294002 was obtained from Jena Bioscience (Jena,
12 Germany). MG132 was obtained from Enzo Life Sciences (Farmingdale, NY, USA).
13 Bis-sulfosuccinimidyl suberate (BS3), a protein crosslinker, and protein A/G agarose
14 were obtained from Pierce Biotechnology (Rockford, IL, USA). Anti-CXCR4
15 monoclonal antibody (clone 44708) and recombinant human SDF-1 were obtained from
16 R&D Systems (Minneapolis, MN, USA). CXCR4-neutralizing antibody (clone 2F1)
17 was obtained from Abnova (Taipei, Taiwan). Anti-Akt (pan) antibodies, anti-phospho
18 Akt (Ser473) antibodies, and phospho p44/42 (Thr202/Tyr204) MAP kinase monoclonal
19 (E10) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).
20 Anti-ubiquitin antibody (clone P4D1) was purchased from Santa Cruz Biotechnology
21 (Dallas, TX, USA). Anti-phosphotyrosine antibody (PY-20) was obtained from BD
22 Biosciences (San Jose, CA, USA). RIPA buffer was obtained from Nacalai Tesque
23 (Kyoto, Japan). Polyvinylidene difluoride (PVDF) membrane and enhanced
24 chemiluminescence prime Western blotting detection reagent were purchased from GE
25 Healthcare (Buckinghamshire, UK).

26

27 **ELISA binding assay**

28 Polystyrene wells were coated overnight with 100 µl of sheep anti-bovine
29 lactoferrin antibody (100 µg/ml) in 50 mM carbonate-bicarbonate (pH 9.6) at room
30 temperature. The wells were blocked with 200 µl of TBS-T [50 mM Tris-HCl (pH 8.0),
31 0.14 M NaCl, and 0.05% Tween 20] containing 1% bovine serum albumin at 37°C for 1
32 hr. After washing five times with TBS-T, the coated plates were incubated with 10
33 µg/ml bovine lactoferrin at room temperature for 1 hr. Biotinylated lipoparticles

containing human CXCR4 were diluted with ELISA diluent solution at 4 µg/ml, then added to the wells and incubated at room temperature for 1 hr. Biotinylated null lipoparticles (without a specific receptor) were used as reference samples to account for any nonspecific binding. The wells were washed five times with TBS-T and incubated for 1 hr at room temperature with streptavidin-HRP at a dilution of 1:1,000 in TBS-T. After five washes with TBS-T, TMB was added for development of color, and the absorbance was read at 450 nm.

Cell culture

HaCaT cells were maintained in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin in an atmosphere of 5% CO₂ and 95% air at 37°C. Caco-2 cells were maintained in MEM supplemented with 10% FBS, 1% NEAA, 100 U/ml penicillin, and 100 U/ml streptomycin in an atmosphere of 5% CO₂ and 95% air at 37°C.

Cycloheximide chase assay

HaCaT keratinocytes were treated with cycloheximide (5 µg/ml) for 15 min, followed by stimulation with bovine lactoferrin (10 µM) or SDF-1 (100 ng/ml) for 3 or 6 hr. At each time point, cells were washed twice by ice-cold PBS and lysed in RIPA buffer supplemented with 1% protease inhibitor cocktail.

Crosslinking

Serum-starved HaCaT keratinocytes (10⁷ cells) were placed on ice and cooled by the addition of ice-cold PBS. After three washes with ice-cold PBS, cells were incubated with bovine lactoferrin (10 µM) or SDF-1 (100 ng/ml) for 60 min at 4°C. After washing with ice-cold PBS, cells were treated with 1 mM BS3 crosslinker for 30 min at 4°C with continuous rocking. The crosslinking reaction was terminated by the addition of 0.02 volume of 1 M Tris-HCl (pH 7.4) for 30 min at 4°C. After washing with ice-cold PBS, cells were lysed in RIPA buffer supplemented with 1% protease inhibitor cocktail. Cell lysates were subjected to immunoprecipitation using anti-CXCR4 antibody.

