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Intracellular labile iron promotes firm adhesion of human monocytes to endothelium under *flow* and transendothelial migration
Iron and monocyte–endothelial cell interactions

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ABSTRACT

Monocyte infiltration across the endothelium is part of the innate immune response, however it may contribute to severity of chronic conditions. We have investigated the effects of iron on the cytokine-mediated recruitment of monocytes to the endothelium, using a physiological *flow* model and a monocyte transendothelial migration model.

Under *flow*, iron loading to endothelial cells promoted an increased number of tumor necrosis factor-α-mediated firm arrest of human monocytes. Similarly, an increased number of firmly adhered monocytes were observed in conditions in which monocytes were iron-loaded, compared to the non-iron-loaded conditions. In both iron-loaded and non-iron-loaded conditions, blockade of the α4 and β2 integrins restored similar number and velocity of monocyte rolling, suggesting that iron did not modulate rolling interactions. However, with the integrin blockade, the number of firmly adhered cells remained higher in iron-loaded conditions than in control conditions, suggesting that iron could have modulated receptors other than the blocked integrins to promote firm arrest. Iron loading indeed upregulated expression of chemokine receptors, CC receptor-2 and CXC receptor-2, but not platelet endothelial cell adhesion molecule-1. This effect concomitantly promoted monocyte chemotactic protein-1-dependent transendothelial migration. In addition, iron-induced firm adhesion and transmigration were counteracted by iron chelation. These data reveal an immunomodulatory function of iron in the cascade of events of cytokine-mediated monocyte infiltration across endothelium, and therefore suggests the role for iron in inflammatory conditions underlying diseases like atherosclerosis and neurodegeneration.

1. Introduction

Recruitment of stimulated leukocytes from the blood stream to the sites of injury and infection is a fundamental mechanism in innate immunity. In many inflammatory conditions, including atherosclerosis and myocardial infarction, excessive leukocyte recruitment is the hallmark of the disease [1]. The infiltrated monocytes initiate and propagate an accumulation of lipid-laden cells that can progress into an atherosclerotic plaque. Moreover, the infiltrated monocytes are capable of producing inflammatory mediators that destabilize pre-existing plaques [2]. Monocyte infiltration also plays a role in the pathophysiology of airway pulmonary diseases, which indeed shares common mechanisms with atherosclerosis [3]. Recently, it has also been extensively proposed that monocyte recruitment across the blood–brain barrier triggers development of neurodegenerative diseases, including multiple sclerosis, Parkinson disease, and dementia [4]. The process of monocyte infiltration through the endothelium consists of consecutive adhesion-mediated events. The first event involves selectins that trigger tethering of the leukocytes to the activated endothelium. This is followed by arrest and firm adhesion of monocytes, a process that occurs with the binding of the integrins, namely very late antigen-4 (VLA-4, α4β1) and lymphocyte function-associated antigen-1 (LFA-1, αLβ2), to their counter receptors on endothelial cells, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), respectively. Firm

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adhesion is followed by transendothelial migration of monocytes, with one of the mediators being platelet endothelial cell adhesion molecule-1 (PECAM-1) [5].

Chemokines, the family of chemotactic cytokines, activate integrins and induce directed migration of the leukocytes. Endothelial cells produce chemokines upon stimulation with cytokines. Monocyte chemotactic protein-1 (MCP-1, a CC chemokine) and interleukin-8 (IL-8, a CXC chemokine), secreted by endothelial cells, bind to their receptors on the monocyte surface, i.e. CCR-2 for MCP-1, and CXCR-1 and CXCR-2 for IL-8, and trigger adhesion as well as infiltration of monocytes [6]. The endothelial surface-bound melanoma growth stimulatory activity-α (GRO-α, a CXC chemokine) together with its receptor on monocytes, CXCR-2 mediates the abrupt arrest of monocytes possibly through an instantaneous conformation change of the integrins as has been shown for lymphocytes [7]. Furthermore, mice lacking either MCP-1, IL-8, or their receptors are resistant to atherosclerosis [8–10].

The role of iron has been previously suggested in atherogenesis [11]. In animal models, iron overload accelerated atherosclerotic lesion formation [12]. Conversely, several animal studies have shown that reduced formation of early atherosclerotic lesions could be achieved by administering either iron chelators or iron-deficient diets [13–15]. Indeed, accumulation of iron has been detected in human atheroma [16]. In its free form, and any other molecular form capable of electron shuttling, iron acts as a catalyst for the formation of hydroxyl radicals that may activate inflammatory response. The contribution of excess iron in combination with risk factors such as hypertension or smoking to cardiovascular death in HFE C282Y heterozygotes [17] was a reason to investigate biological roles of iron during inflammation, specifically the cascade of proinflammatory cytokine-mediated monocyte recruitment that underlies cardiovascular events and also other chronic conditions like neurodegeneration.

2. Materials and methods

2.1. Reagents

To modulate iron levels, Fe(III)citrate (Sigma, St. Louis, USA), and the iron chelators deferoxamine (Novartis, Arnhem, The Netherlands) and deferiprone (Duchefa Biochemie, Haarlem, The Netherlands) were used. Monoclonal antibodies used included: HP2/1 (anti-α4-integrin, Immunotech, Marseille, France) and IB4 (anti-β2-integrin, isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection, Rockville, MD), FITC-conjugated ICAM-1 antibody (R&Dsystem, Minneapolis, USA), FITC-conjugated PECAM-1 antibody (Sigma), PE-conjugated VCAM-1 antibody, Cytochrome-conjugated E-selectin antibody, PE-conjugated CCR-2 antibody and PE-conjugated CXCR-2 antibody (all from BDBiosciences, San Diego, USA),

2.2. Baseline iron level

The iron content of the endothelial growth medium-2 (EGM-2, Clonetics, Wallkersville, USA) was measured by the Vitros® 950 Chemistry System (Ortho-Clinical Diagnostics, Tilburg, The Netherlands) as described previously [18]. To avoid any external iron contamination, in all experiments, plastic materials, which have lower affinity for iron compared to glass were used.

2.3. Cells

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described by Jaffe et al. [19]. Confluent cells from passages two to three were used for all experiments. To minimise donor-to-donor variability, HUVECs were pooled from three to four donors for each experiment.

Peripheral blood mononuclear cells (PBMCs) were isolated from donor blood (Sanquin blood bank, Utrecht, The Netherlands), and monocytes were isolated from the PBMC as described previously [18]. Briefly, monocytes were isolated using the negative immunoselection monocyte isolation kit (MiltenyiBiotech, CLB Sanquin, Amsterdam, The Netherlands) according to supplier's instructions. Before isolation, PBMC was washed by centrifugation at 200 × *g* for 8 min at 4 °C without deceleration for six times, to eliminate most of the platelets. This method resulted in monocyte purity of >90% as analysed by flow cytometry. Purified monocytes were suspended at a concentration of 5×10^6 cells/mL in RPMI1640 (BioWhittaker) supplemented with 2 mmol/L L-glutamine and 0.2% human serum albumin (CLB Sanquin).

2.4. Endotoxin measurements

Iron solutions were tested for the presence of lipopolysaccharide using a limulus amebocyte lysate assay (Cambrex Bioscience, Walkersville, USA). To ensure endotoxin-free conditions, all the materials were kept under pyrogen-free conditions.

2.5. Viability assays

Cellular viability of HUVECs was monitored by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma–Aldrich) method [20]. To monitor monocyte viability, the trypan blue exclusion test was applied. In all experiments, both HUVECs and monocytes were for >95% viable after indicated compound treatments.

2.6. Preparation of iron and chelator-bound iron solutions

The majority of non-transferrin-bound iron (NTBI), the low-molecular weight iron in the circulation is found in the form of Fe(III) complexed to citrate [21]. A 10 mmol/L Fe(III)citrate (1:6 iron–citrate molar ratio) stock solution was made by dissolving the iron crystals in distilled water at 56 °C for 30 min. The iron solution was always freshly prepared and filter-sterilised prior to use. The chelator stocks were 10 mmol/L deferoxamine and 30 mmol/L deferiprone in PBS and stored at −20 °C prior to use. Since six ligands can bind to one iron atom, one deferoxamine molecule, being a hexadantate chelator, or three molecules of a bidentate chelator, like deferiprone, are required to fully chelate one iron atom. The chelator-bound iron solutions at the appropriate ratios were always freshly prepared.

2.7. Dichlorofluorescein assay for measurement of intracellular oxygen-derived radicals

Carboxydichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, USA) is a nonpolar compound that is converted into a membrane-impermeable non-fluorescent polar derivative (DCFH) by cellular esterases after incorporation into cells. The trapped DCFH is rapidly oxidised to fluorescent 2,7-dichlorofluorescein (DCF) by intracellular hydrogen peroxide and hydroxyl radicals. After various compound incubations, the cells were washed, followed by incubation in DCFH-DA at a final concentration of 5 μmol/L for 30 min. After a washing step, emission of the trapped, oxidised DCF in 10,000 cells was analysed by flow cytometry.

2.8. Monocyte perfusion and evaluation

Perfusions under steady *flow* were performed in a modified form of transparent parallel-plate perfusion chamber [22,23]. HUVECs were subcultured to confluent monolayers on a coverslip

(18 mm × 18 mm) precoated with glutaraldehyde-linked gelatin. The micro-chamber has a slit height of 0.2 mm and a width of 2 mm when the coverslip with HUVECs were mounted on a circular plug of the chamber. HUVECs were activated using 2 ng/mL TNF-α (Boehringer Mannheim, Germany) for 4 h prior to each perfusion.