Ubiquitination assay

1 Serum-starved HaCaT keratinocytes were placed on ice and treated with MG132
2 (10 μ M) for 6 hr. Cells were stimulated with bovine lactoferrin (10 μ M) or SDF-1 (100
3 ng/ml) for 30 min at 4°C. After washing with ice-cold PBS, cells were lysed in RIPA
4 buffer supplemented with 1% protease inhibitor cocktail. Cell lysates were subjected to
5 immunoprecipitation using anti-CXCR4 antibody.

6 7 **Immunoprecipitation**

8 HaCaT cells were lysed in RIPA buffer supplemented with 1% protease inhibitor
9 cocktail and centrifuged for 15 min at 15,000 $\times$ g. Protein extract containing 400 μ g of
10 protein was precleared by incubation with 20 μ g of Protein A/G agarose for 60 min.
11 Precleared cell lysate was incubated with anti-CXCR4 antibody (1 μ g), followed by
12 incubation with 20 μ g of Protein A/G agarose for 60 min. All incubation steps were
13 carried out at 4°C under gentle agitation. Protein A/G agarose beads were washed four
14 times with TNE buffer [20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1% Nonidet P-40, 1
15 mM EDTA, and 5 mM mercaptoethanol supplemented with 1% protease inhibitor
16 cocktail]. Immunoprecipitated material was eluted by incubating the beads for 30 min at
17 37°C in Laemmli SDS-PAGE sample buffer.

18 19 **Western blotting**

20 Cells were harvested in RIPA buffer to yield cell lysates. To assess protein
21 expression, cell lysates were resolved by 10% sodium dodecyl sulfate polyacrylamide
22 gel electrophoresis and electrically transferred to a PVDF membrane. Subsequently, the
23 membrane was treated with blocking reagent (Tris-buffered saline containing 0.1%
24 Tween-20 and 5% skim milk) for 2 hr at room temperature. Blocked membranes were
25 incubated with primary antibody, followed by incubation with HRP-conjugated
26 secondary antibody. Immunoreactivity was detected using Western blotting detection
27 reagents.

28 29 **Data analysis**

30 Data are presented as means $\pm$ standard deviation. Unpaired two-tailed t-test was
31 applied to determine statistical significance ($p < 0.05$) for each data set.

32 33 **Results**

Interaction of lactoferrin with CXCR4

We first assessed the ability of CXCR4 to bind bovine lactoferrin. When CXCR4-containing lipoparticles and null lipoparticles (without a specific receptor) were incubated with bovine lactoferrin immobilized to polystyrene wells, the binding of CXCR4-containing lipoparticles was significantly higher (1.7-fold) than that of null lipoparticles (Fig. 1). When CXCR4-containing and null lipoparticles were incubated with uncoated polystyrene wells, only trace amounts of lipoparticles attached to the wells. SDF-1 was previously reported to interact with the extracellular domain of CXCR4 (Brelot et al. 2000). Therefore, we investigated the ability of SDF-1 to compete with lactoferrin for interaction with CXCR4-containing lipoparticles. CXCR4 interaction with bovine lactoferrin was not inhibited by SDF-1 (100 ng/ml), suggesting that bovine lactoferrin interacts with CXCR4 using a different mechanism than SDF-1.