The monocyte suspension at 2×10^6 cells/mL was aspirated from a reservoir through the perfusion chamber during 5 min at a shear stress of 2.0 dynes/cm^2, using a perfusion buffer containing 20 mmol/L HEPES, 132 mmol/L NaCl, 6 mmol/L KCl, 1 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, supplemented with 5 mmol/L glucose, 1.0 mmol/L $CaCl_2$, and 0.5% HAS, pH 7.3. Perfusions were performed as individual runs at 37 °C. For rolling experiments, before perfusion, monocytes were preincubated at 37 °C with the blocking monoclonal antibodies: 10 μg/mL HP2/1 and 10 μg/mL IB4. The incubation was for 15 min at 4 °C. During perfusions, the *flow* chamber was placed on a microscope stage (DM RXE; Leica, Wetzlar, Germany), which was equipped with a B/W CCD video camera (Sanyo, Osaka, Japan). The entire period of perfusion was recorded on a tape using a VHS video recorder connected to the camera. The monocytes in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording. Video images were evaluated for the number of rolling and adherent cells using the image analysis software Optimas 6.1 (Media Cybernetics systems, Silver Springs, MD). The mean number of surface-adherent monocytes was measured after 5 min perfusion at a minimum of 50 randomised high power fields (total surface of at least 2 mm^2). Rolling was measured by the capture of a sequence of 50 frames representing an adjustable time interval (Δt, with a minimal interval of 80 ms). At each frame, the position of every cell was detected, and for all subsequent frames the distance moved by each cell and the number of images in which a cell appears in focus was measured. The velocity of a cell (v) in μm/s was calculated from the equation: $v = L/\Delta t(x-1)$, in which L is the covered distance (μm) and x is the number of images in which a cell appears. The cut-off value to distinguish between rolling and static adherent cells was set at 1 μm/s.

2.9. Fluorescence-activated cell sorting (FACS)

Monocytes were stimulated at cell density of 1×10^6 cells/mL. After incubation with desired compounds, HUVECs were harvested with trypsin, then incubated with 1:50 fluorescence-labelled monoclonal antibodies for 30 min at 4 °C. The cells were washed prior to FACS analysis. Each flow cytometric measurement was performed using a Becton Dickinson FACScan and 10,000 events were analysed.

2.10. Transendothelial migration assay

Monocyte migration through an endothelial monolayer was evaluated using 24-well Costar Transwell chambers (3 μm pore size, Corning, New York, USA). HUVECs were subcultured to confluent monolayers on Transwell inserts precoated with fibronectin (Sigma). Monocytes were labelled with 2 μmol/L 2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester (BCECF-AM, Molecular Probes). 100 μL monocyte suspension ($1–2 \times 10^6$ cells/mL) was seeded in the upper chamber and 600 μL of medium containing 10 nmol/L MCP-1 was added to the lower well. The chamber was incubated at 37 °C in a humidified atmosphere in the presence of 5% CO_2 for 3 h. Fluorescence of monocytes in the lower well was read using a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems, Framingham, USA) at 485 nm excitation and 530 nm emission. The percentage of transmigration was defined as the fluorescence value of the migrated monocytes divided by the value of total fluorescence, multiplied by 100.

2.11. Data analysis

Results are expressed as means ± standard errors of the means. Differences in quantitative measures were tested for significance by using the unpaired two-tailed student's *t*-test. Significance was established when $P < 0.05$.

3. Results

3.1. Baseline iron and endotoxin levels

With no external iron addition, the baseline iron level in basal cell culture medium was 0.36 μmol/L. Lipopolysaccharide contamination in the different iron solutions and cell culture medium was <1 pg/mL.

3.2. Endothelial iron loading enhanced firm adhesion of monocytes under flow

In order to mimic *in vivo* conditions, we studied the monocyte–endothelial interactions using a physiological *flow* model. Under TNF-α stimulation, HUVECs support firm adhesion of monocytes when a high physiological shear stress of 2 dynes/cm^2 is applied [6]. In this study, we stimulated HUVECs with 2 ng/mL TNF-α for 4 h before the perfusion. A twofold increase in the number of adherent monocytes was observed when monocytes were perfused on HUVECs, which had been preincubated with 30 μM Fe(III)citrate for 48 h before TNF-α activation (Fig. 1). Preincubation of HUVECs with either deferoxamine or deferiprone-chelated iron (Fig. 1) or deferoxamine or deferiprone alone (not shown) did not affect adhesion. This indicates that under an inflammatory condition induced by the proinflammatory cytokine TNF-α, iron loading in HUVECs promotes the adhesion of monocytes, under *flow* with shear stress of 2 dynes/cm^2. We have shown previously that incubation of HUVECs with NTBI of as low as 1 μmol/L, increased intracellular labile iron levels [24].

3.3. Monocyte iron loading augmented arrest to endothelium under flow

Likewise, we wanted to know whether iron loading in monocytes could modulate the number of monocyte arrest under this inflammatory *flow* setting. We had observed an increased

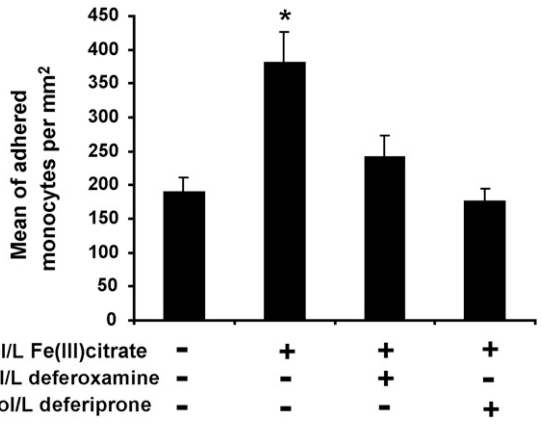

Fig. 1. Endothelial intracellular iron-induced monocyte arrest under *flow*. Human monocytes at 2×10^6 cells/mL were perfused over a HUVEC monolayer for 5 min at a shear stress of 2.0 dynes/cm^2. Before perfusion, HUVECs were incubated with indicated compound(s) for 48 h, and 4 h coincubation with 2 ng/mL TNF-α at the end of each treatment. The number of firmly adhered monocytes on the treated HUVECs was analysed using the Optima 6.1 image analyser ($n = 4$, $^*P < 0.01$).

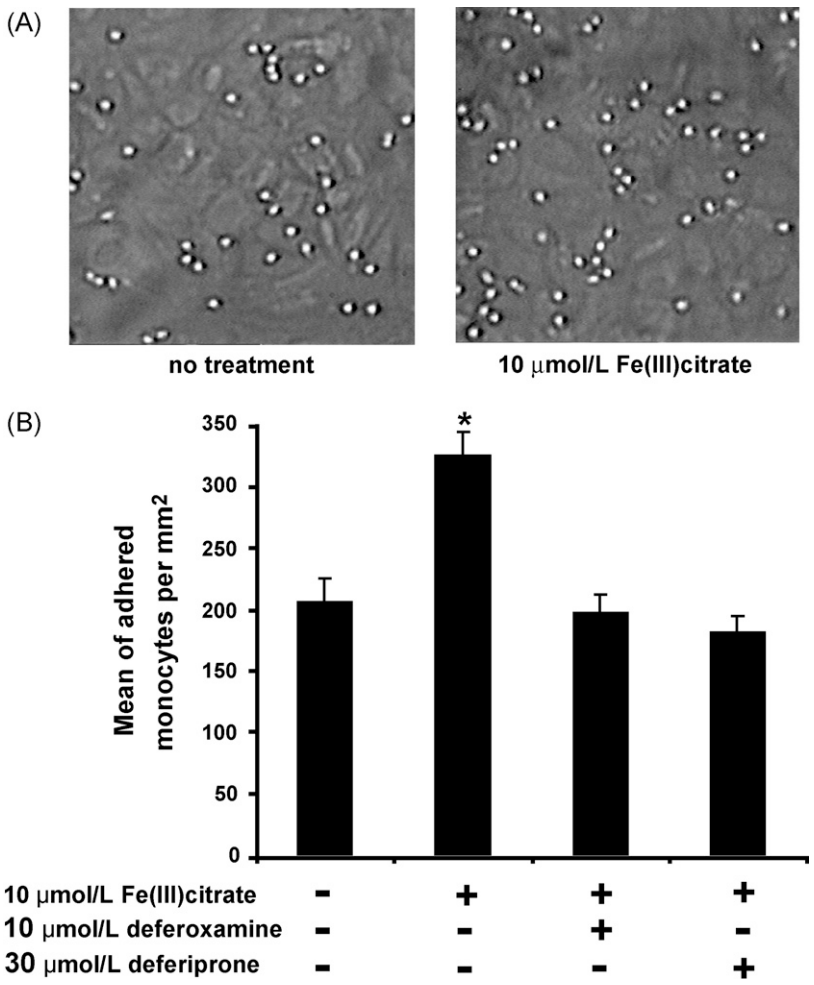

Fig. 2. Intracellular labile iron enhanced monocyte arrest under *flow*. Human monocytes at 2×10^6 cells/mL were perfused over a HUVEC monolayer for 5 min at a shear stress of 2.0 dynes/cm^2. Before perfusion, monocytes were incubated with the indicated compound(s) for 90 min. (A) Representative of images of firm adhesion of monocytes on endothelial monolayer under *flow*, after monocytes were subjected to indicated treatments. Images were taken using a B/W CCD video camera (B) The number of firmly arrested monocytes was analysed using the Optima 6.1 image analyser ($n = 5$, $^*P < 0.01$).