Role of CXCR4 on lactoferrin-induced Akt phosphorylation

To determine whether CXCR4 is involved in lactoferrin-induced activation of intracellular signaling pathways, we assessed the effect of AMD3100, a small molecule inhibitor of CXCR4, on lactoferrin-induced Akt phosphorylation in Caco-2 human intestinal cells and HaCaT human keratinocytes. Treatment of serum-starved Caco-2 cells with SDF-1 (100 ng/ml) or bovine lactoferrin (10 μ M) led to Akt phosphorylation within 5 min, and both SDF-1- and lactoferrin-induced Akt phosphorylation was inhibited by AMD3100 (Fig. 2a). In HaCaT keratinocytes, both SDF-1 and bovine lactoferrin induced Akt and ERK 1/2 phosphorylation. However, pretreatment with AMD3100 inhibited lactoferrin-induced Akt phosphorylation (Fig. 2b). The inhibitory effect of AMD3100 was comparable to that of LY294002, which downregulates Akt phosphorylation. Interestingly, AMD3100 enhanced ERK1/2 phosphorylation in lactoferrin treated HaCaT keratinocytes (Fig. 2b). To exclude any off-target effects of AMD3100, we investigated the effects of a CXCR4-neutralizing antibody on lactoferrin-induced Akt phosphorylation. As shown in Figure 2c, lactoferrin-induced Akt phosphorylation was inhibited by pretreatment of HaCaT keratinocytes with CXCR4-neutralizing antibody (10 μ g/ml). These observations suggest that CXCR4 is involved in lactoferrin-induced Akt phosphorylation in Caco-2 and HaCaT cells.

Lactoferrin-induced dimerization of CXCR4

As with other C-X-C-type chemokine receptors, ligand binding of CXCR4 results in dimerization of the receptors (Vila-Coro et al. 1999; Busillo and Benovic 2007). Therefore, we investigated whether bovine lactoferrin triggers the dimerization of CXCR4 in HaCaT keratinocytes. BS3-mediated crosslinking was performed in keratinocytes stimulated with bovine lactoferrin (10 μ M). Immunoprecipitation with an anti-CXCR4 antibody (clone 44708) followed by Western blotting using anti-CXCR4 antibody (clone 2F1) revealed an 80 kDa band in cells stimulated with bovine lactoferrin (Fig. 3a). The size of this band was close to the expected molecular weight of a CXCR4 dimer, suggesting that binding of bovine lactoferrin triggers dimerization of CXCR4.

Lactoferrin-induced tyrosine phosphorylation of CXCR4

The SDF-1/CXCR4 interaction stimulates tyrosine phosphorylation of CXCR4, followed by the activation of the JAK/STAT pathway (Vila-Coro et al. 1999; Busillo and Benovic 2007). To evaluate the effect of bovine lactoferrin on tyrosine phosphorylation of CXCR4, serum-starved HaCaT keratinocytes were stimulated with bovine lactoferrin (10 μ M) for 5 min. HaCaT keratinocytes were also stimulated with SDF-1 (100 ng/ml) as a positive control. Immunoprecipitation of cell lysates with an anti-CXCR4 antibody followed by Western blotting with an anti-phosphotyrosine antibody showed a rapid increase in CXCR4 tyrosine phosphorylation that peaked at 2 min and then decreased (Fig. 3b). Tyrosine-phosphorylated proteins of a similar molecular weight (40 kDa) were observed in HaCaT cells treated with bovine lactoferrin within 5 min, suggesting that lactoferrin induces CXCR4 tyrosine phosphorylation.

Lactoferrin-induced ubiquitination of CXCR4

Upon stimulation by SDF-1, CXCR4 is mono-ubiquitinated, and this modification acts as an endosomal sorting signal that marks the receptor for lysosomal degradation (Marchese and Benovic 2001; Busillo and Benovic 2007). Therefore, we examined the effect of bovine lactoferrin on ubiquitination of CXCR4. HaCaT keratinocytes were treated with bovine lactoferrin (10 μ M) or SDF-1 (100 ng/ml) for 30 min in the presence of the proteasome inhibitor MG132. Ubiquitination of CXCR4 was detected by immunoprecipitation with an anti-CXCR4 antibody followed by Western blotting

with an anti-ubiquitin antibody. As shown in Figure 3c, CXCR4 underwent ubiquitination in response to treatment with bovine lactoferrin or SDF-1. The extent of lactoferrin-induced ubiquitination was comparable to that of SDF-1-induced ubiquitination.

Lactoferrin-induced down-regulation of CXCR4

The function of CXCR4 is tightly regulated by cell surface expression, which is regulated by receptor endocytosis, intracellular trafficking, and degradation of endocytosed receptors. We performed a cycloheximide chase assay to determine whether endogenous CXCR4 undergoes degradation in response to bovine lactoferrin stimulation. In the absence of an agonist, a modest reduction in immunoreactive CXCR4 was observed in cycloheximide-treated HaCaT keratinocytes. In the presence of bovine lactoferrin (10 μ M) or SDF-1 (100 ng/ml), CXCR4 underwent significant degradation following 3 or 6 hr of agonist treatment (Fig. 4).