intracellular labile iron in monocytes immediately after 10 min of incubation with Fe(III)citrate (not shown). A significantly higher firm adhesion was observed, when monocytes were preincubated with 10 μmol/L Fe(III)citrate for 90 min before perfusion, compared to the non-iron-loaded controls (controls: 189 ± 21 monocytes/mm^2 vs. iron-loaded monocytes: 380 ± 39 monocytes/mm^2, $P > 0.05$, Fig. 2A and B). Preincubation of monocytes with either deferoxamine or deferiprone-bound iron neutralised the iron-induced increase in adhesion (239 ± 29 monocytes/mm^2 and 177 ± 18 monocytes/mm^2, respectively, Fig. 2B). These data indicate that intracellular labile iron in monocytes promotes their firm adhesion to endothelium under the inflammatory condition.

3.4. Intracellular labile iron did not alter monocyte rolling

Next, to investigate whether the iron-induced increase in total adhesion was a result of an increased number of rolling monocytes under *flow* conditions, we treated monocytes with blocking monoclonal antibodies directed against α4 and β2 integrins, HP2/1 and IB4, respectively. Using these antibodies, we were able to restore >60% of rolling interactions. The numbers of rolling monocytes were not altered by iron (controls: 141 ± 17 monocytes/mm^2 vs. iron-loaded monocytes: 128 ± 15 monocytes/mm^2, $P > 0.05$, Fig. 3). However, the levels of firm adhesion of monocytes preincubated with 10 μmol/L Fe(III)citrate for 90 min remained significantly higher than that of controls (controls: 44 ± 10 monocytes/mm^2 vs. iron-loaded monocytes: 125 ± 23 monocytes/mm^2, $P < 0.05$, Fig. 3). The rolling velocities of iron-loaded monocytes were similar to controls (controls: 18.09 ± 0.67 μm/s vs. iron-loaded monocytes: 18.36 ± 0.22 μm/s, $P > 0.05$). Thus, intracellular labile iron did not modulate rolling interactions. We instead observed that the iron-enhanced firm adherence of monocytes was mostly coming from abrupt arrest, independent of the blocked integrins. These data therefore indicate that iron-induced firm adhesion is likely to be mediated by receptors other than α4 and β2 integrins.

3.5. Intracellular labile iron increased CCR-2, CXCR-2, but not PECAM-1 expression on monocytes

Since monocyte adhesion and infiltration is critically mediated by chemokines [6], we investigated whether iron loading modulates expression of CCR-2 and CXCR-2, the main chemokine receptors on monocytes. Incubation with iron at concentration of 10 μmol/L upregulated expression of CCR-2 as well as CXCR-2 (Fig. 4), indicating that intracellular iron modulated the chemokine pathways. Induction of these two receptors could lead subsequently to enhanced transendothelial migration of the monocytes.

Additionally we measured expression of PECAM-1, one of the receptors facilitating monocyte transmigration. In this study, however, PECAM-1 expression remained unaffected by iron loading (Fig. 4).

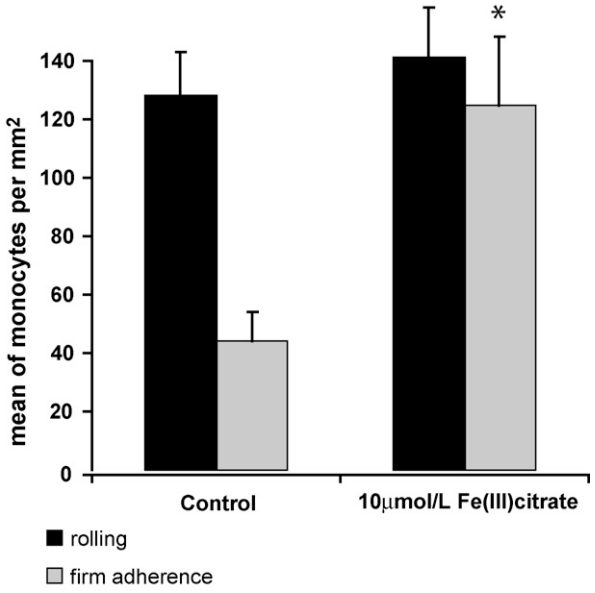

Fig. 3. α4 and β2 blocking modulated rolling and arrest of monocytes under *flow*. Monocytes were treated for 90 min with or without 10 μmol/L Fe(III)citrate before perfusion. After α4 and β2 blocking, the treated monocytes were perfused over a HUVEC monolayer at a shear stress of 2.0 dynes/cm^2. The numbers of rolling and adhered monocytes, were then analysed ($n = 3$, $^*P < 0.01$).

3.6. Intracellular labile iron-induced oxygen-derived radical formation in monocytes

Since chemokine receptor expression could be upregulated by oxygen-derived radicals [25], and given the property of iron as a catalyst to the formation of hydroxyl radicals, we tested whether iron loading in monocytes promotes production of radicals. Using the DCF-fluorescence probe, we indeed observed an increase of intracellular oxygen-derived radicals in iron loaded monocytes compared to controls, shown by an increase in fluorescence intensity (Fig. 5A and B). This effect could be counteracted using either deferoxamine or deferiprone (Fig. 5A and B), confirming that the intracellular redox status is alterable by intracellular labile iron.

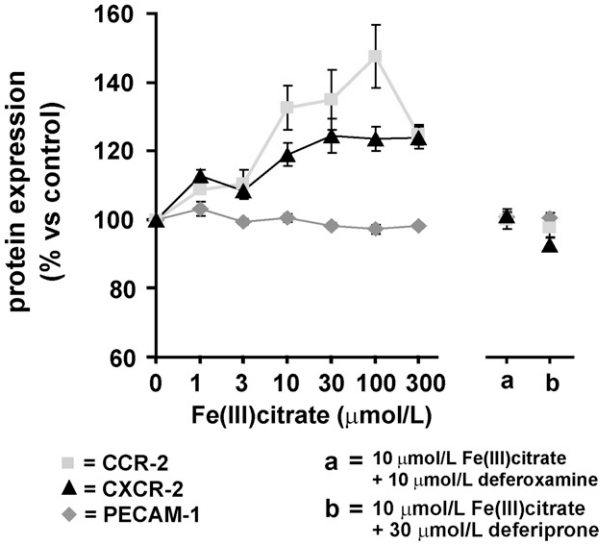

Fig. 4. Intracellular labile iron enhanced CCR-2 and CXCR-2 expression. Human monocytes were treated for 90 min by the indicated treatment. Expression levels of CCR-2, CXCR-2 and PECAM-1 on monocytes were analysed by flow cytometry ($n = 3$–10).

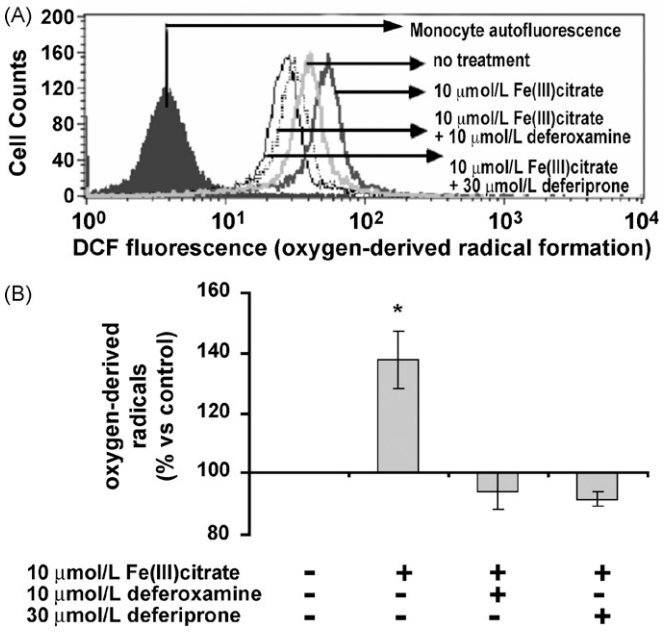

Fig. 5. Iron-induced generation of oxygen-derived radicals. Human monocytes were treated for 90 min using the indicated treatment. Flow cytometry analysis of DCF-fluorescence for oxygen-derived radicals is shown. (A) Representative histogram of flow cytometry analysis. The shift to the right indicates an increase in intracellular radical production in monocytes. (B) The relative levels of oxygen-derived radicals after indicated treatments ($n = 3$–8, $^*P < 0.05$).

After addition of iron chelators, DFC fluorescence was even lower than in non-treated cells (Fig. 5A and B), indicating that the naturally occurring labile iron offers a pro-oxidant environment that may be needed for physiological signalling processes.

3.7. Intracellular labile iron enhanced MCP-1-dependent transendothelial migration of monocytes

Since iron upregulated CCR-2 and CXCR-2 expression, we investigated whether iron loading could promote transendothelial migration of monocytes towards a chemotactic proinflammatory MCP-1 gradient. When monocytes were iron-loaded, the levels of MCP-1-dependent transendothelial migration of monocytes were indeed increased, while deferiprone and deferoxamine could effectively counteract this effect (Fig. 6). Treatment of monocytes with the iron chelators alone did not affect transendothelial migration (not shown). Enhancement of monocyte migration was observed when HUVECs were iron-loaded (Fig. 6), confirming the modulating effects of iron on endothelial activation. The amount of transmigrated monocytes was additively increased when both HUVECs and monocytes were treated with iron (Fig. 6), suggesting that the activation levels of both immune cells may determine the extent of monocyte infiltration. All together the results suggest the role of iron in modulating monocyte transendothelial migration.