Discussion

Previous studies show that lactoferrin inhibits X4-tropic HIV infection by inhibiting attachment of the viral envelope protein gp120 to CXCR4 on the surface of target cells (Berkhout et al. 2002; Berkhout et al. 2004; Saidi et al. 2006). Therefore, we speculated that CXCR4 behaves as a lactoferrin receptor. Here, we present evidence of a novel role for CXCR4 as a receptor for lactoferrin that mediates lactoferrin-induced Akt activation. The best characterized receptor for lactoferrin-induced cell signaling is LRP-1 (Suzuki et al. 2005). However, lactoferrin-induced Akt activation is not inhibited by receptor associated protein (RAP), which inhibits ligand interactions with LRP, suggesting that LRP-1 is not responsible for lactoferrin-induced Akt activation (Grey et al. 2006). In Caco-2 human intestinal cells, intelectin-1 is responsible for lactoferrin uptake and activation of ERK1/2 signaling in response to lactoferrin (Jiang et al. 2011). Nevertheless, lactoferrin-induced activation of Akt was not inhibited by RNA silencing of intelectin-1 (Jiang and Lönnerdal 2012). Therefore, a receptor other than LRP-1 or intelectin-1 must be responsible for lactoferrin-induced Akt activation. Lactoferrin-induced Akt activation was inhibited by a CXCR4 antagonist (AMD3100) or a CXCR4-neutralizing antibody (Fig. 2b, 2c and 5), suggesting that CXCR4 plays an important role in lactoferrin-induced Akt activation. However, unlike in HaCaT

1 keratinocytes and Caco-2 intestinal cells, lactoferrin-induced Akt activation was not
2 inhibited by treatment with a CXCR4 antagonist in normal human dermal fibroblasts
3 (data not shown), indicating that the role of CXCR4 in Akt activation is cell-type
4 specific, and suggesting the involvement of a different receptor in lactoferrin-induced
5 Akt activation in dermal fibroblasts.

6 CXCR4 is an evolutionarily conserved protein (Rossi and Zlotnik 2000). CXCR4
7 expression is heterogeneous among different cells of the leukocyte lineage, suggesting
8 that CXCR4 may act as a lactoferrin receptor in cells other than keratinocytes and
9 intestinal cells. The promoting effect of lactoferrin on CXCR4 expression is reported in
10 dendritic cells (Spadaro et al. 2008). In addition to Akt activation, CXCR4 is also
11 involved in STAT3 activation (Vila-Coro et al. 1999). Further study is required to
12 explore the role of CXCR4 as an lactoferrin receptor.

13 We found that lactoferrin increased tyrosine phosphorylation and dimerization of
14 CXCR4 (Fig. 3). Homo-dimerization of CXCR4 is an initial step for the activation of
15 the JAK/STAT pathway (Busillo and Benovic 2007). In addition, tyrosine
16 phosphorylation of CXCR4 correlated with the activation of the JAK/STAT pathway
17 (Vila-Coro et al. 1999; Busillo and Benovic 2007), implying that lactoferrin activates
18 the JAK/STAT pathway by a CXCR4-dependent mechanism.

19 It was reported that ERK1/2 is downstream of CXCR4, and that SDF-1 triggers ERK1/2
20 activation via a CXCR4-dependent mechanism (Busillo and Benovic 2007, Wu and
21 Yoder 2009). However, we found that a CXCR4 antagonist (AMD3100) increased
22 ERK1/2 phosphorylation in lactoferrin-treated HaCaT keratinocytes (Fig. 2b),
23 suggesting that lactoferrin-induced ERK1/2 phosphorylation is mediated by another
24 receptor and lactoferrin inhibits ERK1/2 phosphorylation as a result of CXCR4 binding.
25 Therefore, the functional outcome of CXCR4 activation is likely determined by the
26 specifics of ligand-CXCR4 interaction.