4. Discussion

The infiltration of monocytes to the site of infection is a crucial event in innate immunity. In the vasculature this event is largely initiated by cytokines (Fig. 7) [6]. In this study, we investigated whether iron plays a role in the event of cytokine-mediated monocyte infiltration. We perfused human monocytes over a monolayer of TNF-α-activated HUVECs. HUVEC iron loading increased the number of firmly adhering monocytes in the *flow* inflammatory model. This may be facilitated by the enhanced adhesion molecule expression, when the intracellular iron levels in HUVECs are increased [26,27]. Further, we asked whether iron could modulate

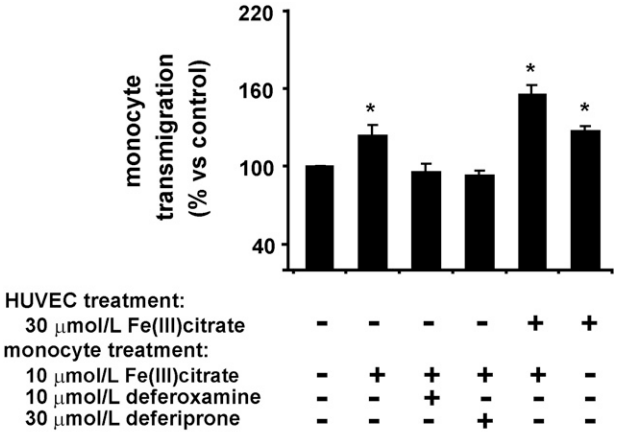

Fig. 6. Intracellular labile iron-induced transendothelial migration of monocytes. HUVECs and monocytes were treated with the indicated treatment, before the monocytes were allowed to transmigrate through the HUVEC monolayer towards an MCP-1 gradient. The fluorescence of transendothelial-migrated monocytes was measured, and the transmigration is expressed as percentages relative to the non-treated controls ($n = 3–7$, $^{*}P < 0.05$).

the activation state of the monocytes and their recruitment to the endothelial layer during inflammation. Under *flow*, we indeed observed that monocyte iron loading leads to a significant induction of abrupt adhesion of monocytes to TNF-α-activated HUVECs. When α4 and β2 integrins were blocked, 60% of the monocytes continued rolling. In this condition, a significantly higher number of iron-activated monocytes was firmly adhering compared to controls, with no change in the average of rolling velocity nor the number of rolling cells. We therefore hypothesised that iron could have affected a set of receptors other than the α4 and β2 integrins. Flow cytometry showed upregulation of surface proteins, CCR-2 and CXCR-2 by iron. These chemokine receptors respond to chemokines, like MCP-1, IL-8 and GRO-α, which are able to support adhesion interaction of leukocytes [6,28], by modulating the potency of integrins and triggering firm arrest of the leukocytes [29]. The data therefore suggest that iron-enhanced abrupt monocyte arrest may be facilitated by the increase of chemokine receptors, lowering the threshold to establish interaction with the activated endothelium.

Independent to PECAM-1 expression, we observed that iron-loaded monocytes compared to controls, transmigrate more effectively towards a gradient of the proinflammatory chemokine MCP-1. Under this condition, iron-induced expression of CCR-2 on monocytes potentiated MCP-1-dependent transmigration, in a PECAM-1-independent manner. Any modulation by iron of other transmigration-mediating molecules on monocytes such as JAM-A, JAM-C and CD99, as well as the secretory molecules, such as cytokines and chemokines, cannot be excluded and certainly warrants further investigation. Given our observations and the observation that in atherosclerosis-prone mice, disruption of CCR-2 caused markedly decreased atherosclerotic lesion formation [9], our results argue for involvement of iron-induced CCR-2 in chronic inflammatory conditions such as atherosclerosis and neurodegenerative diseases.

In a healthy, balanced situation, iron in the circulation is sequestered by transferrin avoiding its involvement in oxidative stress generation, as in its free form or otherwise catalytically active form, iron acts as a catalyst for the formation of oxygen-derived radicals. However, in pathogenic conditions such as hereditary hemochromatosis due to C282Y homozygosity in the HFE gene [30], haemolytic anemias including thalassemias [31], end-stage renal disease patients on hemodialysis [32], and some cases of non-hereditary liver diseases, such as alcoholic liver disease and non-alcoholic fatty liver disease [33], much iron exists as a low molecular weight species, i.e. NTBI. In low concentrations, NTBI may have a normal physiological function for monocyte homing in tissues. However, in higher concentrations, NTBI may cause excessive inflammatory stimulation by promoting monocyte transmigration.

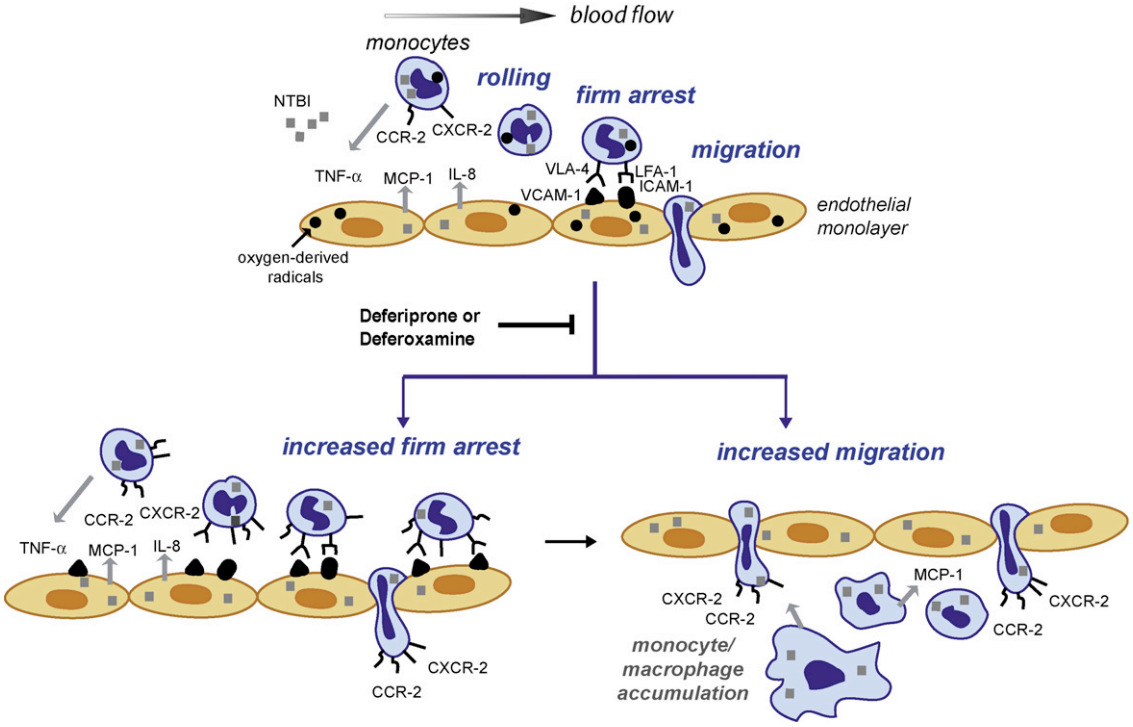

Fig. 7. Illustration of the reported findings with current knowledge showing a sequence of events initiated by NTBI leading to enhanced cytokine-induced monocyte infiltration. NTBI increases the level of intracellular labile iron and oxygen-derived free radicals were generated as a result. The radicals activate the cellular machinery of endothelial cells and monocytes leading to the increased expression of surface adhesion and chemokine receptors. These subsequently promote the inflammatory cascade of events of monocyte recruitment to the endothelium.

This could play a detrimental role in atherosclerosis and neurodegenerative diseases where macrophage infiltration is a key event of pathology [1,4].

Both iron chelators, deferoxamine and deferiprone, could counteract the effects of iron described in this study. This indicates that the effects we observed are iron specific. Moreover, this suggests the potential benefits of iron chelators in conditions with excess NTBI. This also implies that iron chelators may exert anti-inflammatory effects by rendering iron unavailable. This could potentially be useful for managing autoimmune diseases where cytokines play a crucial role in promoting inflammatory reactions.

Oxygen-derived radicals are capable of inducing expression of adhesion molecules [26,27] chemokines [34], and also their receptors [25]. In agreement with these studies, we have observed an increased inflammatory cascade of monocyte infiltration through endothelium as well as oxygen-derived radical formation in iron-loaded monocytes. This finding suggests that enhancement of monocyte–endothelial interactions by intracellular iron is likely mediated by alteration of the redox state of both cell types. The mechanisms whereby iron modulates intracellular redox state which in turn modulates the expression of several atherogenic proteins warrant further investigation. Our findings are particularly important in artherosclerotic and ischemic disease where oxygen-derived radicals aggravate the cytokine-induced inflammatory events as the underlying pathogenesis. The chronic presence of oxygen-derived radicals, possibly occurring in iron-overload conditions, may lead to loss of control of the inflammatory response, which in turn could result in development of persistent inflammatory disorders. Importantly, the impact of intracellular iron in the inflammatory cascade of monocyte infiltration through endothelium may not only be deleterious in chronic inflammation, but also during acute infection.

We have previously shown that iron promotes monocyte adherence in static conditions [26]. In this study, using a physiological *flow* model that mimics *in vivo* leukocyte extravasation in the vasculature, we have demonstrated the role of intracellular labile iron in enhancing the cytokine-mediated consecutive processes of monocyte–endothelial interactions (Fig. 7). Taken together, our study establishes an immunomodulatory function of iron in the complex process of monocyte recruitment and transmigration during inflammation, and supports the role of iron in inflammatory conditions.

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