27 Nevertheless, lactoferrin mimicked many aspects of CXCR4 activity induced by SDF-1
28 binding, including receptor dimerization, tyrosine phosphorylation, and
29 mono-ubiquitination (Fig. 3). Ubiquitination of CXCR4 is a common way of mediating
30 internalization and degradation of CXCR4 by the lysosomal pathway (Busillo and
31 Benovic 2007). In addition, tyrosine phosphorylation of CXCR4 drives endocytic
32 internalization of CXCR4 (Busillo and Benovic 2007). We observed that lactoferrin
33 treatment accelerated the downregulation of CXCR4 to the same extent as SDF-1 (Fig.

4), suggesting that lactoferrin promotes the CXCR4 degradation by increasing tyrosine phosphorylation and mono-ubiquitination of CXCR4.

The mechanism through which SDF-1 induces the endocytosis and degradation of CXCR4 is not fully understood. A region in the extracellular N-terminal domain of CXCR4 is responsible for the initial binding of SDF-1, and this is followed by its interaction with extracellular loops and the membrane-spanning domains of CXCR4 (Brelot et al. 2000). This second interaction is required for the activation of intracellular signaling. HIV-1 infection via CXCR4 seems to be dependent on interaction of SDF-1 with the residues of CXCR4 involved in activation of intracellular signaling (Brelot et al. 2000). It will be valuable to characterize the lactoferrin-binding site of CXCR4 and assess its binding properties.

In this study, we found that the amount of CXCR4-containing lipoparticles bound by lactoferrin was 1.7 times the number of null lipoparticles bound (Fig. 1), suggesting that bovine lactoferrin interacts with the extracellular domain of CXCR4. At the surface of mammalian cells, the sulfated chain of the proteoglycan is the main lactoferrin-binding site and occupies approximately 80% of the total binding (Legrand et al. 2006). Lactoferrin bound to null lipoparticles may represent the binding of lactoferrin to heparan sulfate proteoglycans (HSPGs).

SDF-1 blocks CXCR4-dependent HIV-1 infection in T-lymphocytes (Bleul et al. 1996; Oberlin et al. 1996; Valenzuela-Fernández et al. 2001). This inhibitory effect of SDF-1 is due not only to competitive interaction of SDF-1 and gp120 with CXCR4, but also to the down-regulation of CXCR4 at the cell surface by induction of CXCR4 endocytosis (Amara et al. 1997). We found here that lactoferrin also enhanced CXCR4 down-regulation by enhancing CXCR4 turnover (Fig. 4). This lactoferrin-induced CXCR4 down-regulation implies that lactoferrin might inhibit HIV infection of target cells by accelerating CXCR4 endocytosis.

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14 **Figure legends**

15 **Figure 1. Binding of CXCR4-containing lipoparticles to immobilized bovine**
16 **lactoferrin.** Biotinylated lipoparticles containing CXCR4 and null lipoparticles
17 (without a specific receptor) were incubated with bovine lactoferrin immobilized to
18 polystyrene wells. Uncoated polystyrene wells served as negative controls. Binding of
19 biotinylated lipoparticles was detected by incubation with horseradish
20 peroxidase-labeled streptavidin, followed by a tetramethylbenzidine (TMB) peroxidase
21 substrate. Absorbance measured at 450 nm was used to assess lipoparticle binding to
22 bovine lactoferrin. For the competition assay, CXCR4-containing lipoparticles were
23 incubated with bovine lactoferrin in the presence of 100 ng/ml SDF-1. Data are
24 presented as the mean $\pm$ standard deviation. ***, $p < 0.001$; NS, no significant
25 difference.

27 **Figure 2. Involvement of CXCR4 in lactoferrin-induced Akt phosphorylation.** (a)
28 Effect of a CXCR4 antagonist on lactoferrin-induced Akt phosphorylation in Caco-2
29 human intestinal cells. Serum-starved cells were stimulated with bovine lactoferrin (10
30 μ M) or SDF-1 (100 ng/ml) in the presence or absence of AMD3100, a CXCR4
31 antagonist, for 5 min. Akt phosphorylation was assessed by Western blotting. (b) Effect
32 of a CXCR4 antagonist on lactoferrin-induced Akt and ERK1/2 phosphorylation in
33 HaCaT human keratinocytes. Serum-starved cells were stimulated with bovine

lactoferrin (10 μ M) in the presence or absence of AMD3100 (10 μ M) or LY294002 (20 μ M) for 5 min. Akt and ERK1/2 phosphorylation were assessed by Western blotting. (c) Effect of a CXCR4-neutralizing antibody on lactoferrin-induced Akt phosphorylation. Serum-starved HaCaT keratinocytes were pretreated with CXCR4-neutralizing antibody (CXCR4 Ab: 10 μ g/ml) for 30 min, followed by stimulation with bovine lactoferrin (10 μ M) for 5 min. Akt phosphorylation was assessed by Western blotting. Western blotting using an anti-pan Akt antibody was performed to confirm equal loading. Band intensity was measured with ImageJ 1.50i software and normalized against Akt, and the protein levels are shown at the bottom of each blot.

Figure 3. Modification of CXCR4 by bovine lactoferrin. (a) Lactoferrin-induced dimerization of CXCR4. Serum-starved HaCaT keratinocytes were cooled on ice and stimulated with bovine lactoferrin (10 μ M) or SDF-1 (100 ng/ml) at 4°C for 60 min. Cell surface proteins were crosslinked with a membrane impermeable crosslinker (BS3) for 30 min at 4°C. Cell lysates were subjected to immunoprecipitation with an anti-CXCR4 antibody (clone 44708), followed by Western blotting using anti-CXCR4 antibody (clone 2F1). (b) Lactoferrin-induced tyrosine phosphorylation of CXCR4. HaCaT keratinocytes were stimulated with bovine lactoferrin (10 μ M) or SDF-1 (100 ng/ml) for 5 min at 37°C. Tyrosine phosphorylation of CXCR4 was detected by immunoprecipitation with anti-CXCR4 antibody (clone 44708) followed by Western blotting with an anti-phosphotyrosine antibody (PY20). (c) Lactoferrin-induced mono-ubiquitination of CXCR4. HaCaT keratinocytes were treated with MG132 (10 μ M) for 6 hr, followed by stimulation with bovine lactoferrin (10 μ M) or SDF-1 (100 ng/ml) for 5 min. Cell lysates were subjected to immunoprecipitation with an anti-CXCR4 antibody (clone 44708), followed by Western blotting with an anti-ubiquitin antibody (clone P4D1). Band intensity was measured with ImageJ 1.50i software and normalized against CXCR4 in the immunoprecipitate, and the protein levels are shown at the bottom of each blot.

Figure 4. Lactoferrin-induced degradation of CXCR4. HaCaT keratinocytes were treated with cycloheximide (5 μ g/ml) for 15 min, followed by stimulation with bovine lactoferrin (10 μ M) or SDF-1 (100 ng/ml) for the indicated times. The reduction in CXCR4 was detected by Western blotting with an anti-CXCR4 antibody (clone 2F1).

1 Western blotting using an anti-GAPDH antibody was performed to confirm equal
2 loading. Band intensity was measured with ImageJ 1.50i software and normalized
3 against GAPDH, and the protein levels are shown at the bottom of each blot.
4
5 **Figure 5. Diagram illustrating CXCR4 as lactoferrin receptor that activates the**
6 **PI-3K/Akt signaling pathway.** Bovine lactoferrin (BLF)-induced activation of the
7 PI-3K/Akt signaling pathway was inhibited by AMD3100, a CXCR4 inhibitor,
8 suggesting that BLF activates the pathway by a CXCR4-dependent mechanism. Both
9 BLF and stromal derived factor-1 (SDF-1) were bound to CXCR4 in a non-competitive
10 manner and the specifics of binding resulted in activation of non-identical signaling
11 pathways.

Draft

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